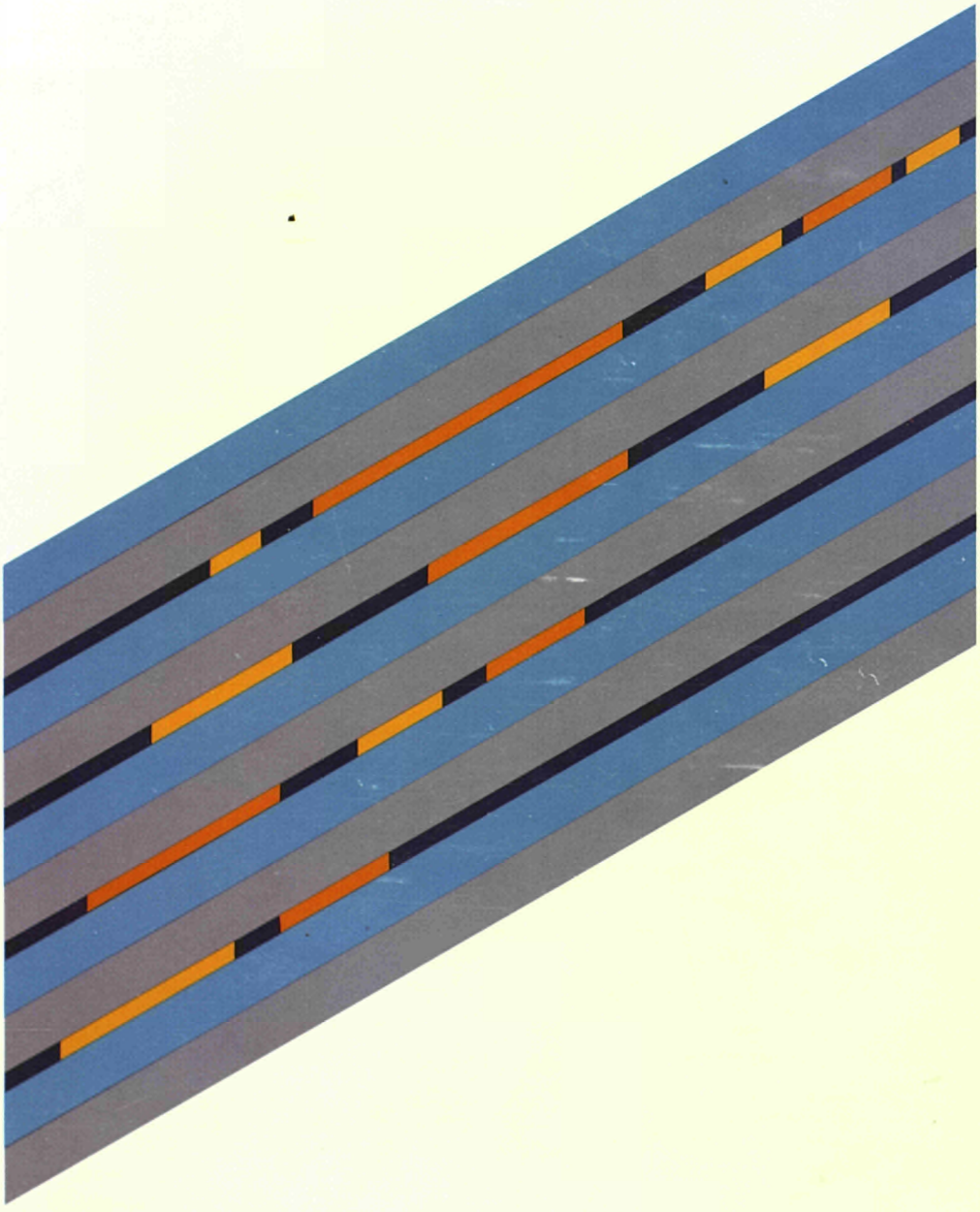
BAP

Biotechnology Action Programme

PROGRESS REPORT 1987

Volume 2: Scientific reports from participating laboratories



Commission of the European Communities
EUR 11138 EN

Biotechnology Action Programme

BAP

1985-89

PROGRESS REPORT 1987

Volume 2: Scientific reports from participating laboratories

Edited by :

E. MAGNIEN
Commission of the European Communities
Rue de la Loi 200
B-1049 Brussels

Directorate-General 'Science, Research and Development'
Directorate 'Biology'
Division 'Biotechnology'

Commission of the European Communities
EUR 11138 EN

Published by the
COMMISSION OF THE EUROPEAN COMMUNITIES
Directorate-General
Telecommunications, Information Industries and Innovation
Bâtiment Jean Monnet
LUXEMBOURG

Cataloguing data can be found at the end of this publication

Luxembourg, Office for Official Publications of the European Communities, 1987

ISBN 92-825-7672-8 Catalogue number: CD-NB-11138-EN-C

Printed in Belgium

TABLE OF CONTENTS

Volume 1

Page

TABLE OF CONTENTS

Volume 2

Page

SCIENTIFIC REPORTS FROM PARTICIPATING LABORATORIES

BIO-INFORMATICS

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Rijksuniversiteit Gent

Contract no.: BAP - 0138 - B

Project leader: K. KERSTERS

Scientific staff: B. Pot

Address: Rijksuniversiteit Gent
Lab. voor Microbiologie
K.L. Ledeganckstraat 35
B - 9000 GENT

Telephone no.: +32.91.227821.EXT.442

Telex no.:

Other contractual partners in the joint project:

L.R. Hill, National Collection of Type Cultures (London)
M.J. Dunn, Jerry Lewis Muscle Research Centre (London)
R.G. Whalen, Institut Pasteur (Paris)

Title of the research activity:

Electrophoresis of proteins : Data capture, analysis and construction of databanks.

Key words:

SDS-PAGE, Protein electrophoresis, Taxonomy, Xanthomonas, Data-processing

Reporting period: July 1986 - June 1987

I GENERAL OBJECTIVES OF THE JOINT PROJECT:

1) Improve existing and/or develop new software for data capture, data reduction, data storage and comparison of 1- and 2-dimensional polyacrylamide gel-electrophoretic protein patterns of bacteria and eukaryotic cells. High flexibility will be persued to accommodate for variation in hardware configurations.
2) Develop databanks for 1-D and 2-D protein patterns of pathogenic bacteria, biotechnologically important bacteria and eukaryotic cells. Areas of exploitation: screening and identification of bacterial strains, recognition of new groups and study of human and animal disease-related protein abnormalities. Full exploitation of PAGE technology across the Community will be possible.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

1) Improve methods for the preparation of highly reproducible gel-electrophoretic protein patterns of biotechnologically important bacteria.
2) Improve existing software for data capture, data reduction and data correction (for gel to gel variation) of the raw digitized patterns.
3) Develop new techniques and software for the evaluation of the new processing steps, applied to bacterial groups from the LMG (Laboratorium Microbiologie Gent) collection. In order to evaluate the taxonomical results two bacterial groups were chosen that were previously investigated by polyphasic approach at the LMG (Xanthomonas / Pseudomonas, and Moraxella / Psychrobacter).

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY

Sodiumdodecylsulfate (SDS) polyacrylamide gel-electrophoresis (PAGE) was carried out in a Protean dual slab cell (Bio-Rad, USA) as described by Laemmli (1970), modified by Jackman (1985). The gels were photographed and dried in a Gel-Slab-Dryer Model 224 (Bio-Rad, USA). Dried gels were scanned with a LKB 2202 ultroscan laser densitometer (LKB, Sweden), and raw digitized data were captured on an Apple //e microcomputer (Apple Computer Inc., USA), equipped with a Transwarp accelerator (Applied Engineering, USA) and a A/D converter interface (LKB, Sweden), using the modified GELSCAN software (LKB, Sweden). The Apple 1.2 Pascal program GELSCAN was modified to store all necessary strain and gel lane information with the digitized profile. Raw digitized data were stored on 51/4 inch floppy disks (Apple Duodisk, Apple Computer Inc., USA). After interpolation and alignment by the Apple 1.2 Pascal program INTFILE (Casteleyn, P. and Pot, B., LMG) data were transferred to the Siemens 7570-C mainframe (Centraal Digitaal Rekencentrum (CDR), State University Gent, Belgium) using the Apple 1.2 Pascal program TERMTRANS (Coppieters, C., CDR). The final numerical analysis was performed as described by Kersters (1985) on the Siemens mainframe computer. The processed data were also stored on a Apple Profile 10Mb harddisk (Apple Computer Inc., USA) using the OMNIS 3 Database Manager (Blyth

Computers Ltd., England). Raw data and interpolated data can be visualized and compared by plotting on a Roland DXY-990 plotter (Roland DG corporation, Japan) using the Apple 1.2 Pascal program PLOTCURVES (Pot, B., LMG).

2. RESULTS & DISCUSSION

2.1. Preparation of cell-free extracts

We investigated the influence of growth temperature, growth medium and growth time on the protein pattern. These factors are important for the high reproducibility, necessary for the construction of a data base.
The conservation of the protein extract at -80° C was excellent over the ten months test period. Immediately after preparation, extracts are stored in small aliquots (200 µl) in order to reduce the number of freezing and defreezing steps.

2.2. The gel-electrophoretic technique

The gel-electrophoretic technique was further standardized in cooperation with the National Collection of Type Cultures (NCTC, London), another BAP-contractor of this project. We changed to BDH-products to enhance comparability. The quality of the protein gels was improved by keeping the quantities of the extracts applied on the slots as constant as possible, as this increased the reproducibility of the low molecular weight zone of the protein profile. It was further noticed that the polymerization time of the stacking gel was correlated with the overall sharpness of the protein bands. Also more care was taken to the final destaining procedure, giving more consistent background.

2.3. The use of a reference extract

In each gel (18 data lanes, 2 edge lanes) the extract of the bacterial strain LMG 1125 (Psychrobacter sp.) was included 3 to 5 times in order to (i) detect small shifts within each gel and (ii) compare gels over a longer period of time. The profile of strain LMG 1125 was chosen because of a number of well-separated, sharp protein bands, spread over the entire protein profile (Fig. 1).

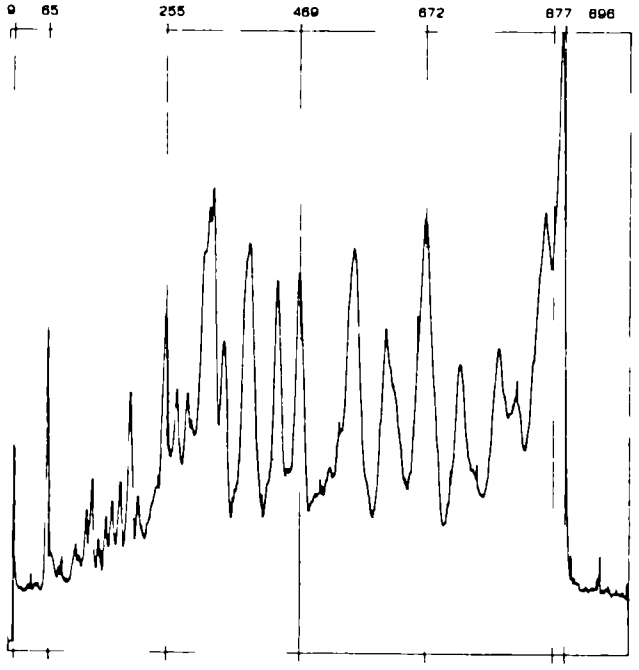


Fig. 1: Protein electrophoretic trace of the reference extract LMG 1125. The 'interpolation intervals' are delineated by vertical lines.

The extract is easily prepared and besides being much cheaper than molecular weight markers it also produces protein peaks which are much sharper, allowing a more precise location of peaks. The extract was used with good results over a 10 months period and it allowed to investigate the reproducibility of the gel-electrophoretic technique over that period. The reproducibility of the reference profile (LMG 1125), used for interpolation of these gels was > 0.94, whereas the reproducibility of a non-reference strain was > 0.87.

2.4. The coupling of data reduction and normalization of profiles

A time-saving algorithm on the basis of the Lagrange interpolation method was worked out to normalize the gels and to reduce the 1000 measured points to a more manageable 400 point trace. Using the well-localized sharp protein bands of the reference extract (strain LMG 1125) each 4-5 neighbouring gellanes were subdivided in usually six 'interpolation-intervals' (Fig. 1). Each interval was separately interpolated with the Lagrange interpolation algorithm to yield a constant number of interpolated points. The number of points to be kept in each interval was calculated statistically from ± 100 experiments with strain LMG 1125 over a long period of time. For each interval the reduction ratio (i.e. number of raw points / number of interpolated points) was calculated. For the reduction to a 400 point trace this number is only influenced by (i) the length of the profile (i.e. the number of points between start- and end point of the trace) and (ii) the shift of the profile against the standard LMG 1125 curve. If the reduction ratio is calculated in function of the total trace length, it is a measure of the gel quality and it can be used as a built-in control-parameter for the interpolation process.

2.5. The program for data reduction and interpolation: INTFILE

1) Read raw data and info of reference (Ref.) trace.
2) Search position of start- and end point.
3) Calculate expected position and standard deviation of Ref. peaks using trace length and start position.
4) Look for Ref. peak in the calculated regions, using standard maxima.
5) Delineate the different intervals using the detected Ref. peak positions.
6) Calculate reduction ratio per interval.
7) If ratio's OK then read raw data and info of non-reference (non-Ref.) trace, else give message and partial printout.
8) Interpolate non-Ref. lane within each interval using Lagrange's algorithm.
9) Write out interpolated curve with info in sequential output file (OFL-file).
10) Until new Ref. trace: read raw data and info of next non-Ref. trace and go to 8).
11) Until all lanes are interpolated: go to 1).
12) End of program.

The complete processing of a 1000 point trace by a microcomputer takes only 45 seconds. This is due to the use of the Pascal computer language, the use of an accelerator card, and the combination of data-reduction and normalization into one single processing step. As a reference trace is automatically marked by the modified GELSCAN program (on the basis of the LMG number stored with the info), the reduction and normalization process is carried out automatically for one complete gel.

2.6. The taxonomic results obtained within the genus Xanthomonas

The SDS protein profiles of approximately 270 strains of the plant pathogenic xanthan producing bacterial species Xanthomonas campestris (X. c.) were registered and compared. We also included the type strains of the 5 other Xanthomonas species. Of the 72 pathovars (pv.) of X. campestris dealt with, two important plant pathogenic pathovars X. c. pv. pelargonii and X. c. pv. begoniae were investigated in more detail (32 and 31 strains, respectively). The numerical analysis revealed 9 main clusters above a correlation coefficient of r = 0.66 (Van Nieuwenhuyse D., 1987).

X. campestris can electrophoretically be differentiated from the other Xanthomonas species. They form clusters 1 and 2, the latter being subdivided in 5 smaller subclusters. Clusters 3, 4 and 8 correspond to X. albilineans, X. axonopodis and X. fragariae, respectively. The other clusters comprise strains of the electrophoretically heterogeneous species X. maltophilia. "X. populi" belongs to cluster 2, but cannot be located in one of the 5 subclusters.

From some pathovars in clusters 1 and 2 we found that almost all strains within each pathovar yielded very similar or identical protein electrophoregrams. These pathovars are: arracaciae, arrhenatheri, barbareae, bauhiniae, cajani, campestris, cassiae, cerealis, coracanae, coriandri, cyamopsidis, desmodii, desmodigangetici, graminis, guizotiae, holcicola, hordei, khayae, mangiferaeindicae, manihotis, nigromaculans, oryzae, oryzicola, patelii, pelargonii, phlei, phleipratensis, pisi, poae, secalis, taraxaci, translucens, undulosa, vasculorum, viticola, vitiscarnosae, vitistrifoliae and vitiswoodrowii. It is striking that subcluster 2E comprises only strains from pathovars that are pathogenic on plants of the family Poaceae. Likewise the pathovars pathogenic for plants of the Vitaceae also form one dense cluster (cluster 1). Fig. 2 represents a similarity matrix of two X. campestris pv. campestris strains and several strains of the two pathovars begoniae and pelargonii. X. campestris pv. begoniae is electrophoretically heterogeneous, whereas pv. pelargonii forms one dense cluster. From the similarity matrix it is also clear that both pathovars can electrophoretically be differentiated and that pv. campestris is closer to pv. pelargonii than the different subgroups of pv. begoniae are related to one another.

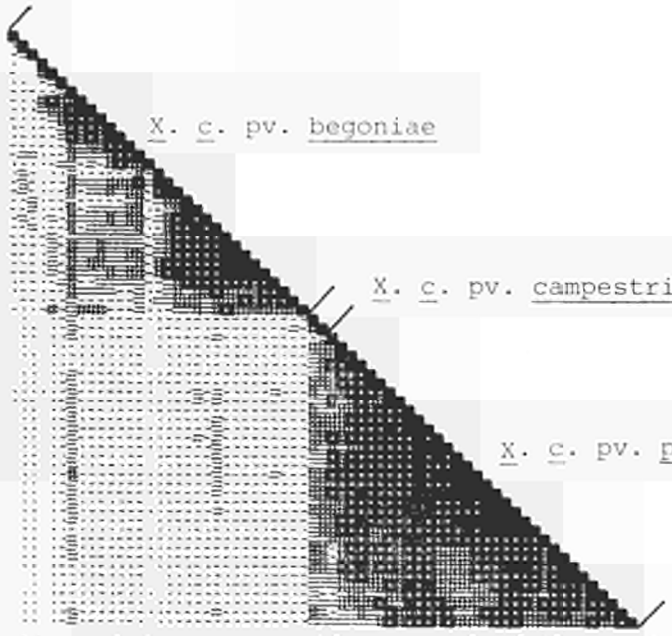


Fig. 2: Similarity matrix representing a part of the protein-electrophoretic results obtained with strains of the genus Xanthomonas. Three pathovars of the species X. campestris are shown.

SDS-PAGE can thus be used to identify Xanthomonas strains. Some pathovars within X. campestris can definitely be differentiated. DNA:DNA hybridizations should confirm these findings. Other pathovars are electrophoretically heterogeneous, and their present taxonomic status is therefore uncertain. More strains should be investigated in order to test the homogeneity of the other pathovars.

3. REFERENCES

- Jackman, P.H.J. In: "Chemical methods in bacterial systematics" (M. Goodfellow and D.E. Minnikin, eds.), pp. 115-128. Academic Press, London (1985).

- Kersters, K. In: "Computer-assisted bacterial systematics" (M. Goodfellow, D. Jones and F.G. Priest, eds.), pp. 337-368. Academic Press, London (1985).

- Laemmli, U.K. (1970). Nature (London) 227: 680-685.

- Van Nieuwenhuyse, D. (1987). Fijnstructuur van het plantpathogeen species Xanthomonas campestris aan de hand van computer-geholpen analyse van eiwit electrophoregrammen. (Finer relationships of the plant pathogenic species Xanthomonas campestris using computer-aided analysis of protein electrophoregrams). Verhandeling ingediend tot het verwerven van de graad van Licentiaat in de Dierkundige Wetenschappen, Biotechnologische richting. Laboratorium voor microbiële Genetica, Faculteit Wetenschappen, Rijksuniversiteit Gent.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1. PUBLICATIONS IN SCIENTIFIC JOURNALS, MONOGRAPHS, ...

B. Pot, D. Van Nieuwenhuyse, J. De Ley and K. Kersters. Improved acquisition and processing of SDS-PAGE protein patterns applied to Xanthomonas strains. In: Enzyme engineering: Protein design and applications in biocatalysis. First sectorial meeting BAP, Capri, Italy 2-6 May 1987, pp. 25-26.

IV.2. SHORT COMMUNICATIONS, INTERNAL REPORTS, ...

IV.3. PATENTS DEPOSITED IN CONNECTION WITH THE RESEARCH PROGRAMME

IV.4. DOCTORATE THESIS (Ph.D) AND DEGREE THESIS AWARDED DURING THE PERIOD OF CONTRACT

The programs developed were used for the final processing and interpretation of the protein electrophoretic work accomplished in the following degree theses.

1) Delplancke, Steven. (1987). Genotypische en electrophoretische verwantschappen van bakteriën uit de "Moraxella paraphenylpyruvica" groep. (Genotypic- and protein electrophoretic relationships of bacteria from the "Moraxella paraphenylpyruvica" group). Verhandeling ingediend tot het verwerven van de graad van Licentiaat in de Dierkundige Wetenschappen, Biotechnologische richting. Laboratorium voor microbiële Genetica, Faculteit Wetenschappen, Rijksuniversiteit Gent.

2) Van Nieuwenhuyse, Dries (1987). Fijnstructuur van het plantpathogeen species Xanthomonas campestris aan de hand van computer-geholpen analyse van eiwit electrophoregrammen. (Finer relationships of the plant pathogenic species Xanthomonas campestris using computer-aided analysis of protein electrophoregrams). Verhandeling ingediend tot het verwerven van de graad van Licentiaat in de Dierkundige Wetenschappen, Biotechnologische richting. Laboratorium voor microbiële Genetica, Faculteit Wetenschappen, Rijksuniversiteit Gent.

3) Vauterin, Luc. (1987). Taxonomische studie van Xanthomonas campestris pv. pelargonii en Xanthomonas campestris pv. begoniae, van belang in de Belgische sierplanten teelt. (Taxonomic study of Xanthomonas campestris pv. pelargonii en Xanthomonas campestris pv. begoniae, important in the Belgian ornamental plant industry). Verhandeling ingediend tot het verwerven van de graad van Licentiaat in de Plantkundige Wetenschappen, Fysiologisch-Biochemische richting. Laboratorium voor microbiële Genetica, Faculteit Wetenschappen, Rijksuniversiteit Gent.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)	Yes	
Joint meeting(s)	Yes	

Descriptive information for the above data.

A) Exchange of materials and joint experiments:

1) In order to compare the results of the SDS-PAGE technique performed at NCTC, London and at LMG, Gent protocols and bacterial strains were exchanged. Gels are being run at both labs, photographed and analysed numerically. Exchange and comparison of results should allow to track the main causes of differences. New protocols should then be worked out which allow the SDS-PAGE technique to be exported to other labs, yielding results that are compatible with other data, available in PAGE-pattern databanks.

2) The electrophoretic data obtained, will be merged with the data base developed at LMG in the context of "Microbial Information Network Europe" (MINE), a joint project of 5 nations and their culture collections in the CEC Biotechnology Action Programme (BAP 0134).

B) Joint meetings:

1) 5-9 january 1987: Working visit of K. Kersters and B. Pot to NCTC, London. The complete SDS-PAGE procedure was closely followed, and differences with the procedure at LMG, Gent were noticed and discussed. The equipment at NCTC, London was compared with the equipment in LMG, Gent. Software principles were exchanged and discussed.

2) 8 january 1987: Meeting at NCTC, London. Present: L.R. Hill, R.J. Owen, M. Costas (NCTC, London), M. Dunn (Hammersmith, London) and K. Kersters and B. Pot (LMG, Gent). Program: Administrative and technical matters of the consortium; progress of the scientific working visit of K. Kersters and B. Pot at NCTC; near-future plans for further cooperation; the Capri-meeting of the CEC in May 1987.

3) 1 april 1987: Meeting at Unilever Research Laboratories, Vlaardingen, Nederland. Present: K. Kersters, B. Pot (LMG, Gent), T. Verrips, A. Ledeboer (Unilever, Vlaardingen) and several members of the scientific staff of Unilever Research. Program: An exposition by K. Kersters on polyphasic taxonomic research on the bacterial genus Pseudomonas. Arrangements were made for a taxonomical analysis using SDS-PAGE of a variety of lactic acid bacteria, important in the agro-food industry.

4) 2-6 may 1987: First sectorial meeting of BAP, Capri, Italy. Present: All members of the consortium. Program: Besides the official program of this first sectorial meeting there was time to discuss preliminar results and to plan future experiments.

5) 10-11 june 1987: Participation of B. Pot on the worhshop "The Construction, Applications and Benefits of Protein Databases Developed from Computer-Analyzed Two-Dimensional Gels" at the Institut Pasteur, Paris. This workshop was organized by R. Whalen, contractor in the consortium, together with Protein Database Inc., USA. Hardware and software for the analysis of two-dimensional gels was demonstrated. Arrangements were made for the 2-D analysis of some well-chosen bacterial strains from LMG, Gent.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: P. H. L. S., London Contract no.: BAP - 0133 - UK

Project leader: L.R. HILL
Scientific staff: M. Costas, L.L. Sloss (nee Rodger)

Address:
National Collection of Type Cultures
Central Public Health Laboratory
61 Colindale Avenue
UK - LONDON NW9 5HT

Telephone no.: +44.1.2004400 EXT.3707/3708

Telex no.: 8953942 DEFEND G

Other contractual partners in the joint project:

K. Kersters, Rijksuniversiteit Gent
M.J. Dunn, Jerry Lewis Muscle Research Centre (London)
R.G. Whalen, Institut Pasteur (Paris)

Title of the research activity:

Electrophoresis of proteins : Data capture, analysis and construction of databanks.

Key words:

Electrophoresis, Identification, Bacterial proteins, Classification, Computer analysis

Reporting period: August 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

1. Improve existing and/or develop new software for data capture, data reduction, data storage and comparison of 1 and 2-dimensional polyacrylamide gel electrophoretic protein patterns of bacteria and eukaryotic cells. High flexibility will be pursued to accommodate for variation in hardware configurations.
2. Develop databanks for 1-D and 2-D protein patterns of pathogenic bacteria, biotechnologically important bacteria and eukaryotic cells. Areas of exploitation, screening and identification of bacterial strains, recognition of new groups and study of human and animal disease related protein abnormalities. Full exploitation of PAGE technology across the community will be possible.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

1. Sub-classification of species of Providencia and Campylobacter.

2. Improvement of data capture and expansion of database.

3. Improvement of standard protocol.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

METHODOLOGY

Reproducibility of preparation of whole cell protein extracts is being maximised to allow direct comparison of profiles. Protein separation is, for 1-D, in 10% SDS-PAGE gels using the Bio-Rad system and staining with PAGE Blue 83. Molecular weight markers are included in each gel. The gels are dried and scanned with an LKB 2202 Ultrascan Laser densitometer linked to an Apple IIe microcomputer. Corrections for gel to gel, based on calibration patterns, are made and traces stored on computer discs as files in a database. Software now includes identification routine. A new system using a Compaq 386 computer, software written in Turbo Pascal with automated stretch/ shrink facility is currently under development to link with LKB XL laser densitometer.

RESULTS

Providencia Spp.

Providencia rustigianii - 20 strains of P. rustigianii from clinical, veterinary and environmental sources were characterised by SDS-PAGE of their total cell proteins. The pr files were highly reproducible and contained 45 to 50 discrete bands. 2 different computer assisted numerical analyses were carried out:-
a) All protein bands included. The protein profiles of the 20 P. rustigianii strains were arranged into 6 clusters at the 85% S-level. One of these clusters contained the type strains of both P. friedericiana and P. rustigianii which confirmed a recent report that these two species are synonymous. There was no apparent correlation between the protein profiles of each cluster and the clinical source of the isolates.
b) Principal protein bands (34-41 kDa range) excluded. At the 86% S level the 20 P. rustigianii strains formed a single cluster and the reference strains of P. stuartii, P. rettgeri, P. alcalifaciens and Morganella morganii remained unclustered.

P. alcalifaciens - 25 strains of P. alcalifaciens from clinical and veterinary sources were treated as for P. rustigianii above. Again 45 to 50 discrete, highly reproducible bands were obtained and again 2 studies were carried out:-
a) All protein bands included. The 25 strains were arranged into 10 clusters at the 85% S level.
b) Principal protein bands excluded. At the 83% S level the 25 P. alcalifaciens strains formed a single cluster and the reference strains of P. stuartii, P. rettgeri and P. rustigianii remained unclustered.

Campylobacter Spp.

Urease Positive Thermophylic Campylobacters and C. laridis.

Protein profiles of 9 C. laridis strains and 15 allied isolates, including members of the Urease Positive Thermophilic (UPTC) group were prepared and scanned. Approximately 40 discrete bands were discernible with the major bands being found in the 40-48 kDa range. C. laridis and UPTC strains could be separated at the 77% S level. The C. laridis subclustered into 2 groups and the UPTC's into at least 5.

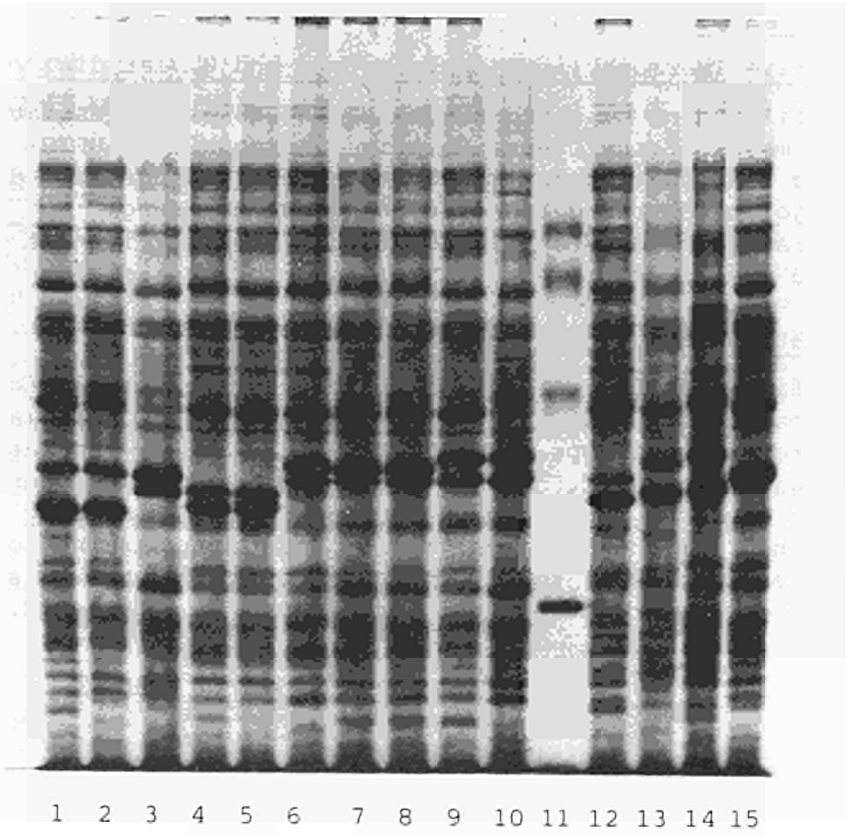
Nitrate Negative Campylobacters.

Protein profiles of 25 strains of Nitrate Negative Campylobacters (NNC's) were prepared and scanned. The 25 strains consisted of isolates from 3 different predefined groups - NNC-1, NNC-2 and NNC-3, the latter being catalase negative or weak. Again 40 or so discrete bands were obtained. The 3 groups could be separated at the 80% S level with only 4 strains not conforming. Biochemical analyses showed these strains to differ from the other members of their groups in certain tests; eg strength of catalase and oxidase reactions and aerobic growth or at 42°C.

DISCUSSION

Whole cell protein patterns offer considerable potential for typing bacterial strains of clinical interest, especially for those for which there are no other widely available typing methods. The 1-D PAGE protein patterns are a reproducible means of grouping strains since, under well-defined and standard conditions they can be considered as "fingerprints" with closely related bacteria displaying similar or identical patterns. The Pearson product-moment correlation coefficient and UPGMA taxonomic clustering used takes into account both qualitative and quantitative data which can cause problems when a few principal bands account for up to 20% of the total protein, as was found with the Providencia. Parallel analysis omitting principal bands can be used to discriminate between organisms. With the Providencia study the protein results were consistent with previously published DNA-DNA hybridisation data. The technique of PAGE could provide a basis for a rapid identification system for many bacteria, especially once a database of profiles is complete.

Fig. Example of a typical SDS-PAGE 1-D gel.
Tracks of 1-10 and 15 Providencia alcalifaciens
Track 11 Molecular weight standards.
Track 12-14 Reference strains of other Providencia species.



IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

1. PUBLICATI NS IN SCIENTIFIC JURNALS.

a) "Numerical Analysis of electrophoretic protein patterns of Providencia rustigianii strains from human diarrhoea, other clinical specimens and the environment"
M. Costas, B. Holmes and L.L. Sloss.
submitted for the Journal of Applied Bacteriology.

b) "Numerical Analysis of electrophoretic protein patterns of Providencia alcalifaciens strains from human faeces and veterinary specimins."
B. Holmes, M. Costas, L.L. Sloss
submitted for the Journal of Applied Bacteriology.

2. SHORT COMMUNICATIONS:

a) "One-dimensional electrophoretic protein fingerprints in Campylobacter identification and biotyping".
R.J. Owen, M. Costas, L.L. Sloss and F.J. Bolton.
Abstract for the IVth International Workshop on Campylobacter Infections.
June 16-18th. 1987. Gotenborg, Sweden.

b) "Technical aspects of 1-D SDS-PAGE of bacterial proteins; Application to identification of pathogens".
Abstract for the Commission for the European Communities, BFIP Meeting.
Capri, Italy. 2-6 May 1987.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff	Yes	
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

EXCHANGE OF MATERIALS, with Dr. Kersters, Gent.

Samples both chemical and bacterial, including bacterial standards. Protocols and information on results.

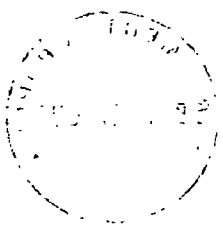
EXCHANGE OF STAFF.

Karl Kersters and Bruno Pot from the Rijkuniversiteit, Gent visited the National Collection of Type Cultures in London, in January 1987. Lesley Sloss from the NCTC visited the Jerry Lewis Muscle Research Centre in July 1987.

JOINT MEETINGS.

8th. Jan: at NCTC: attended by Kersters, Pot (Gent), Dunn (Hammersmith), Smith (Queen Mary College), Whalen (Paris).

C.E.C., BAP Meeting. "Enzyme Engineering: Protein Design and Applications in Biocatalysis". Attended by L.R.Hill and M. Costas. Capri, Italy. 2-6 May 1987.



BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Royal Postgraduate Medical School, London

Contract no.: BAP - 0142 - UK

Project leader: M.J. DUNN

Scientific staff:

Address: Jerry Lewis Muscle Research Centre
Royal Postgraduate Medical School
DuCane Road
UK - LONDON W12 OHS

Telephone no.: +44.1.7432030 EXT. 2124

Telex no.: 8951182 GECOMS G

Other contractual partners in the joint project:

K. Kersters, Rijksuniversiteit Gent
L.R. Hill, National Collection of Type Cultures (London)
R.G. Whalen, Institut Pasteur (Paris)

Title of the research activity:

Electrophoresis of proteins : Data capture, analysis and construction of databanks.

Key words:

Electrophoresis, Databases, Computer analysis, Human proteins, Genetic disorders

Reporting period: August 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

(1) Improve existing and/or develop new software for data capture, data reduction, data storage and comparison of 1- and 2-dimensional polyacrylamide gel electrophoretic protein patterns of bacteria and eukaryotic cells. High flexibility will be pursued to accommodate for variation in hardware configurations. (2) Develop databanks for 1-D and 2-D protein patterns of pathogenic bacteria, biotechnologically important bacteria and eukaryotic cells. Areas of exploitation: screening and identification of bacterial strains, recognition of new groups and study of human and animal disease-related protein abnormalities. Full exploitation of PAGE technology across the Community will be possible.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

(a) Collection of 2-D gel patterns
(b) Install and validate computer equipment
(c) Prepare timetable for and begin PDQUEST software implementation
(d) Begin to examine advanced image processing algorithms.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Methodology

Fibroblast and muscle cell cultures are being established from skin and skeletal muscle biopsies taken from normal individuals and from patients with a variety of genetic neuromuscular disorders (including Duchenne, Becker, and other muscular dystrophies; spinal muscular atrophy and other neuropathic disorders; Prader-Willi syndrome). Samples of these cultures are radiolabelled with [^{35}S]-methionine and the proteins subsequently separated by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE). The 2-D patterns are visualised by autoradiography. The patterns are digitised at 100 µm resolution using the Datacopy linear diode array camera, controlled by an IBM-XT microcomputer, which has been installed in the laboratory. The data is then transferred to the Orion supermicrocomputer system which is now running a full-time service in the laboratory. At present this data transfer is achieved

using the programme "Kermit", but it is planned that in the forthcoming period this will be replaced with a faster and more reliable "Ethernet" network connection. The Orion computer system has also been connected to the international packet switching system (IPSS) so that it is available for exchange of information and data with other members of the consortium and also for communication with other BAP contractors (eg via EUROKOM).

RESULTS

(a) Collection of 2-D gel patterns

A large number of 2-D gel patterns of [^{35}S]-labelled proteins of human skin fibroblasts and muscle cells from normal individuals and patients with a variety of neuromuscular disorders have been collected. Autoradiographs of these 2-D gels have been digitised using the Datacopy camera system and the raw, digitised image files have been transferred to the Orion computer system. They are, therefore, available for analysis and database construction when the PDQUEST implementation is completed (see (b) below).

Some [^{35}S]-labelled protein samples of fibroblast and muscle cells have been sent to Protein Databases Inc (PDI) in the USA. There they have been separated by 2-D PAGE and analysed using the PDQUEST software. This has substantiated that the software system is capable of analysing these 2-D gel patterns successfully. Perhaps more interestingly, this analysis has also suggested some differences in the 2-D protein patterns of cells from normal individuals and patients with Duchenne muscular dystrophy.

(b) Implementation of PDQUEST software

A timetable for the conversion of the PDQUEST software has been agreed with PDI, which should ensure that a fully functional release of the software will be available for use on the Orion computer system by the end of September 1987. Since PDQUEST is implemented in "C" under Unix, no incompatibility is expected at the code, system call, or file system levels. The major obstacle to implementation is the graphics interface as these are usually proprietary to individual computer hardware systems. A decision has been taken to consolidate the graphics calls in PDQUEST in such a way that only a single routine will require modification to facilitate implementation of future releases of PDQUEST. Several standard graphics interfaces have been investigated and it has been decided that "X-windows" from MIT will provide the best basis for

conversion. High Level Hardware plan to have X-windows implemented on the Orion system by Autumn 1987, so that this should be available for use with the PDQUEST system. PDI have agreed to provide a software source licence for the PDQUEST system, so that we will also have the ability to test the alternative Starpoint proprietary graphics interface of the Orion system. Moreover, this will facilitate integration of any new algorithms for analysis of 2-D gel patterns which we develop (see (c) below).

(c) Advanced image processing algorithms

Alternative statistical methods and parallel processing techniques are being explored in collaboration with K. Smith (Institute F) for their suitability for analysis of 2-D gels. Recent advances in the theory of Markov random fields (MRF) provide a consistent and usable probabilistic framework for the analysis and matching of 2-D gel images. Techniques such as simulated annealing and iterated conditional nodes exist for finding solutions to appropriately formulated problems on MRFs and these approaches are currently under investigation.

CONCLUSIONS

The PDQUEST software system will soon be available on the Orion computer system to begin database construction of 2-D gel patterns of fibroblast and muscle proteins in human genetic disorders. The system is being implemented in such a way that any new algorithms which are developed to improve data analysis can be readily integrated.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

(a) Proceedings of Meetings

Smith, K. and Dunn, M.J. (1986). Computer analysis of 2-D gels using statistical methods and parallel processing. In: Electrophoresis '86 (ed. Dunn, M.J.), VCH Verlagsgesellschaft, Weinheim, pp. 560-562.

(b) Short Communications

Dunn, M.J. and Smith, K. (1987). Computer analysis of two-dimensional gels. Abstract for the Commission of the European Communities, BAP meeting, Capri, Italy, 2-6 May 1987.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff	Yes	
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

Exchange of materials

Samples of bacteria from the NCTC are being analysed by 2-D PAGE.

Exchange of staff

Lesley Sloss from the NCTC visited the Jerry Lewis Muscle Research Centre to learn 2-D PAGE procedures.

Joint Meetings

C.E.C., BAP Meeting. "Enzyme Engineering: Protein Design and Applications in Bioanalysis", Capri, Italy, 2-6 May 1987.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Inst. Pasteur, Paris

Contract no.: BAP - 0145 - F

Project leader: R.G. WHALEN

Scientific staff:

Address:
Institut Pasteur
Dept. of Molecular Biology
25, rue du Dr. Roux
F - 75724 PARIS CEDEX 15

Telephone no.: +33.1.45688483

Telex no.: 250609 PASTEUR F

Other contractual partners in the joint project:

K. Kersters, Rijksuniversiteit Gent
L.R. Hill, National Collection of Type Cultures (London)
M.J. Dunn, Jerry Lewis Muscle Research Centre (London)

Title of the research activity:

Electrophoresis of proteins : Data capture, analysis and construction of databanks.

Key words:

Two-dimensional electrophoresis, PDQUEST, Cell differentiation, Protein databases, Numerical classification

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

1) Improve existing and/or develop new software for data capture, data reduction, data storage and comparison of 1- and 2-dimensional polyacrylamide gel electrophoretic protein patterns of bacteria and eukaryotic cells. High flexibility will be pursued to accomodate for variation in hardware configurations.
2) Develop databanks for 1-D and 2-D protein patterns of pathogenic bacteria, biotechnologically important bacteria and eukaryotic cells. Areas of exploitation: screening and identification of bacterial strains, recognition of new groups and study of human and animal disease-related protein abnormalities. Full exploitation of PAGE technology across the Community will be possible.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

1) Install the commercial PDQUEST™ system on a MassComp computer.
2) Perform initial analysis on results obtained from an experiment to compare various mouse cell lines representing different stages of differentiation.
3) Attempt to compare data obtained in different laboratories using the sample electrophoretic and software systems.
4) Begin analysis of new means of classifying the data obtained from the PDQUEST™ system in an attempt to create new software as a basis for expert system analysis.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Methodology

Electrophoresis of complex protein mixtures by two-dimensional gel electrophoresis is recognized as one of the most powerful separation methods for comprehensive study of protein metabolism. However, the quantity of data obtained currently requires the use of powerful minicomputers and software.

A software system has been made available through Protein Databases Inc. (Huntington Station, New York) under the name PDQUEST™. This system includes: (i) spot detection routines, (ii) automatic conversion of film optical density into DPM values, (iii) routines for manual and automatic matching of gel images, (iv) various graphical presentations of quantitative data, (v) statistical analysis, and (vi) a structured database allowing textual information to be associated with individual polypeptides.

The PDQUEST™ software system is implemented on a MASSCOMP MC-5500 32-bit virtual memory minicomputer, employing a 68010 CPU with 3 MB of memory, a hard disk capacity of 385 MB, and an array processor connected

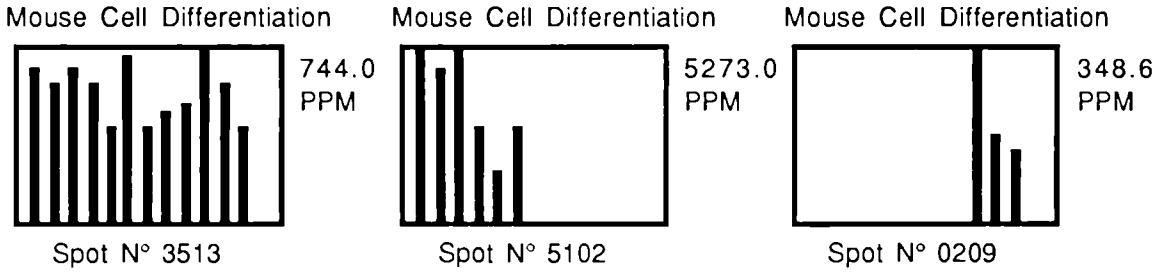
to the CPU bus. Two-dimensional gel results, usually in the form of radiographic film images, are first scanned with an Optronics rotating drum densitometer at a resolution of 200 X 200 m. Raw scan data (approx. 1.2 MB) is then read into the MASSCOMP computer via magnetic tape. After spot detection, the reduced gel image file is ca. 100-200 KB.

2. Results

For our initial biological studies carried out with the PDQUEST™ system, we have chosen three mouse cell lines, all derived from the C3H strain. These lines can be taken to represent three distinct phases of differentiation. A teratoma cell line, 1003, is derived from embryonic cells and has the capacity to differentiate into various epidermal cell types. A second line, called 3T101/2, is apparently a mesodermal stem cell, since it can be converted into myoblasts, chondroblasts and adipocytes by treatment with azacytidine. Finally, the C2 myoblast line can be maintained in a non-differentiated state, but can be induced to form multinucleate myotubes which express several characteristic muscle-specific proteins.

The four cell types were analyzed after labelling triplicate samples with ^{35}S-methionine. The resulting twelve gel images were then alligned to a standard image (a representative gel chosen from the series of twelve) using the PDQUEST™ software by designating about 100 landmark spots (i.e. polypeptides identifiable in all images). The matching function then allows a given polypeptide in each individual gel image to be considered as identical and all quantitative or textual data can be accessed by referring to that spot in the standard gel image.

The quantitative results can be presented in different ways, and a histogram-like presentation is a convenient way to examine the potential differences. This presentation is shown below.



Each bar of the histogram represents the intensity of a single spot (given an arbitrary spot number) in each of the gel images in the order: 1003 triplicates, 3T101/2 triplicates, C2 myoblast triplicates, and C2 myotube

triplicates. The heights of the bars are normalized to the spot of greatest intensity, and data are presented as PPM values. Spot 3513 is an unknown protein which is clearly present in all gel images. Spot 5102 is also an unknown protein, but is only found in 1003 and 3T101/2 cells. Spot 0209 is a muscle-specific isoform of tropomyosin, and as expected is found only in fused, differentiated C2 myotubes.

Textual information can be associated with each spot number and accessed along with the quantitative data. The database currently has a simple structure with 38 predefined categories, including for example: amino acid composition, chromosomal location, protein name, tissue distribution, etc. These annotations can be entered as new information becomes available for each polypeptide species, and they can be accessed by indicating a given spot in the standard gel image. In a collaborative experiment with S. Blose (Protein Databases Inc.), we have compared our C3H mouse results with those of a database created from NIH 3T3 cells and have found that it is possible to match many of the spots in the standard images from the two sets of data thus demonstrating that it will be possible to compare results between different laboratories.

We have also begun to develop programs for the analysis of the quantitative data, in collaboration with P. Nugues (Centre de Recherche en Informatique de Nancy). After first filtering the data to retain only those data sets that are complete and coherent, comparison and classification is carried out to search for groups of proteins whose metabolism varies in similar ways.

3. Discussion

We have completed installation of the PDQUEST™ system and analyzed a biological experiment designed to examine mouse cell development. A number of large differences have been found among the cell types analyzed, and the numerical classification approach should provide more automated means of examining the data. Our experience in comparing our gel images with those obtained at Protein Databases Inc. demonstrates that it is possible to compare and exchange data obtained from the PDQUEST™ analysis provided that the initial electrophoretic experiments are performed using standardized protocols.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

No articles were published during this first year.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)	Yes	
Joint meeting(s)		No

Descriptive information for the above data.

The electrophoretic data obtained by Dr. Dunn has been examined on the PDQUEST™ system in our laboratory.

Joint experiments are planned with the laboratories of Dr. Hill and Prof. Kersters concerning two-dimensional electrophoretic analysis of medically and agriculturally important microorganisms.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: UMIST, Manchester

Contract no.: BAP - 0029 - UK

Project leader: M.S. BECK

Scientific staff:

(Project Manager : W.J. MARTIN - Ext. : 2588/2123)

Address:

Manchester Biotechnology Centre
UMIST - Dept. of Instrumentation
and Analytical Science
P.O. BOX 88
UK - MANCHESTER M60 IQD

Telephone no.: +44.61.2363311 EXT. 2589/2123

Telex no.: 666094 UMIST G

Other contractual partners in the joint project:

F.M. Pohl, Universität Konstanz
J.E. Bateman, Rutherford Appleton Laboratory (Didcot)
R. Massen, T. C. I. D. P. (Konstanz)

Title of the research activity:

Automation of DNA sequencing.

Key words:

Automation of DNA sequencing for Genomic charting

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

To accelerate biotechnology data-capture by automating aspects of DNA sequencing processes. The front-end molecular biology is being improved and a machine vision directed robot is under construction to automate bacterial plaque/colony selection, harvesting and culture inoculation. An improved direct blotting electrophoresis apparatus will be built with a specially constructed multi-wire proportional counter - for direct imaging of blotted radiolabelled bands. Deconvolution and smoothing MWPC image improvement methods will be developed together with a knowledge-based sequencing software package

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Software programmes to separate, compute the co-ordinates of and automatically classify bacterial "plaques" on petri dishes are being devised. This information will be passed to a robot X-Y-Z system to automatically select white plaques (from a lawn of blue and white plaques) and transfer them individually for separate culture inoculation. The robot is being produced in conjunction with collaborators at TCCIDP (who are developing the robot's vision system) and a major European instrument manufacturer. Image processing and knowledge based sequence abstraction software for the MWPC is also being developed.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

METHODOLOGY A MAGISCAN 2 computer has been programmed to identify white plaques on a lawn of blue and white plaques and to distinguish plaques from hairs, bubbles condensation, bacterial colonies defects in the petri dish and the dish edge. The positional coordinates of each event of interest can be computed. A robot system to pick candidate plaques has been designed and the system patent protected. Software was written in PASCAL on a MAGISCAN 2 image processing computer and the system presently has four phases as follows:-

(a) Image capture A simple black and white Vidicon camera was positioned above a sample petri dish which itself was illuminated from below. An image consisting of 512 by 512 picture elements or pixels is captured from the camera by the MAGISCAN 2 hardware. Each pixel is stored as a 6 bit number in the range 0 to 63 which represents a digital approximation to the brightness at that point.

(b) Segmentation To minimise the shading error, regions or windows of a petri dish image, large enough to contain mostly lawn yet small enough to have a low shading error, were histogrammed. The histograms were parametrized by the fitting of a Gaussian curve with the distribution mean being taken to be the histogram mode. Then the curve shoulders are located and from these a low and a high threshold computed. The value of 2.55 sigma was chosen as this cuts at the 1% tails of the Gaussian distribution and would eliminate 99% of the lawn points. The image was scanned with the two local thresholds obtained in this way and any points brighter than the high threshold or darker than the low threshold are stored away as being likely plaque pixels.

(c) Feature extraction The number of features is an important decision, as too few will result in a high error rate, whereas too many will result in sluggish program performance. Of the nine features finally selected four relate directly to harvesting. These are the x,y plaque centre co-ordinates and the x,y extent, that is the size of the enclosing rectangle, of each item. The remaining five were selected on the basis of estimated disciminating power and are as follows: (i) area (ii) form factor (iii) mean shade (iv) core shade (v) standard deviation in shade

(d) Classification The classifier implemented compares the distances in 7 dimensional feature-space, computed by a Euclidean metric, between the item being classified and each of four class templates held in a database. The item is allocated to the class of the closest template, and a figure of merit dependant on the relative distances is computed. The features are normalised according to the range of values encoutered to avoid weighting any feature unfairly. The templates are stored in the database which the user must initially generate by operating the recogniser program in a manual mode. The class templates are simply the mean of all the features of each class.

RESULTS Preliminary results using the recogniser programmme on a sample dish other than the one it was trained upon provide the following information with regards to execution speed and accuracy:- The segmentation pass on a 512 by 512 6 bit pixel image took 60 seconds. Feature extraction on a 256 by 256 pixel subwindow took an additional 60 seconds. In the trial performed, 64 items were detected and classified. This took a further 1 to 2 minutes. On a trial run classification was monitored and 4 mistakes were noticed, resulting in 60/64 or 93% accuracy. It is important to point out that none of these mistakes involved the misclassification of a white plaque and so were of the least serious kind. In a second trial the MAGISCAN 2 was linked to an X-Y plotter which successfully

demonstrated successive movement of a pointer to imaged and selected white plaque coordinates.

A design excercise to identify a suitable X-Y-Z cartesian coordinate system for plaque harvesting from petri dishes and culture inoculation in discrete containers has been carried out resulting in the selection of the combined Unimatic table and rotating disc system illustrated below. Provision has been made for extending the robot to a continuous operation mode.

Several systems to prevent contamination and cross-contamination of the plaque harvesting probe have been developed and patent protection sought for the technology. (see attached patent details)

DISCUSSION

The two major problems involved in the development of an APSCIR system have been largely resolveed, viz plaque selection, and discrete plaque harvesting without contamination. A vision system and X-Y-Z cartesian coordinate robot are being designed to implement these solutions. A major European Instrument Manufacturer has become involved and it is hoped to produce a first working robot prototype within 12 months.

Figure 1. Overall Design of the automatic plaque selection and culture inoculation robot - The APSCIR System

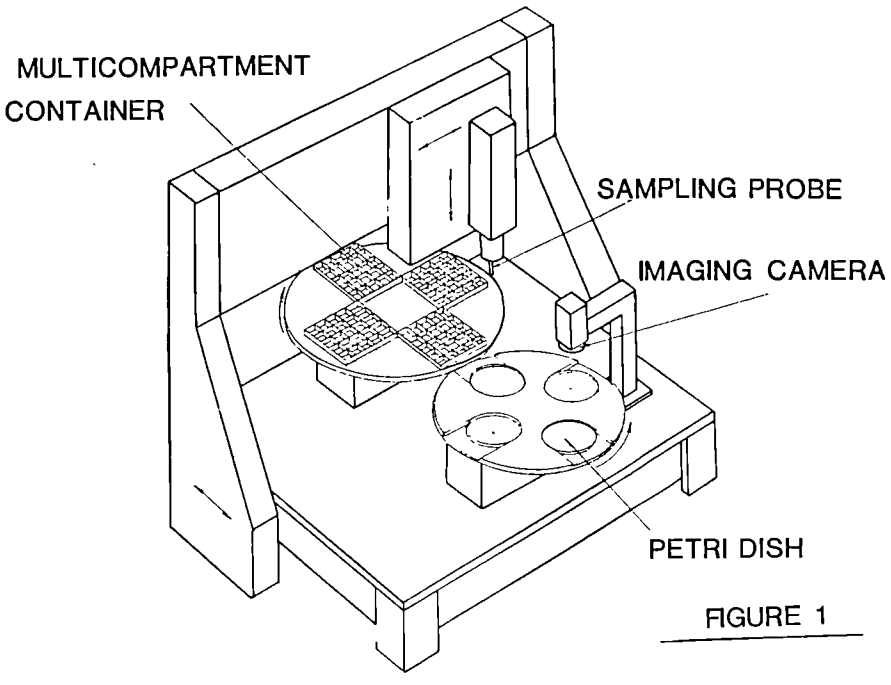


FIGURE 1

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

1. PUBLICATIONS IN SCIENTIFIC JOURNALS

William J Martin and R. Wayne Davies. 1986.
Automated DNA Sequencing: Progress and Prospects.
Bio/Technology, 4 : 890-895.

Martin.W.J. 1987.
Charting the Human Genome - A design role for the life-science laboratory instrument engineer. J.Phys.E. Commissioned ISAT review (in press)

Martin.W.J 1987 **Instrumenting Access to the Human Genome** Proceedings Biotec87. Online International

Courtney P 1987 **A Vision System for an Automatic Plaque Harvesting Machine** Joint INSTMC/SERC Control and Instrumentation Research Symposium 6-7 April 1987 Cambridge. Abstracts. The Institute of Measurement and Control

Martin W.J. and Courtney P. 1987 **Automation of DNA Sequencing: Microlitre Reagent Manipulation and Automatic Plaque Harvesting** Proceedings, EEC Sectoral Workshop, Capri, Italy.

2. Internal Reports

Courtney P 1986 **Automation of Plating out and Culture Inoculation** Feasibility Study Report. DIAS UMIST.

3. Patent Applications

Martin, W.J., **Sampling of Microbiological Material.** U.K. PCT Patent Application No 8605140 of February 27, 1987.

Martin, W.J., **Resilient Strip** U.K. Patent Application No. 8615048 of June 20 1986.

4. Degree Thesis

Courtney P 1987 **A Vision System for an Automatic Plaque** Harvesting Machine MSc Thesis UMIST

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Exchange of Materials Petri dish samples, of the type to be imaged by the plaque selection device, were supplied to TCCIDP and nylon matrix materials employed in blotting were presented to RAL to aid in the design of the MWPC. Flexible matrices with radiolabelled DNA bands were prepared for RAL by the Manchester Group and by Dr Stephan Beck (formerly of University of Constance). An extensive technical literature and technical specification exchange took place between UMIST, RAL, TCCIDP and University of Constance regarding the joint designs of the automatic sequencing systems.

Exchange of Staff Mr D Q Xu and Mr M Tso have worked on MWPC experiments at RAL to optimise band resolution. Mr Courtney will be visiting TCCIDP shortly to familarize himself with the vision system technology under development in Constance and test the software developed at UMIST. He will transfer the computer and camera concerned to UMIST for use on the APSCIR system pictured in Figure 1.

Joint Experiments Special sequence and RFLP radiolabelled gels and blots have been produced in Manchester for imaging in RAL with a view to determining suitable radio-markers and gel conditions for MWPC imaging. Image data produced by the MWPC in RAL has been modified at UMIST, to improve the resolution, by applying deconvolution and smoothing routines to the raw data. Software produced in UMIST for plaque selection will be incorporated in the vision system under development at TCCIDP.

Joint Meetings Dr Martin, Dr Browne and Mr Courtney have visited Constance for meetings at UC and TCCIDP with Professors Pohl and Massen and their groups.. A second meeting was held in Italy (Professors Massen and Pohl with Dr Martin and Mr Courtney) after the EEC sectoral workshop in May 1987. The minutes of these two meetings are attached. Dr Martin, Mr Courtney Mr Hyslop (UMIST) and Professor Massen (TCCIDP) have had a joint and several separate meetings with the European Instrument Manufacturer interested in producing and marketing the APSCIR robot.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: University Konstanz

Contract no.: BAP - 0135 - D

Project leader: F.M. POHL

Scientific staff: J.D. Hoheisel

Address:
Universität Konstanz
Fakultät für Biologie
Postfach 5560
D - 7750 KONSTANZ

Telephone no.: +49.7531.882134

Telex no.: 733359 UNIV D

Other contractual partners in the joint project:

M.S. Beck, U. M. I. S. T. (Manchester)
J.E. Bateman, Rutherford Appleton Laboratory (Didcot)
R. Massen, T. C. I. D. P. (Konstanz)

Title of the research activity:
Automation of DNA sequencing.

Key words:
Direct blotting electrophoresis, Nucleic acids, Sequencing data collection, Exonuclease III

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Acceleration of data-capture in biotechnology by automating different aspects of the DNA sequencing processes. The front-end molecular biology and biochemistry is being improved and a machine vision directed robot is under construction to automate colony selection, harvesting and culture inoculation. An improved direct blotting electrophoresis apparatus with a specially constructed multi-wire proportional counter will be built for direct imaging of radioactive bands. Deconvolution and smoothing MWPC images together with knowledge based sequencing software will be developed.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The biochemistry for the preparation of suitable DNA for sequencing will involve the digestion with enzymes for the creation of ordered subclones, the preparation of single stranded DNA from plasmids with helper phage or with exonuclease treatment. Improved designs for the direct blotting electrophoresis apparatus in collaboration with the development of a new multi-wire proportional counter should simplify the automatic collection of quantitative data from sequencing gels. Beside software developments, the testing and feasability studies of different sequencing schemes are actively persued.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

METHODOLOGY

The enzyme exonclease III from E.coli provides a very convenient tool for the generation of very defined single stranded DNA. Such single stranded DNa can be used for the preparation of unidirectional deletions of predetermined size. This allows the cloning of ordered subclones which simplyfies considerably the sequencing of larger DNA fragments.

Since enzymatic sequencing with DNA-polymerase requires single stranded DNA and primer hybridisation, the use of exonuclease III for generating the proper substrate has been studied. Since the use of this enzyme can be of considerable help in different aspects of biotechnology the enzymology has been studied in detail and the gene has been cloned into a plasmid.

First experiments with helper phages are encouraging for obtaining single stranded template molecules from plasmid DNA.

The instrumental development with respect to the direct transfer (or blotting) electrophoresis was mainly into two directions. One was to settle on certain dimensions which are important for the automation of the consecutive steps, like reading of the data by the multi-wire proportional counter. The other was the improvement of the discontinous transport of the conveyor belt by an improved way to establish the tension of the nylon net.

In order to gain flexibility in the on line treatment of the blotting membrane after and/or before the transfer electrophoresis, a prototype which includes a number of solution modules has been built in collaboration with Prof. M. Eigen as shown schematically in Figure 1.

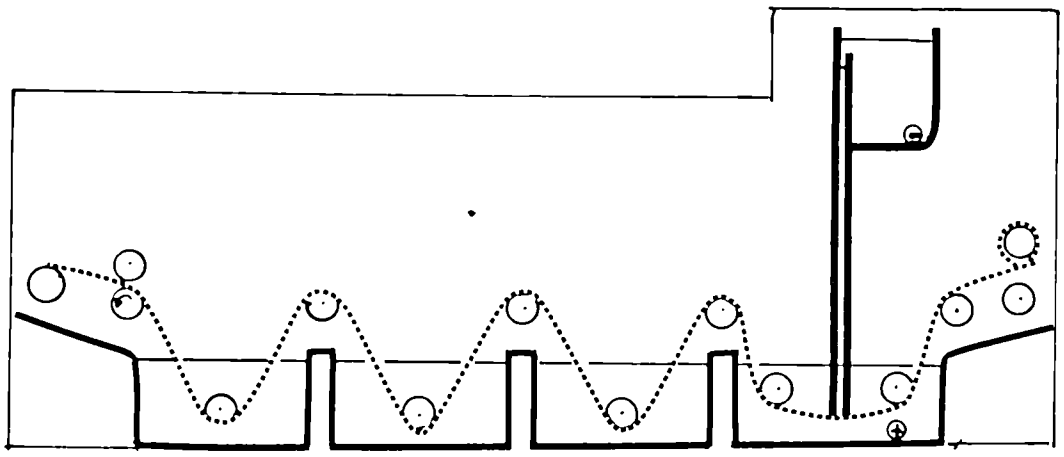


Figure 1: Schematic drawing of a direct blotting electrophoresis apparatus with three solution modules integrated.

The automatic reading of sequencing gels is at present done by using autoradiographic examples, which are digitized by a simple reflection reader, steered by a commercial X-Y plotter. This allows to gain experience in the use of different alogarithms for the extraction of reliable sequence data from the direct blotted sequencing runs. The built up of a library of sequencing errors which can occur in reality seems to be an important prerequisite in the development of "expert sequence reading systems".

RESULTS AND DISCUSSION

An integrated approach to the automation of sequencing of nucleic acids, starting from the prepartion of individual subclones with suitable inserts, via DNA-preparation, sequencing reactions and electrophoretic separation to the automatic evaluation of the experimental data by an "expert system" using a modular design strategy appears as a very promising way to simplify this important task.

The different strategies of improving the biochemistry do require rather extensive enzmatic investigation in order to evaluate the importance of external variables on the experimental outcome. In the case of exonuclease III the dependence on the temperature, salt concentration, pH and DNA concentration was studied by time dependent product formation (J.Hoheisel, in preparation).

Different problems encountered with different designs of the direct blotting electrophoresis apparatus appear now to be solvable. A major emphasis will be to provide interested groups with a simple but reliable design in order to obtain a broad basis for experimentation. Since the method will be of value not only for sequencing, but also for mapping purposes, considerable efforts are still devoted to improve the basic design.

The ease of an automatic reading of sequencing runs depends largely on the quality of the sequencing reactions, whereby the quality of the DNA-preparation is a major factor. Every improvement in the chemistry and/or biochemistry of the sequencing reactions will therefore simplify the automatic reading process. Here the use of different enzymes will be of help. The development of vision systems is already of considerable help in other parts of this project and will be persued with high priority for sequencing.

The integration of real sequencing projects into the biochemical and instrumental developments should give an important feeback with respect to the different options possible and their implementation within the system. Therefor it is planned to supplement this project with sequencing efforts directed towards DNAs of biotechnological importance.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

1. PUBLICATIONS IN SCIENTIFIC JOURNALS

Jörg D. Hoheisel and Fritz M. Pohl (1987) Searching for Potential Z-DNA in Genomic Escherichia coli DNA.
J. Mol. Biol. 193, 447-464.

2. INTERNAL REPORTS

Fritz M.Pohl (1987) Direct Transfer Electrophoresis.
Proceedings of EEC Sectorial Workshop, Capri, Italy

3. PATENTS

Fritz M. Pohl (1986) "Electrophoretic apparatus employing a collecting belt moving in contact with a gel" US-patent 4-631-120 (Dec.23rd,1986)
Fritz M. Pohl (1986) "Method in which elementary particles electrophoetically migrate through a gel onto a collecting surface of a moving belt."
US-patent 4-631-122 (dec.23rd,1986)

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff	Yes	
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

Exchange of Materials:
Materials used for direct blotting electrophoresis, like the nylon nets used for the conveyor belt have been given to RAL. Petri dish samples and autoradiographs have been given to TCCIDP for examination in vision systems.

Exchange of Staff:
A former coworker, Stephan Beck, established up to now the contacts with UMIST. Detailed discussions with G. Tappen of RAL in Constance about specifications of MWPC.

Joint Meetings:
Dr.Martin, Dr.Brown and Mr. Courtney from England attended a meeting here in Constance (Sept. 1986.) familiarizing themself with the possibilities at UC and TCCIDP.
A further meeting was held in Capri after the EEC sectoral workshop (Courtney, Martin, Massen and Pohl).
Next meeting planned for end of September in Manchester.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Rutherford Appleton Lab., Didcot Contract no.: BAP - 0035 - UK

Project leader: J.E. BATEMAN
Scientific staff:

Address:
Rutherford Appleton Laboratory
Chilton
UK - DIDCOT, OXON OX 11 OQQ

Telephone no.: +44.235.21900 EXT.6262/6614

Telex no.: 83159

Other contractual partners in the joint project:

M.S. Beck, U. M. I. S. T. (Manchester)
F.M. Pohl, Universität Konstanz
R. Massen, T. C. I. D. P. (Konstanz)

Title of the research activity:
Automation of DNA sequencing.

Key words:
Digital, Autoradiography, MWPC, DNA, Sequencing

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

To accelerate biotechnology data-capture by automating aspects of DNA sequencing processes. The front-end molecular biology is being improved and a machine vision directed robot is under construction to automate bacterial plague/cclony selection, harvesting and culture inoculation. An improved direct blotting electrophoresis apparatus will be built with a specially constructed multi-wire proportional counter - for direct imaging of blotted radiolabelled bands. Deconvolution and smoothing MWPC image improvement methods will be developed together with a knowledge-based sequencing software package.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

To redesign the RAL MWPC autoradiographic system to accommodate the DNA blotting matrices produced by University of Constanz for Electronic imaging. To institute production of this system. To redesign the computer interface hardware and software of the original system to permit the use of the IBM PC/AT in place of the Apple II. To manufacture the new interface and establish a working data-capture system based on the IBM PC/AT. To carry out tests to show the compatibility of the blotting matrices and the MWPC.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

METHODOLOGY

The work program at RAL during the first year of the contract has concentrated in 2 main areas: design of the special MWPC auto-radiographic system to accept the nylon blotting electrophoretograms and the assembly and programming of the computer system which controls the MWPC and displays the data. In addition we have carried out some essential tests of the compatibility of the nylon mats and the MWPC.

(a) The MWPC

After discussion with the other contractors we have progressed through a design sequence which finds us at the present time with a definitive version of the proposed apparatus. This is designed to accept a roll of dried blotting matrix from the electrophoresis process and digitise it into the computer with minimal attention from an operator. The manipulation of the matrix within the gas space of the counter has been entirely motorised so that complete computer control will eventually be possible.

The commencement of manufacture has been delayed because the necessary finance for the project has not been forthcoming. However, we anticipate that this problem will be solved in time to avoid any significant slippage in the overall programme.

(b) The computer

The IBM PC/AT has been purchased and commissioned with a Digisolve graphics system to display the images. A new interface card for the IBM PC/AT has been produced and commissioned. A completely new software system for data-taking and display has been written and commissioned by connecting it to an old MWPC system. The software gives a 1024 x 512 digital image format resident in the AT memory with a maximum pixel count of 256. When a pixel is full the image is stored on the fixed disc and concatenated with any further data sets at the end of the exposure. One half of the image is displayed on the Digisolve at a time. Fast histogram routines are included to give sections through the image.

(c) Blotting matrix tests

Using a previous MWPC system it has been possible to image some DNA blotting matrices supplied to us by Dr S Beck at MRC Molecular Biology Lab. Cambridge. The handling of the nylon mats has not proved difficult and the problem of discharging the positive ion currents has been solved by either coating the mat with a thin layer of carbon or covering it with a nickel mesh.

RESULTS

The computer system is essentially completed and has performed to expectation. Data can be accumulated at rates approaching 12 kHz which is much higher than we have ever observed from a DNA plate. The data capture, storage and display software (and hardware) have been thoroughly tested.

The tests with the blotting matrices produced no unpleasant surprises and there is every reason to believe that the mechanical design will cope with the observed properties of the nylon mats. A fine nickel mesh laid on top of the nylon lost no observable efficiency for ^{35}S beta particles and gave stable operation of the MWPC (ie discharged the ion current).

The conceptual mechanical design is now complete and ready for manufacture.

DISCUSSION

The progress on all fronts has been satisfactory. The computer system is essentially complete and ready for connection to the hardware. The conceptual mechanical design has now been complete for some time as have been the plans for the production of the front-end electronic systems. However, the funding expected from the SERC Bioengineering Directorate has not yet materialised. This has required that we reorganise the programme to meet the long term objectives. We nevertheless expect to be able to deliver an MWPC system to UMIST against the original timetable.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

1. No publications.

2. Introduction to the RAL/UMIST MWPC Autoradiography System. (User Guide).

3. No patents

4. No theses.

TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	No
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Exchange of Materials: Samples of various types of electrophoretic media labelled with beta emitters have been supplied by UMIST and other centres for use in our existing autoradiographic system. This in turn has produced data for the analysis software package being developed at UMIST. Collaboration is close so that these exchanges amount to joint experiments.

Formal meetings are held quarterly to review the progress of the RAL contribution at which a representative from UMIST attends. Informal visits are exchanged several times a year when discussions seem to be required.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Steinbeis-Stiftung f. Wirtschaftsförderung Baden-Württemberg

Contract no.: BAP - 0036 - D

Project leader: R. MASSEN

Scientific staff: A. Kolb

Address:
Transfer Centre for Image Data Processing - TCIDP
Reichenaustrasse 81c
D - 7750 KONSTANZ

Telephone no.: +49.7531.57502

Telex no.: 733355

Other contractual partners in the joint project:

M.S. Beck, U. M. I. S. T. (Manchester)
F.M. Pohl, Universität Konstanz
J.E. Bateman, Rutherford Appleton Laboratory (Didcot)

Title of the research activity:
Automation of DNA sequencing.

Key words:
Machine vision, Culture harvesting, Robot, Image processing

Reporting period: Juli 1986 - Juni 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The techniques of DNA sequencing are of central importance in modern biotechnology. The present manual methodes are tedious and time consuming and prohibitive to the sequencing of large genomes. Under the joint project "Automation in DNA sequencing" two groups in England and two in Germany work together to automate important steps in the SANGER and in the MAXAM-GILBERT DNA-sequencing method. The TCIDP provides its expertise in developping fast and low-cost machine vision hardware for a vision controlled plaque and culture harvesting robot involved in several of these automating steps.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Development of low-cost VME-BUS based digital image processing hardware for real-time feature extraction from culture grey-level images taken with a CCD-matrix camera. Evaluation of colour cues using a 1-chip, CCD-matrix colour camera.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

3.1 Methodology

Based on current own work in industrial real-time symbol extraction from grey-level images taken with CCD-array cameras, a VME-BUS vision system was developped for the processing of images from petri-dishes containing different sort of cultures. This system uses a fast preprocessing pipeline composed of two large kernel programmable real-time convolvers for horizontal and vertical edge detection, fast look-up tables for the computation of edge gradient magnitude and orientation and a 2-D 2-stage recursive neighbourhood processor for noise-free edge contour thinning.

A colour CCD-camera is used for near real-time colour cue extraction using fast look-up table based colour system transformation and colour pixel classification.

The vision controlled picking robot (Fig.1) is selecting cultures and plagues based on two sets of features:

1. geometric features like centroid, shape, connectivity etc. using edge contour images.
2. grey-level and colour intensity features for discriminating between cultures and plagues of nearly identical shapes

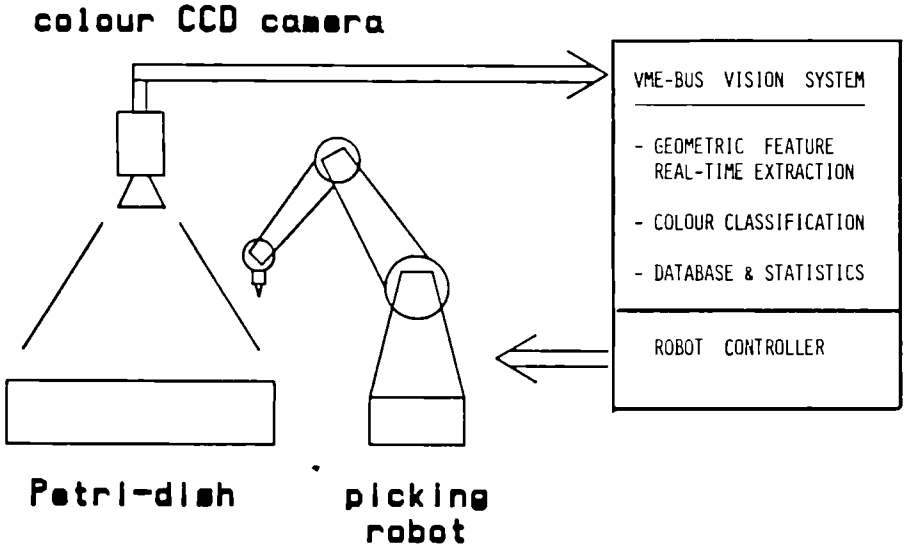


Fig. 1

3.2 Results

A VME-BUS vision system with a 68000 processor was developped and a 4 board real-time preprocessor for thinned edge contour extraction inserted as a pipeline between the video ADC and a SRAM-based image frame grabber. Due to the large kernel of 8 by 8 pixels this preprocessor works very satisfactorily in extraction contours from plagues and cultures even with very unequally illuminated and poor contrast petri dishes (Fig.2). The rather unsharp culture edges can be extracted as 1-pixel wide noise-free contours in real-time, i.e. at a pixel clock rate of 10 MHz. Different convolution kernels were tested and optimized for petri-dishes with blue and white cultures illuminated at a flat angle, the camera being placed in the dark field orthogonal to the gel surface.

First results in classifying cultures using a 1-chip CCD-colour camera are giving promising results. The rather poor colour channel seperation of these cameras and their poor spatial resolution still leed to dubious results for low saturated pixels (near black or near white). This phenomenon has to be studied further on.

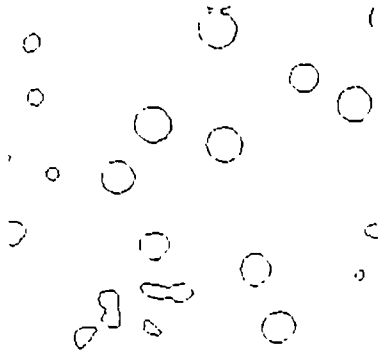


Fig. 2a

Fig. 2b

3.3 Discussion

The locating of plaques and cultures for a vision controlled harvesting robot using both geometric features and colour/grey-level intensity cues prooves to be a sound approach. The very performant 2-D preprocessing using a real-time pipeline processor based on 4 VME-BUS boards however leads to a production cost level tolerable only for large industrial systems.

Based on the grey-level feature extraction algorithms currently under development at UMIST we plan to reduce this preprocessing to a less expensive near-to-real-time scheme. The image frame grabber is enhanced by an on-board 68020 32-bit processor. This "intelligent frame grabber" can easily be parallelized so as to reach any required degree of performance (number of plagues analyzed per second) without the need of developping new specialized hardware. A german manufacturer of biotechnology equipment has already decided to use this approach in the near future.

The investigation in robust colour feature extraction will emphasize the problem of low saturated pixel classification and analyze in more detail the performance of low-cost colour CCD cameras.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV 1.

Massen, R.
Simnacher M.
Janke, P.
Rösch, J.
Kehrle, K.

Real-time color and greylevel preprocessing for the control of a biotechnical robot. (in german) to be presented at the 9. DAGM-Symposium 29.9.-1.10.87, Braunschweig

IV 2.

Massen, R

Real-time symbol extraction from grey-level images (in german)
3rd workshop GI Fachgruppe 1.2.6 "image analysis and understanding", Munich 25.5.87

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff	Yes	
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

A low-cost VME-BUS vision systems has been assembled and will be delivered to UMIST at the end of the first period. A researcher from UMIST is introduced to the hardware and software during a several days stay in Constance.

Two joint meetings have been held during the first period:

a) a 2-day workshop has been organized in Constance by the University of Constance and by the TCIDP the 22/23 September 1986 joining 11 researchers from the UK and Germany working on the project.

b) a meeting was arranged at the BAP-session in may 87 in Capri to discuss the current state of research and to set up the goals for the 2nd years period.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Dutch Royal Academy of Science Contract no.: BAP - 0005 - NL

Project leader: M.A.A. SCHIPPER
Scientific staff: K.W. Gams, J.A.J.M. Stalpers, G.J. Stegehuis, D. Yarrow, J. Smith (Univ. of Amsterdam)

Address:
Centraalbureau voor Schimmelcultures
P.O. BOX 273
NL - 3740 AG BAARN

Telephone no.: +31.2154.11841

Telex no.:

Other contractual partners in the joint project:

J. de Brabandère, S. P. P. S. (Brussels)
D. L. Hawksworth, C. A. B. (Kew)
D. Claus, D. S. M. (Göttingen)
N.J. Van Uden, Gulbenkian Institute of Science (Oeiras)

Title of the research activity:

A European network of microbial culture collection databanks : integrated catalogue.

Key words:

Databank, Microorganisms, Culture collection, Networking, MINE

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The establishment of a Microbial Information Network in Europe (MINE) on data in Culture Collections. National cq regionally coordinating Information Centers, containing databanks of one or more Collections, form the backbone of the Information Network System. The knowledge based microbial information, which content develops rapidly, necessitates to locate the Centers close to the mainnational Culture Collection(s). The On-line Information Service of MINE will be supported by an integrated catalogue.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

1. Choise of hardware and software for compatible and uniform database management and retrieval in each node and for network-communication.
2. Developing of a uniform format for microbial data on fungi, yeasts and bacteria, for constructing a common Data Definition.
3. Input of Dutch catalogue information for on-line use. Developing a microbial data acquisition PC-oriented workstation for affiliated Culture Collections.
4. Preparation of thesauri for fungal names, chemical compounds, enzymes etc.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

METHODOLOGY.

From the start the United Kingdom and the Dutch nodes agreed, as a matter of hardware and software compatibility, to use VAX-hardware and BASIS-software for database management and retrieval, while networking are provided by DECNET. Lateron the German node followed this initiative.
In the Netherlands the CBS in Baarn is the Dutch Coordinating Center for all microbial data, which is stored in a Micro-VAX/BASIS system. Affiliated collections use IBM PC/AT3 and ORACLE for their database management, and DECNET-DOS for communication purposes.
During several meetings of the MINE participants, agreements on the principal features of the format were reached. The field definitions were elaborated in general terms, so that an implementation was possible. Subsequently a BASIS-DDL was developed in the Netherlands and distributed to the other MINE members.
The previous CBS catalogue file was converted from a sequential ASCII file using the VMS editor to introduce field tags for BASIS FORMS. An instruction manual for a data typist is written for further input from the CBS-card system, using the SCREEN modules of BASIS. Affiliated collections in the Dutch network are now using IBM PC/AT3 workstations

for yeast and bacteria data acquisition. These data are integrated into the MINE node at the CBS in Baarn with FORMS.

RESULTS

The conversion of the Dutch Catalogue was completed at the end of December 1986. The latest updates were entered in March 1987. A report form was written so that the final double column print could be produced directly on the laser printer.
DECNET has been installed on the Micro-VAX and a connection with DATANET1 will be established soon.
For smaller collections like the CBS yeast division housed in Delft, an ORACLE yeast databank is implemented on a IBM PC/AT3 microcomputer. Up- and down load procedures were written to transfer data from the IBM PC/AT3 to the Micro-VAX and vice versa.
At the Biotechnica in Hanover '86 and the 4th ECB in Amsterdam '87, the on-line accessibility of the Dutch MINE node was demonstrated. At the ECCO-6 meeting in Budapest '87, the state of the art of MINE was introduced and discussed.
Following the agreements on the formats for fungi,yeast and bacteria a publication on MINE was submitted this year: "Structuring strain data for storage and retrieval of information on fungi and yeast in MINE, the Microbial Information Network in Europe".
Thesaurus files have been produced for nutrient media by a compilation from the ATCC and DSM catalogues, for enzymes using the standard enzyme nomenclature and for geographical data.

DICUSSION

Studies are underway:
- to improve the usersfriendly accessibility of MINE by way of a Menu-driven Query System.
- to reduce the on-line costs through the use of a Referral Information System located in one host computer or distributed on the national MINE nodes.
- to improve the networking capabilities between the MINE nodes.
- to investigate the service capabilities of MINE as an Information Center for Microbial data.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

1. Exchange of materials: formats
   DDL´s
   Manuals
2. Exchange of staff: advising and instruction of staff members of participating nodes.
3. Joint experiments: on-line access demonstration of Dutch node and U.K. node and vice versa.
4. Joint meetings: regularly general assembly MINE meeting
   " MINE computer experts meeting

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: L'Etat Belge (S.P.P.S.) Contract no.: BAP - 0134 - B

Project leader: J. DE BRABANDERE

Scientific staff: Mr. CASTELEYN, Prof. DE LEY, Mr. FALISSE, Prof. HENNEBERT, Dr. JANSSENS, Dr. NOLARD, Mr. VUNCKX (= team 86/87 of the "Belgian Coordinated Collections of Microorganisms")

Address: Services de Programmation de la Politique scientifique
8, rue de la Science
B - 1040 BRUXELLES

Telephone no.: +32.2.2304100

Telex no.: 24501 PROSCI B

Other contractual partners in the joint project:

N.J. Van Uden, Gulbenkian Institute of Science (Oeiras)
M.A.A. Schipper, Centraalbureau voor Schimmelcultures (Baarn)
D.L. Hawksworth, C. A. B. (Kew)
D. Claus, D. S. M. (Göttingen)

Title of the research activity:

A European network of microbial culture collection databanks : integrated catalogue.

Key words:

Microorganisms, Culture collections, Catalogue (integrated), Database (distributed), Network

Reporting period: April 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The establishment of an European computernetwork on microbial strain information, called "Microbial Information Network Europe".
The major purposes of the distributed database system are to facilitate a fast and reliable (on-line) exchange of information among culture collections from the different participating countries on the one side, and between MINE-nodes and the potential users on the other side.
The so called electronic integrated catalogue which will be the result, aims to become a practical working instrument for as well fundamental as application oriented microbiologists working in institutions or companies allover the world.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The main objective for the past period was the setting up of firm foundations to construct MINE on. It was generally understood that a crucial and basic point for the future success of MINE will be constituted by the mutual compatibility between different MINE-nodes, and this on different levels. In these lines several meetings have been organised with the aim to develop on the one side compatible and interchangeable formats and on the other side compatible hard- and software which will facilitate mutual communication with(in) MINE.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Methodology

As stated in point II a lot of meetings were organised in view of the development of joined agreements on different subjects.
In the general MINE-meetings the tasks were divided and decisions or resolutions were taken upon the results of the jobs that had been worked out by different subgroups of MINE-collaborators.
The Belgian MINE-node has actively collaborated in these meetings, and has prepared multiple worknotes as inputs for these workshops.

2. Results

a. Public Relations material

- A MINE-logo (fig. 1) was designed in August 86 by the Belgian Science Policy Office.
  This logo permits the boosting of the notority and the recognisability of MINE. As such it appears yet on the MINE-poster designed and printed in Belgium and on the catalogues of some of the MINE-collections. It's use and dispersion will

certainly increase in the future.

- A MINE-brochure - wherein a special part is focused on the Belgian Collections of Microorganisms - was drawn up under the coordination of the Dutch MINE-node; and has been distributed yet in different international meetings. Adaptation and uniformisation of this brochure is actually worked upon.

fig. 1

b. Formats

In order to achieve compatibility between the different data-bases and national nodes, a consensus was reached about a common format for introduction and storage of data in different fields.
This format had to be flexible since frequent updating will be necessary due to new data resulting from further research on the strains, and due to new fields resulting from changes in the areas of interest of the users.
A subset of fields was extracted from the full-format resulting in a minimal data-set (MDS) comprising all classical catalogue-data supplemented with the indication of the areas in which more information can be obtained. This MDS will unlimitedly be available through the network, directing each user to the appropriate cultures and collections for his demands.
The content of the remaining fields of the format, comprising the actual research data from literature and more especially from own unpublished research, will only be available on request.

Within these lines, two working groups - one for fungi and yeasts and another for bacteria - have obtained a consensus on formats.
Publication of the formats is foreseen in the near future.

c. Computerrelated aspects

The MINE-computerexperts have been working on the technical implementation of MINE; that's to say they've started the assessment of two alternative options. The Dutch and Belgian experts are actually dwelling on the feasability of distributed databases with a referral system (the original idea agreed upon), while the German node investigates the so called 1-computersolution.

As the Belgian subnodes haven't acquired actually their definitive hard- and software a call for tender was made.
Here upon nine offers were received, which are actually assessed.

Like all other BAP-contractants will the Belgian MINE-members join the Eurokom electronic mail system.
The appropriate preparations are made.

d. The state of the art of the B.C.C.M.

The Belgian Coordinated Collections of Micro-organisms harbours actually one bacteria- and two fungi service collections. They constitute together an unique stock of microbial genepool and allied know-how.

The LMG-collection (curator Prof. Dr. J. DE LEY, Ghent) holds over 7.000 bacterial strains representing some 125, mainly gram -, genera. It invests special effort in the constitution of a large biotechnological database, an activity which is fuelled by research in different areas as taxonomy (DNA : rRNA and DNA : DNA hybridizations, polyacrylamide gel electrophoresis of soluble and total cell proteins, ...), phytobacteriology and biotechnology (fermentations).

The MUCL-collection (curator Prof. Dr. G.L. HENNEBERT, Louvain-la-Neuve), includes all groups of fungi (over 15.000 strains), especially Hyphomycetes, but also yeasts. Most strains are original, collected from all parts of the world and are of industrial, agricultural, forestal, biochemical, taxonomic and biodeteriorative interest. Research topics cover fundamental an applied mycology (food stock preservation, fermentation abilities, fermented foods, mycotoxins, deteriorating activities, ...).

The IHEM-collection (curator Dr. N. NOLARD-TINTIGNER) comprises nearly 4.000 strains, including filamentous fungi and yeasts of interest for public health; allergenic, mycotoxigenic and pathogenic fungi for man and animals. Research fields covered are : morphology, epidemiology and pathogenicity, fungal allergies and immunology, fungal metabolites, ...

All BCCM-collections provide special services, like : identification, preservation, safe-deposit, information retrieval, contract research, training.

e. Printed catalogue

A printed BCCM-catalogue will be available in the beginning of 1988. It will be edited conform the MINE-guidelines, in case those will be established by time.

3. Discussion

- Where it has been clearly demonstrated that MINE raises high interest by the potential users of it, it is generally felt that there remains still a communicationgap to be filled between MINE (and the CEE culture collections in general) and the demandside. Mailing of an information brochure, combined with a small questionary, to a broad sample of the potentially interested European industrial and institutional community would certainly provide both the crucially needed marketing formation and the attended amplified demands from the outside world.
- The bottleneck for the implementation of MINE is considered to be the technical realisation of the network. The MINE-computerexperts, supported by all other MINE-colleagues, feel that an outside expert should perform a feasibility study on the different alternatives, and this with their active cooperation.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV. 1. Publications in scientific journals, monographs, ...

- Structuring strain data for storage and retrieval of information on fungi and yeasts in MINE, the Microbial Information Network Europe (MINE technical publication 1). In press.

IV.2. Short communications, internal reports ...

- The Belgian Coordinated Collections of Microorganisms - the Belgian nodes of MINE. Book of abstracts p. 93 of the meeting of BAP-contractors on "Culture Collections and genetic engineering of microorganisms", Ioannina, 23-25 April 87. (CEC-edition)

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

A. In the frame of the MINE-project

a. Exchange of material (s)

- Public relations material (poster, brochure)
- Numerous working documents as inputs for the different meetings

b. Joint meetings

- General MINE-meetings
  - . 28 May 1986 (BAARN)
  - . 4 November 1986 (BAARN)
  - . 19 March 1987 (BAARN)
  - . 11 June 1987 (BRUSSELS)
  - . 21-22 september 1987 (KEW)
- BAP-contracter meeting
  - . 23-25 April 1987 (IOANNINA)
- Computer expert meeting
  - . General
    - . 21 May (BAARN)
    - . 3 August (KEW)
  - . Bilateral
    - . 8-9 december 1986 (visit of Belgian Computer experts to Dutch node)
- Format meetings
  - . Bacteria
    - 3 November (DSM)
  - . Fungi-yeast
    - . 19 March 1987 (BAARN)
    - . 8 April 1987 (BAARN)

B. Out the scope of the MINE-project

- Prof. Dr. K. KERSTERS from the LMG (Ghent) participates at the contract BAP-0138 on "Electrophoresis of proteins : Data capture, analysis and construction of databanks"
- Prof. Dr. G.L. HENNEBERT (MUCL) and Dr. N.M. NOLARD-TINTIGNER (IHEM) participate at the contract BAP-0028 on "Development of improvement techniques for the preservation of fungal strains of biotechnological importance".

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: C. A. B., Kew Contract no.: BAP - 0004 - UK

Project leader: D.L. HAWKSWORTH
Scientific staff: R.D. Platt, D. Allshopp, J.C. Gilmore, R. Ibboison, D. Smith

Address: CAB - International Mycological Institute
Ferry Lane
UK - KEW, SURREY TW9 3AF

Telephone no.: +44.1.9404086

Telex no.: 847964 COMAG G (mark "ATTN CMI")

Other contractual partners in the joint project:

M.A.A. Schipper, Centraalbureau voor Schimmelcultures (Baarn)
J. de Brabandère, S. P. P. S. (Brussels)
D. Claus, D. S. M. (Göttingen)
N.J. Van Uden, Gulbenkian Institute of Science (Oeiras)

Title of the research activity:
A European network of microbial culture collection databanks : integrated catalogue.

Key words:
Culture collection, Microbiology, Databases, Biotechnology, Fungi

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The objective of this project is to produce an integrated computerized catalogue of the holdings of culture collections in Europe by a computer network in order to improve awareness of strains available and facilitate ordering

A node will be based in each participating country, holding collection data of that country in a standard format and of a compatible structure. Establishing the MINE referral database and inputting national data is scheduled to be completed at the end of 1989.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

By the end of 1988, it is intended that the minimum data set (MDS) for all UK collections participating in the UK node will be input. Data wil be transferred either by computer or keyboarded as appropriate to the CAB International VAX 11/780 Computer.

A database for every participating collection is being established, each of which will contain the relevant Minimum Data Set for fungi, yeast, bacteria, etc. Information to be keyboarded will be taken directly from that latest catalogue of that collection. Machine-readable data will be transferred by modification from either individual collections own software or down-loaded from MiCIS, subject to software limitations.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY

(a) MINE UK Node Agreements were sent out to all main collections and some institutions in the UK, following a UK Node Meeting with representatives from those collections. All major UK service collections have now signed this agreement except one small collection which is expected to sign shortly.

(b) The Project Administrator appointed has requested participating collections for copies of their latest edition catalogues, as well as hardware/software information from those collections which already have their data computerised. Replies to these requests are being examined in consultation with CABI Systems Group.

(c) A database is being set up for every participating collection. Fields contained in these databases are those of the agreed MDS's.

(d) Data incorporation will be either by computer methods or keyboarding, as appropriate.

(e) Establishment of mechanisms for electronic mail and data transfer between all participating nodes and formulation of agreements on data availability.

2. RESULTS

(a) The following culture collections have agreed to participate in the UK Node:

NCTC	National Collection of Type Cultures	4 000 strains
NCYC	National Collection of Yeast Cultures	2 000 strains
NCIB	National Collection of Industrial Bacteria	3 800 strains
NCMB	National Collection of Marine Bacteria	1 500 strains
NCPF	National Collection of Pathogenic Fungi	830 strains
NCFB	National Collection of Food Bacteria	2 000 strains
CCAP	Culture Collection of Algae & Protozoa	2 000 strains
NCWRF	National Collection of Wood Rotting Fungi	530 strains
ECACC	European Collection of Animal Cell Cultures	320 strains
CMI	CAB International Mycological Institute	12 000 strains
	TOTAL	28 980 strains

The NCPPB, National Collection of Plant Pathogenic Bacteria, is expected to join shortly.

(b) Data currently held on computers is as follows in participating UK collections:

NCTC	ITL Momentum "Typelink" computer	8" diskette
NCPF	ITL Momentum "Typelink" computer	8" diskette
NCYC	GEC LGC Computer	
NCIB	Prime Computer	
NCMB	Aberdeen Univeristy Press Computer	
ECACC		IBM compatible disk
CCAP		IBM compatible disk

(c) Terminals, lines and laser printer have been installed, at CMI and this has enabled six databases to be set up on the VAX, 3 fungal and 3 bacterial. Keyboarding has begun on the NCFB database and a sample of the computerized data has been sent to NCFB for approval; it is likely to take up to four months to keyboard the whole collection, but this will be the quickest method of computerizing the data in this case, as NCFB will not be on-line through MiCIS for at least six months. Keyboarding will also be the quickest alternative for NCWRF.

(d) As details of collection software are recieved, CABI Systems Group are looking into possibilities of data transfer. Secondly, the possibilities of down-loading from MiCIS are being analysed. As long as machine-readable data can be transferred, it is anticipated that the deadline of 24 months for data entry of all UK collections will be met.

(e) CAB International is linked to IPSS facility, has been accepted to join EuroKom and a password has been issued.

(f) Various exhibitions, conferences and meetings have been attended to promote the MINE project including the International Union of Microbiological Societies (IUMS) meeting in Manchester in September 1986, and the ECE Contractors Meeting, Ioannina, in April 1987. Formal discussions took place with possible users and participants. The UK Node made a contribution to the MINE folder produced by CBS and the MINE project will next be promoted at the Mycological Society of America meeting in Ottawa in June 1987 and the International Biodeterioration Symposium in Cambridge in September 1987.

(g) CAB International Systems Group moved to the CAB International Centre, Wallingford, Oxfordshire OX10 8DE in May 1987; tel. (0491) 32111. The UK Node of MINE will continue to run on CAB INternational's DEC VAX 11/780 computer which has 10 megabytes of memory and 2.7 gigabytes of on line storage; a VAX 8250 has been added to make a VAX cluster, able to accommodate over 100 on-line users simultaneous with access via the packet switching services PSS and IPSS CMI will have eight direct links to the VAX cluster via ETHERNET.

3. DISCUSSION

The work is progressing satisfactorily and targets will be met on schedule provided that the electronic data transfer of data from some collections does not present any unforeseen problems.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV 1 PUBLICATION IN SCIENTIFIC JOURNAL

"Focus on CMI", Dennis Allsopp and Rhonda Platt. MiCIS News Vol.1, No.6, June 1987. (pp. 2-7).

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

Meetings of the MINE contractors including representatives from all nodes have been held in Baarn and Brussels; CMI has had 2-4 delegates at each of these meetings. The next meeting to be held will be at CMI in September 1987. Formats have been exchanged and discussed at these meetings, the MDS and a full format are now agreed for fungi and yeasts; that for bacteria is now in an advanced stage. A working party of computer specialists has been established with the main object of determining the most appropriate methods of networking or storing the MINE database(s); the first meeting of the group took place in April 1987 in Baarn and the second is to be held at CMI in Kew in August 1987.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Ges. für Biotechn. Forschung mbH, Braunschweig
Contract no.: BAP - 0003 - D
Project leader: D. CLAUS
Scientific staff: M. Kracht, W. Lehnberg

Address: Deutsche Sammlung von Mikroorganismen - DSM
Gesellschaft für Biotechnologische
Forschung mbH
Grisebachstrasse 8
D - 3400 GÖTTINGEN

Telephone no.: +49-551-393822

Telex no.: 952667 GEBIO D

Other contractual partners in the joint project:

N.J. Van Uden, Gulbenkian Institute of Science (Oeiras)
M.A.A. Schipper, Centraalbureau voor Schimmelcultures (Baarn)
J. de Brabandère, S. P. P. S. (Brussels)
D.L. Hawksworth, C. A. B. (Kew)

Title of the research activity:

A European network of microbial culture collection databanks : integrated catalogue.

Key words:

Culture collection, Microorganisms, Database, MINE, Network

Reporting period: May 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The objective of MINE is to facilitate a fast and reliable on-line exchange of information among culture collections from the different participating countries on the one side, and between the MINE-nodes and the potential users on the other side. By this way, fundamental as well as application-oriented microbiologists will have access to a very broad scope of available strains and related data of biological and biotechnological importance.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The DSM will be the German node of MINE. A catalogue is published regularly containing information on the available strains. For the reporting period the aim was to computerize these catalogue data and to choose and install a suitable database management system including first practical experience with it.

A cooperation between DSM and national research institutions as well as commercial companies - including data transfer to and from DSM - is aimed.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY

Computer Equipment:

The DSM database will be established on a DEC VAX 8600 (memory: 72 MB, harddisk: 3.2 GB) at the GBF in Braunschweig using BATTELLE's database management system BASIS. At DSM in Göttingen 3 IBM AT 03 and 2 HP LaserJet are used mainly for administrative tasks but also for preparing the input of strain data in the BASIS system.

Computerization of catalogue data:

A KURZWEIL reading machine (scanner) available at the GWDG (Gesellschaft für Wissenschaftliche Datenverarbeitung Göttingen) was used to transform the printed catalogue in machine readable data, because it turned out to be impossible to

convert disks with the catalogue data used with a memory typewriting machine into another format.

2. RESULTS

Computerization of strain data:

The catalogue plus supplements including the more detailed information on media and literature was scanned by means of the KURZWEIL reading machine. The resulting ASCII files are currently edited by means of the word processing system WordPerfect. The correction of all strain data is finished and further information on media and literature will be edited till the end of July.

In a second step the responsible scientists have to check the catalogue data for name changes, false or additional informations etc. This work has been done for the yeasts.

To make use of the computerization of strain data for building up the database a special PASCAL conversion program has been written which scans the catalogue file for keywords. Herewith most of the catalogue information can be automatically assigned to the defined fields of the database management system. This program was successfully tested using some yeast data. Some modifications in respect to bacteria will enable the program to convert this major part of catalogue data.

Database management system:

The BASIS database software was chosen because of

flexibility
interfaces to external programs and data
existence of a PC version (retrieval only)
preference of this system by other MINE members.

It was installed in July 1986. Databases on informations about biotechnology in German speaking countries (BIKE), on enzyme data (BRENDA), and on literature (for GBF members only) have been established.

By means of the slightly changed BASIS DDL (Data Definition Language) from the Dutch node in Baarn it was possible to create a well working test MINE database containing the yeast data gained by the PASCAL conversion program.

National cooperation:

A meeting was held on 22 January at the DSM in Göttingen with representatives of national research institutions and from industry to discuss possible cooperation between DSM and these institutes. Main items were possible database systems others than BASIS, communication problems, and database format. Further meetings are planned.

3. DISCUSSION

An important item of the MINE meetings was the format of the database. The participants agreed on minimal data sets for fungi and yeasts on the one hand and bacteria on the other hand. The full format for fungi and yeasts will be published in July 87. The discussion on the full format of bacteria has just begun. The best solution would be to define as much fields as possible which can be used for information on all groups of organisms.

- The functioning of MINE as a European network is not quite clear. It is generally agreed that each node should hold his minimum data set or even better the minimum data set of all collections. A solution has to be found for more complicated questions of users who want to have an organism with several certain properties. The easiest solution would be to combine the whole information of all collections on one mainframe (or more); so if the contacted node cannot answer the question it will link the users to the mainframe holding all data. Another solution could be a referral system which knows where information is available and will gather the desired information from all concerned nodes. DSM favors the one-computer-solution and would be willing and possibly able to hold the combined full data set.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

M. Kracht and D. Claus: The German Collection of Microorganisms (DSM), a node of the MICROBIAL INFORMATION NETWORK EUROPE (MINE), Biotechnology Action Programme, Meeting of Contractors, Ioannina, Greece, April 1987 (Poster)

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

Meetings:

Name	Place	Date	Participants of DSM/GBF
MINE meeting	CBS, Netherlands	28 May 1986	D.Claus, W.Lehnberg
MINE meeting	CBS, Netherlands	4 November 1986	D.Claus, M.Kracht, W.Lehnberg
MINE meeting	CBS, Netherlands	19 March 1987	M.Kracht, W.Lehnberg
CEC Contractors meeting	Ioannina, Greece	23/25 April 1987	M.Kracht
Computer experts meeting	CBS, Netherlands	21 May 1987	M.Kracht, W.Lehnberg
MINE meeting	PPS, Belgium	11 June 1987	M.Kracht, W.Lehnberg

The Dutch node gave strong support to the development of the BASIS database by providing the data definition language (DDL) and by discussing some general problems with DSM members. Further cooperation concerning menues and help files is aimed.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: C. R. I. M., Montpellier

Contract no.: BAP - 0136 - F

Project leader: J.F. SALLANTIN

Scientific staff: J. Haiech, M. Manard, J.F. Sallantin, R. Terrat

Address:
Centre de Recherche en Informatique de Montpellier
U.A. C.N.R.S. no. 815
860, rue de Saint Priest
F - 34100 MONTPELLIER CEDEX

Telephone no.: +33.67.63.04.60

Telex no.:

Other contractual partners in the joint project:

P.M. Sharp, Trinity College (Dublin)

Title of the research activity:

Research and diffusion for a portable European system of access and analysis of biosequences.

Key words:

Workstation editor, Machines communication, Biosequences, Learning, Homologies

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Research and diffusion for a portable European system of access and analysis of biosequences : Conception and implementation of a computing environment which allows to have access and analyse biosequences in an intelligent workstation (the BIOSTATION) and to transmit or receive operations results or sequences, i.e COMMUNICATE with other computers or laboratories.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Conception of this Workstation on IBM PC, AT or XT :

- Development of the Editor;
- Development of the Communication;
- Specification of the software tools to be implemented in order to obtain a first biostation prototype.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

III.1 METHODOLOGY

The biotechnologies recent developpement leads to a new necessity : provide biologists with the tools required to manipulate sequential objects, i.e. genetic sequences. The BIOSTATION (Workstation dedicated to sequences analysis) can be defined as a set of functionalities allowing to:

a) Represent, generate, consult or modify genetic sequences : Machine-man communication.
Thus, **a sequences editor** has been written using the Microsoft Pascal on IBM PC, AT or XT (for a wide spreading, the computing equipement portability is essential). In order to obtain a pleasant tool, this communication uses an EGA graphic interface and a mouse.

b) Transmit or receive operations results or sequences to/from other computers or laboratories : Machine-machine communication.
Thus, **a communication card** has been realized; it is able to concurrently manage several asyncronous serial channels. This communication board is equipped with its own processor (Intel 80188) and memory (256 Kbytes, RAM). A software environment, under development, allows the coprocessor to manage communication links independently from the host machine and to share critical ressources (disk, screen, ...).
These two main aspects (edition & communication) are, at the present time, original and low cost solutions.

c) Integrate the A.I software to sequences analysis in order to solve two kinds of methods: Learning and plan generation (sight directed mutagenesis). These tools, separately tested at the present time, allow biologists to solve problems such as generalized homologies and patterns recognition in a class of functional homologous biological sequences.

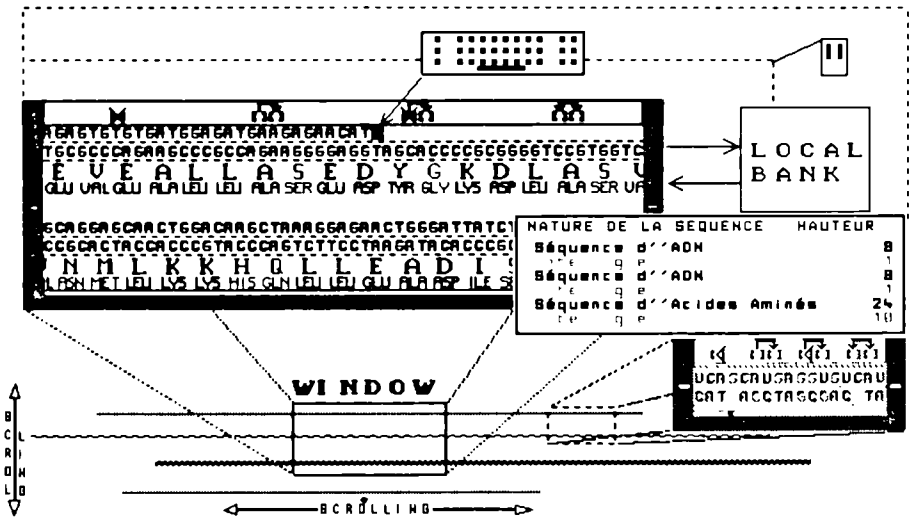
Such tools allow biologists to make methods suitable for the problems they are working out. Nevertheless, the editor is realized in order to allow the classical softwares use, as UWGCG or SASIP (with communication tools).

III.2 RESULTS

A) THE EDITOR

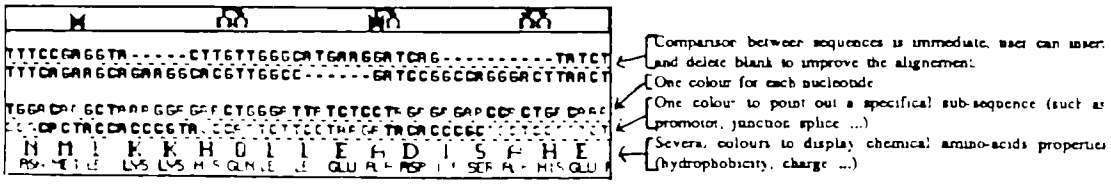
The sequences editor is a tool dedicated to genetic sequences management. Its main characteristics are :
- The objects perception is interactive (their representation is only linear and allow editing functionalities) ;
- Display of sequences features and properties (using application methods or not using them) for a visual analysis;
- A friendly user environment (to manage the different applications dialogue).

Edition functionalities :



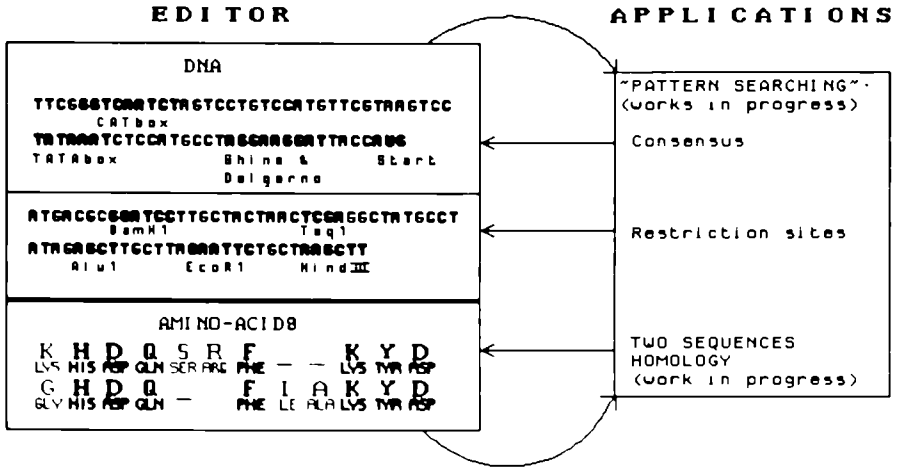
- Modification of window size and shifting ;
- Choice of window parameters (lines type : DNA, RNA, AA, results) ;
- Conception of new sequences (input from keyboard)
- Access to private or public banks of sequences in order to manage existing or new sequences
- Display of several windows on the same screen ;
- Scrolling of the sequences in a window on one or several lines ; standard position, zoom;
- Modification of a sequence : deletion, insertion, cut/paste, exchange of sub-sequence ...

We emphasize on the fact that this editor allows a visual elicitation of the sequences structural properties by the use of editor facilities and human reasoning :

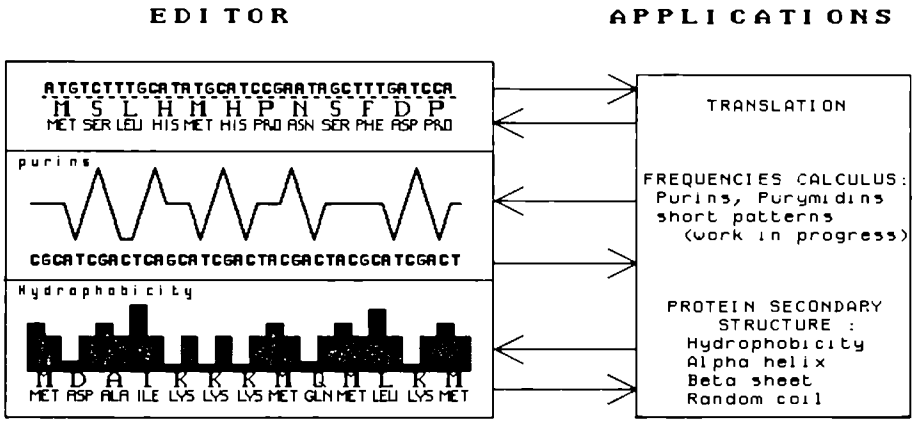


B) EDITOR AND METHODS

a. Methods can use the sequence display facilities :

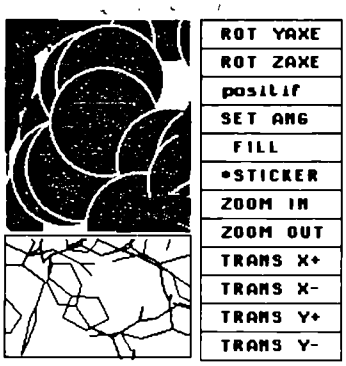


b. Methods can send their results on "visual" result lines:



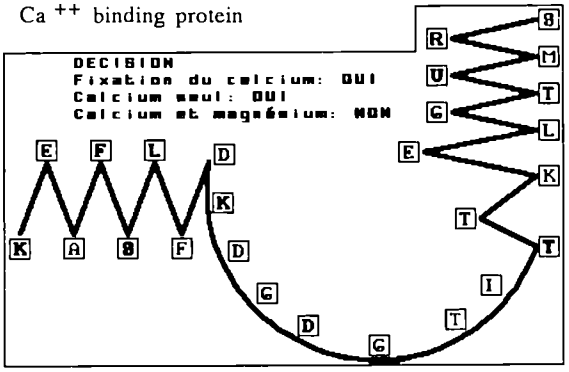
C) OTHER DISPLAY of THE SEQUENCES (only accessible by the editor but not completely compatible)

Geometrical representation of a protein tertiary structure (Hydra-like):



realistic representation

Sequences structures properties determined known by the A.I. software : learning system on the Ca^{++} binding protein



symbolic representation

These two structural editors have been realized using the Microsoft Pascal in order to make them easily portable.

D) COMMUNICATION

General purpose tools (operating system, ...) have been realized for the communication board in order to make the implementation of communication oriented applications be easier. More generally, the access, through international networks which run different standards (EARN, EUROCOM, TRANSPAC ...), to softwares such as genetic data banks (GenBank, EMBL ...) and A.I programs running on heterogeneous hardware (the laboratory VAX and PS300 and the CNUSC ...) form the current goals.

III.3 DISCUSSION

We realized necessary but not sufficient software tools : we have to link the homology program to the editor and the learning system to the editor; furthermore a modification on a sequence line must imply a modification of the associated result line.

In order to have an idea of such a software difficulties and imperfections, we need to work in a more concerted way with biologists (beta testing of the biostation prototype). Thus, the installation of the dialogue between BIOSTATIONS will be realized. It will be based on the reference model of open system interconnection (X400 series of CCITT).

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1 PUBLICATIONS IN SCIENTIFIC JOURNALS, MONOGRAPHS, ...

BARBOUX C., LIQUIERE M. & SALLANTIN J.
"Argumentation d'un apprentissage à l'aide d'expression visuelle."
Numéro spécial d'Intellectica, décembre 1986.

BOUCHERON S., QUINQUETON J., SALLANTIN J. & SOLDANO H.
"CALM : Contester pour Apprendre en Logique Modale."
Numéro spécial d'Intellectica, décembre 1986.

NANARD M. & NANARD J.
"La Biostation et l'édition guidée par la sématique."
TSI, Vol. 5, N° 4, 1986.

NANARD M. & NANARD J.
"The Biostation : bioinformation for biotechnologists."
Ed. A.S. Kolaskar, CCMB, Hyderabad 500 007, India.

QUINQUETON J. & SALLANTINJ.
"CALM : Contestation for Argumentative Learning Machine."
in Machine Learning : a guide to current research, Michalski Carbonnel Mitchell Eds., Kluwer and Sons, janvier 1986.

IV.2 SHORT COMMUNICATIONS, INTERNAL REPORTS

NANARD J., NANARD M. & RASPAL L.
"Une interface conviviale pour une base de données biologiques."
Colloque Inforsid, Fontevraud, France, mai 1986.

QUINQUETON J, SALLANTIN J. & HAIECH J.
"SEQUOIA : Concept formation from sequential data."
EWSL 86, Orsay, France, février 1986.

BACQUET O., BOUCHERON S., BARBOUX C., HAIECH J., HENAUT A., LIQUIERE M., MOISY J.L., NANARD J.,NANARD M., QUINQUETON J., RASPAL L., SALLANTIN J. & SOLDANO H.
"Artificial Intelligence and Genetic Sequences Analysis, towards an intelligent workstation : the Biostation."
Report for Cube realized in the framework of the contract GS DIALR/CCE n° BAP-0136 F, 1986.

BARBOUX C., BOUCHERON S., LIQUIERE M., QUINQUETON J., ROULLE A., SALLANTIN J., SOLDANO H. & SZCZECINIARZ J.J.
"Système d'apprentissage CALM."
R.R. CRIM N° 28, novembre 1986.

RASPAL L., NANARD J. & NANARD M.
"Une interface conviviale pour une base de données biologiques."
R.R. CRIM N° 24, février 1986.

HAIECH J.
"La Biostation : Environnement informatique d'une méthodologie d'analyse des relations structure/activité d'une protéine."
Rapport interne GS DIALR, 1987.

MOISY J.L., SOLDANO H., BARBOUX C. & HAIECH J.
"Learning and knowledge representation on sequences : a biological application."
R.R. CRIM N° 34, janvier 1987.

QUINQUETON J., SALLANTIN J. & SOLDANO H.
"CALM : A learning engine in modal logic."
R.R. CRIM N° 30, janvier 1987.

BACQUET O., BARBOUX C., BOUCHERON S., HACQUIN F., NANARD J., PATENNE T., SALLANTIN J. VILAREM M.C.
"The Biostation : A computing environment for scientific teams working on protein engineering."
Report for Cube realized in the framework of the contract GS DIALR/CCE n° BAP-0136 F, april 1987.

IV.4 DOCTORATE THESIS (Ph.D) AND DEGREE THESIS AWARDED DURING THE PERIOD OF CONTRACT

PINGAND P.
"Utilisation d'outils I.A. dans la prédiction des structures tertiaires des protéines."
Rapport de DEA, 1986.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)		No
Exchange of staff	Yes	
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

Dr. Desmond HIGGINS's visit (3 days) in march, 1987.
Dr. Chris SANDER's visit during the I.A. BIOMED 86 Congress (3 days), novembre 1987.
Dr. Shoshana WODAK's visit during the I.A. BIOMED 86 Congress (3 days), november 1987.

Before starting an international collaboration, our purpose was to dispose of an equipment (experimental prototype to be improved). A diffusable realization will be finished for november 23rd, 1987.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Trinity College, Dublin

Contract no.: BAP - 0137 - IRL

Project leader: P.M. SHARP

Scientific staff: D.G. Higgins, D.C. Shields

Address:
Trinity College
Dept. of Genetics
IRL - DUBLIN 2

Telephone no.: +353.1.772941 EXT.1035

Telex no.: 25442 TCD EI

Other contractual partners in the joint project:

J.F. Sallantin, C. N. R. S. (Montpellier)

Title of the research activity:

Research and diffusion for a portable European system of access and analysis of biosequences.

Key words:

Sequence databases, Sequence analysis, Homology searches, FORTRAN 77

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

To develop an integrated, portable system of nucleic acids and protein sequence analysis software, for use by molecular biologists who do not have immediate access to special biocomputing facilities.

This system should meet the perceived requirements of end-users. It is also the aim to incorporate statistical analyses, and to develop knowledge-based systems for sequence analysis.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

To use the Irish molecular biology community as a model system for the diffusion of biosequence data and analysis software. This is to be achieved by setting up a centrally based facility, on a mainframe computer, comprising the major sequence databases with software to retrieve and analyse sequences. Access to the facility is to be provided by computer network and the distribution of some software for local use.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

In Ireland, there are approximately 50 full-time academic staff involved in Biotechnology/molecular biology research (plus over twice this nuber of postdoctoral and postgraduate researchers) from five universities and two institutes for higher education. All have access to local mainframe/mini computing acilities and are connected to each other by a network (HEANET) allowing remote logins, file transfers and electronic mail. Thus Ireland serves as a model system for the diffusion of biosequence data and analysis software.

METHODOLOGY

Our approach has been to assemble a package of software for biosequence analysis bearing in mind the following criteria: the needs of users, portability of software, and ease of use/flexibility. We make this package available from one centrally based mainframe

computer (an ICL 3900 running VME in Trinity College, Dublin), access being provided by computer network.

We have circulated a detailed questionnaire among the potential users of our system, asking for information regarding the availability of computer facilities, computing experience, sequence analysis software already available or in use, and research being undertaken that would benefit from computer analysis.

RESULTS

The main conclusion to be drawn from the questionnaire is that while most users have readily available access to both mainframe and microcomputers (particularly IBM PC's and compatibles), few have extensive computer experience and little sequence software is in use. The types of service most desired are (i) access to the sequence databases, (ii) sequence comparisons and homology/similarity searches, and (iii) a range of simple analyses, such as open reading frame prediction, restriction digests, protein hydropathy profiles, and RNA secondary structure prediction.

The first and most useful piece of software we installed was ACNUC (1), a relational database system for accessing the GenBank, EMBL and NBRF sequence databanks, developed mainly at the Institut d'Evolution Moleculaire, Lyon. All three sequence collections are converted to a common format which can be accessed by a single retrieval program. By making extensive use of hash coding, direct access files and bit manipulation, the ACNUC query program provides almost instant access to any sequence or group of sequences using 13 different selection criteria. The ACNUC system is written in standard FORTRAN 77 and has very low core memory requirements (approximately 80 kbytes), making it very portable. Indeed, we are working on a microcomputer version to be used with subsets of the databases which we can distribute on floppy disk or by network.

We have developed an efficient, direct method for interacing analysis software with ACNUC (2). This has proved to be invaluable for homology searches (2) where a query sequence is compared to some or all of the sequences in a database. A direct interface is simple to program (it can be used for any method requiring access to large volumes of sequence data), efficient with regard to disk space usage and input output, and allows one to take full advantage of the ACNUC

selection criteria. We have written programs to perform homology searches for nucleic acid and protein sequences using the methods of (3), (4) and (5) with such an interface.

For general sequence analysis, we have written a range of programs, the most widely used being those for sequence alignment and restriction digests. The general purpose package ANALSEQ (written by Christian Gautier, from the I.E.M., Lyon) is being installed and we will link our one-off programs to it. ANALSEQ interacts directly with ACNUC. WE have also installed the shotgun sequencing programs of Staden (6).

REFERENCES

1. Gouy, M., Gautier, C., Attimonelli, M., Lanave, C. and di Paola, G. (1985) Comp. Appl. Biosci. 1:167-172
2. Higgins, D.G. and Gouy, M. (1987) Comp. Appl. Biosci. 3 (in press)
3. Lipman, D. and Pearson, W. (1985) Science 227:1435-1441
4. Bishop, M. and Thompson, E. (1984) Nucleic Acids Res. 12:5471-5474
5. Wilbur, W. and Lipman, D. (1983) Proc. Natl. Acad. Sci. USA 80:726-730
6. Staden, R. (1987) in Bishop, M. and Rawlings, C. (eds.) Nucleic acid and protein sequence analysis: a practical approach. IRL Press.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)		No
Exchange of staff	Yes	
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

One member of our staff (D.G. Higgins) visited the group of J.F. Sallantin (C.N.R.S., Montpellier), in April/May 1987, to discuss progress and future collaboration.

Additionally, the two project leaders (P.M. Sharp and J.F. Sallantin) have met for discussion at the BICEPS meeting (Brussels, December 1986) and again at the BAP meeting in Capri, May 1987.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Stamicarbon bv Contract no.: BAP - 0034 - NL

Project leader: W.J. DE WIJN
Scientific staff: DSM, Research

Address:
Stamicarbon B.V.
P.O. BOX 53
NL - 6160 AB GELEEN

Telephone no.: +31.4494.65417

Telex no.: 36058 DSM NL

Other contractual partners in the joint project:

H.J. Lepers, Fachhochschule Aachen

Title of the research activity:

Simulation of an enzymatic process using an immobilized biocatalyst.

Key words:

Enzymatic process, Immobilized enzyme, Modelling, Process simulation, Scientific education

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

A) Obtaining an understanding of the kinetics and physical properties of the immobilized enzyme complex.
B) Developing a dynamic process-simulation-model of a biochemical process with immobilized enzymes.
C) Application of this model for optimization of industrial research programmes and scientific education.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

A) Implementation of the Graphic Interactive Dynamic Symulation system (GIDS) for educational purposes on the MicoVAX II computer of the Fachhochschule Aachen (FHA), the other contractual partner in this joint project.
B) Laboratory research into an enzymatic process using an immobilized catalyst.
C) Mathematical modelling of a bioreactor.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

A) Implementation of GIDS on the MicroVAX II computer of the FHA.

The GIDS software was introduced to the Fachhochschule Aachen by means of a training course at Stamicarbon in Geleen (NL), using a specially developed educational GIDS-application.
After acceptance, the GIDS-system software and the application were installed on the MicroVAX II computer of the Fachhochschule Aachen.
Preliminary discussions were held on the subject of educational aspects of the implementation of a bioreactor simulation.

B) Laboratory research into an enzymatic process using an immobilized catalyst.

Introduction

The process selected as model for the simulation of an enzymatic process using an immobilized biocatalyst is the hydrolysis of inulin to fructose.
Inulin is a polyfructose extracted from chichory roots by a process simular to the sugarbeet extraction.
The enzyme, inulase (EC 3.2.1.7) is extra-cellularly produced by the mould Aspergillus phoenicis.

Fermentation data of Aspergillus phoenicis.

A. phoenicis is cultivated in a medium with inulin as inulase-inducer. The composition of the culture medium is:

- yeast extract	2.	% w/v,
- Na(NH4)HPO4	0.1	% w/v,
- (NH4)2SO4	0.05	% w/v,
- Inulin	1.	% w/v,
- pH	5	
- Cultivation time	8	days
- Cultivation temperature	28	°C.
- Extra-cellular inulase yield	23	Units/g broth

Standard assay conditions for inulinase are:

pH	: 4.5,
T	: 60 °C,
reaction time	: 1 hour,
substrate	: 10 % w/w inulin Sigma (MW:4550)

Enzyme recovery

The cells of A. phoenicis grown in pellets are removed from the fermentation liquid by filtration. The yeast cells of yeast extract origin are removed by ultrafiltration (0.1 micron).
The enzym inulase (M $\simeq$ 300.000 D) is concentrated by ultra filtration (Amicon DC2 with a H1P 10 - 8 hollow fiber cartridge). The final preparation has an activity Vm $\simeq$ 2 * 10^{-3} kg fructose/kg concentrate.s

Enzyme properties

The characteristic of the free enzyme have been determined:

Km value	90 g inulin/kg (2.0 * 10-2 M)
T optimum	64°C (fig 1)
pH optimum	4.0 (fig 2)
Thermostability	see fig 3
Product inhibition	inhibition by the product fructose is observed Ki = 20 g fructose/kg ($1.1 * 10^{-1}$ M)
I/S-ratio	0.66
MW	about 300.000

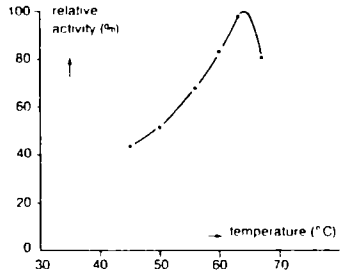


Figure 1 Effect of temperature on the activity of the free enzyme

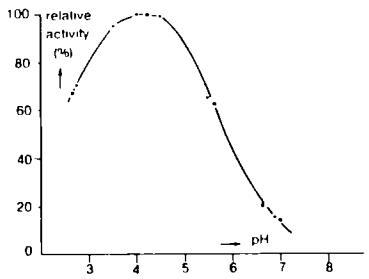


Figure 2 Effect of pH on the activity of free (.....) and immobilized (-.-.-) enzyme

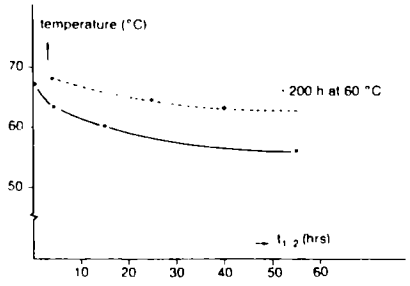


Figure 3 Stability of free (•——•) and immobilized (•- -•) enzyme

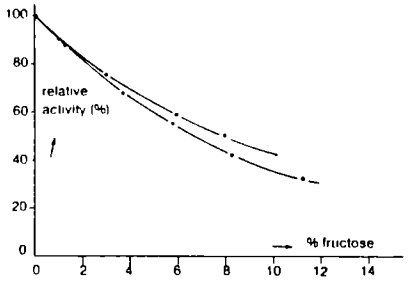


Figure 4 Inhibition by fructose of the activity of free (•-•-•-•) and immobilized (• • • •) enzyme (batch experiment)

Enzyme immobilization

The soluble enzyme is immobilized by covalent attachment to weak base anion exchange resin Duolite A7.
At the non activated resin surface the enzyme should be attached by ionic-binding. To achieve a covalent attachment the resin surface is activated by glutaraldehyde.
The resin particles are activated in a fluid bed reactor by recycling a 5 % w/w glutaraldehyde solution (pH 5.0).
After washing, the concentrated enzyme solution is recycled for 24 hours at 10 °C, 70 % of the original activity was found to be immobilized.
Initial reaction rate:

0.4 kg fructose/m^3 immobilized enzyme · s

Properties of the immobilized enzyme

The properties (T and pH optimum) of the immobilized enzyme differ apparently from those of the free enzyme. But if the initial reaction rate is determined and the actual pH is measured, the properties of the immobilized biocatalyst do not differ significantly from those of the free enzyme.
An exception is the enhanced apparent thermostability.

Hydraulics of the immobilized biocatalyst

The hydraulic properties of the immobilized biocatalyst are investigated in a fluid bed reactor.
Particles with dp= .3 - 1.0 * 10^{-3} m and ρp=1125 - 1100 kg/m^3 show in a liquid with ρl = 1020 kg/m^3 and η l = 5 * 10^{-3} N.s/m^2, a Vmf and Vt of respectively 10^{-4} and 8 * 10^{-3} m/s.
at vl = 4 * 10^{-4} m/s, the expansion is about 15 %.

In a small scale laboratory reactor (dr=1.7 *10-2 m and He=0.15 m), the variation from the ideal plug flow is 0.04, so the backmixing is neglectable.

Mass transfer in the immobilized catalyst

The influence of the mass trasfer in the immobilized bio-catalyst particle on the overall reaction rate of the inulin hydrolysis has been investigated in a fluid bed reactor. The total reactor is simulated by recirculating the output of the reactor.
In a quasi steady state mass transfer equals the reaction rate, so r=

$$D_e \left(\frac{\partial^2 S}{\partial R^2} + \frac{2}{R}\frac{\partial S}{\partial R}\right) = V_m \frac{S}{S + k_m (1+P/K_I)}$$

The thermal deactivation of the enzyme is of a first order:

$$V_m = V_{mo}\, e^{-kd.t} \qquad \text{in which} \quad kd = kdo\, e^{-Ed/RT}$$

The thermal deactivation rate is small related to the diffusion rate, so a quasi steady state is assumed.

C) Model development

To achieve a realistic operating simulation the following aspects were considered in the mathematical model of the bioreactor:
- Plug flow with calculated backmixing
- Mass transfer by convection from the liquid bulk to the enzyme particle
- Mass transfer by diffusion in the enzyme particle
- Adsorption and reaction kinetics by a Michaelis-Menten mechanism, including:
  . product inhibition
  . effect of pH
  . infuence of the temperature on the reaction-deactivation rate of the enzyme

This leads to:

Macro mass balance:

$$U \frac{dSb}{dh} = k_{LS} (S_b - S_{R=1}) \qquad (1)$$

boundery condition $h=0 : S_b = S_{feed}$

Micro mass balance:

$$\mathbb{D}_e \left(\frac{\partial^2 S}{\partial R^2} + \frac{2}{R} \frac{\partial S}{\partial R} \right) = \tfrac{1}{4} d_p^2 \cdot r_s \qquad (2)$$

boundery conditions $R=0 : \frac{\partial S}{\partial R} = 0$

$$R=1 : \mathbb{D} \left(\frac{\partial S}{\partial R} \right)_{R=1} = k_{LS} (S_b - S_{R=1})$$

The differential equations are solved using the orthogonal collocation method, so the concentration profiles in the particle and the liquid can be decribed in relation to the substrate as well as to the product.
With this model parameter fitting has been carried out against the results of the laboratory experiments, in order to determine the numerical values of the physical and chemical parameters.

To cope with fast dynamic phenomena, equations (1) and (2) should be extended with accumulation terms:

$$(1)\quad El \cdot \frac{dSb}{dt}$$

$$(2)\quad Ep \cdot \frac{\partial S}{\partial t}$$

List of simbols:

Sb	concentration of substrate in the bulk
S	,, ,, ,, ,, ,, pores
u	liquid velocity
k_{LS}	mass transport parameters
$\mathcal{D}$	diffusion coefficient
R	radius
h	reactor hight
r_s	reaction rate
E_L	holdup of liquid
E_P	fraction porevolume

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

Poster on BAP-meeting at Capri

TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)		No
Joint meeting(s)	Yes	No

Descriptive information for the above data.

Exchange of material:

The GIDS system software and an application were implemented on the Fachhochschule Aachen.

Joint meetings:

There have been several meetings between the project managers and coworkers of the Fachhochschule and Stamicarbon/DSM.

a) Defining the hard- and software for the simulation computer.

b) Discussions about the progress in the research and development and about the educational aspects of the bioreactor simulation.

c) Meetings at Stamicarbon in Geleen (NL) for intoduction into the GIDS system and for discussion.

d) Participation of both parties in the BAP-sectorial-meeting at Capri

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Fachhochschule, Aachen

Contract no.: BAP - 0033 - D

Project leader: H.J. LEPERS

Scientific staff: Fachbereich Chemieingenieurwesen

Address:
Fachhochschule Aachen
FB 3 - Chemieingenieurwesen
Kurbrunnenstrasse 22
D - 5100 AACHEN

Telephone no.: +49.241.80.65.00 or 80.65.37

Telex no.:

Other contractual partners in the joint project:

W.J. de Wijn, Stamicarbon B.V. (Geleen)

Title of the research activity:

Simulation of an enzymatic process using an immobilized biocatalyst.

Key words:

Enzymatic process, Immobilized enzymes, Modelling, Process simulation, Scientific education

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

a) Obtaining an understanding of the kinetics and physical properties of the immobilized enzyme complex.

b) Developing a dynamic process-simulation-model of a biochemical process with immobilized enzymes.

c) Application of this model for optimization of industrial research programmes and scientific education.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

a) Introduction of didactic aspects into the concept and the structure of the simulation model.

b) Setting up the process-simulator for the later implementation of the simulation model.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

a) Concept of the simulation model

After gathering experimental data and formulation of the basis model by DSM/Stamicarbon (see there) discussions were be held to define the static and the dynamic properties of the model.

On the one hand it is important to have an exact static model for industrial research. On the other hand the dynamic model for education purposes must allow real-time computing and time-acceleration.

b) Setting up the process-simulator

The process-simulator consists of a MICROVAX II computer with the operating system, the GIDS (Graphic-Interactive-Dynamic-Simulation)-system and the simulation model.

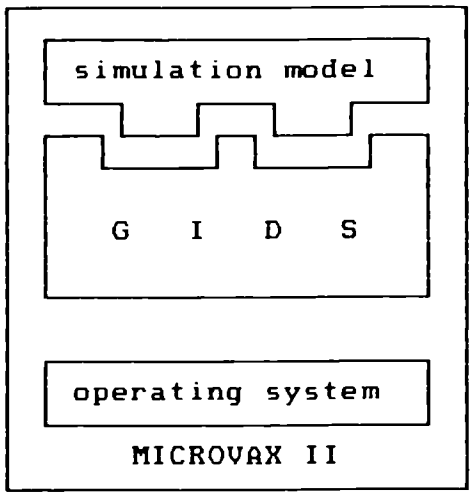


Fig. 1: simulator

In the reporting period the configuration of the hard- and software were difined.

After that the basic configuration of the computer with 5 MB memory, 71 MB disk unit, a dual-floppy and one printer was ordered and installed.

Because there were announced new graphic-colour-terminals (VT340), we are waiting for them and are working with two VT241-terminals in the meanwhile.

The GIDS-programme-system was developed by Stamicarbon and tested and accepted by Fachhochschule. Beyond that personnel of Fachhochschule was trained on the process-simulator at Stamicarbon in Geleen (NL). For training purposes GIDS was connected to an application model which later on will be exchanged for the simulation model of the enzymatic process.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

Poster on BAP-meeting at Capri

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

Exchange of material

A preliminary version of the GIDS-programme-system, an application for training purposes and the related manual were given to the Fachhochschule.

Joint meetings

There were several meetings between the projekt managers and coworkers of Fachhochschule and DSM/Stamicarbon for:

- defining the hard- and software of the process-simulator
- discussions about the progress in the research and the development of the simulation model of the enzymatic process and educational aspects
- training on the GIDS-programme-system and discussions about possibilities of improvement
- participation in the BAP-sectorial-meeting at Capri.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: I. N. P. G., St. Martin d'Hères

Contract no.: BAP - 0030 - F

Project leader: A. CHERUY

Scientific staff: R. Montellano, M.P. Bernier (1/2 part time)

Address:
Laboratoire d'Automatique
E.N.S.I.E.G. - I.N.P.G.
B.P. 46
F - 38402 ST. MARTIN D'HERES

Telephone no.: +33.76448245

Telex no.: 320 205 F

Other contractual partners in the joint project:

G. Bastin, U. C. L. (Louvain-la-Neuve)
J.P. Bovée, I. N. R. A. (Thiverval-Grignon)
A.G. Rozzi, IRSA/CNR (Bari)

Title of the research activity:

Advanced monitoring and computer control of biotechnological processes.

Key words:

Bioprocess, Mathematical modelling, Estimation, Process control, Monitoring

Reporting period: September 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

General objectives of the project are to develop and to provide "bio-informatics" tools (algorithms, software and hardware) for automatic monitoring and computer control of bioreactors and biotechnological processes with a view to yield optimization and products quality improvements. These tools will be rationalized so as to accomodate a broad class of biological models and industrial processes. They will be validated by simulation and on pilot plan.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

To design and to begin the realization of a computer aided modelling software.
At the present time, this software allows :

- an interactive design of functional scheme of bioprocesses.
- an automatic generation of a balance equation for every state variable defined in the functional scheme.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

INTRODUCTION

The software system for computer aided modelling we are developping has to enable bioengineers to easily and interactively design an appropriate model directly from their perception of the process. Such a system has to take into account the model designer approach and to be based on a modelling methodology.

I.- MATHEMATICAL MODELLING METHODOLOGY

A modelling methodology has been proposed [1,2] whose main steps are:

1) **Definition of the aim at mathematical modelling.**
2) **System analysis.**
3) **Mathematical formulation.**
4) **Experimentation and data acquisition.**
5) **Model identification.**
6) **Model validation.**

This methodology results from our ten years experience in modelling, analysis and control of biological processes; it shows that a model cannot be fully and automatically done by computer. However software tools can be provided in order to make easier the model designing. Here we will consider the aid in system analysis and in mathematical formulation.

II.- AID IN SYSTEM ANALYSIS: DESIGN OF A FUNCTIONAL SCHEME

System analysis has to take stock of knowledge and experimental results about a process and to summarize them in a functional scheme exhibiting all the relevant variables with their interactions to be taken into account by a model. The main problem in elaborating such a scheme is to define all the relevant variables with their relations. To make this task easier, we propose software tools offering to user graphic facilities to interactively design the functional scheme.

We have classified all the relevant variables of any bioprocess in 7 types corresponding to 7 functions and a graphic symbol has been associated to a variable class (figure 1). If a component has two functions (for instance substrate and inhibitor), both graphics symbols are superimposed.

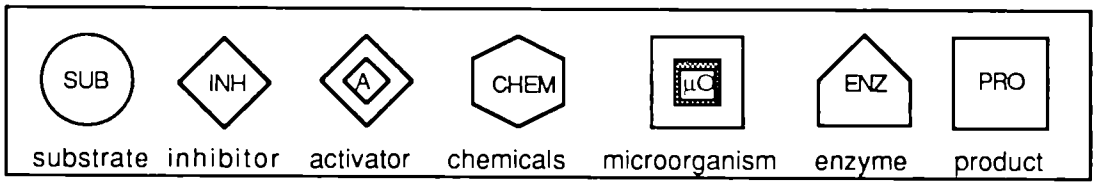


Figure 1 : Relevant variables of a functional scheme

In the same way, the relevant reactions implied in any bioprocess have been classified in 4 types: growth reaction, biosynthesis reaction, enzymatic reaction, physicochemical reaction. The software system present all the facilities to choose and to manipulate components and relations required in any functional scheme. A result of functional scheme is presented in figure 2.

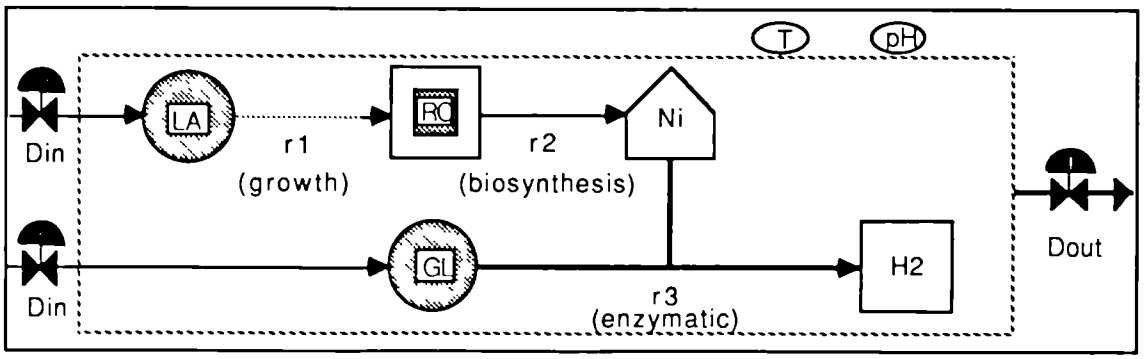


Figure 2 : A functional scheme of a bioprocess

III.- AID IN MATHEMATICAL MODELLING

From the functional scheme of a process, mathematical modelling is performed through 2 steps.

In the first one, for every component (variable) exhibited by the scheme, one defines a first order differential equation expressing the balance between its formation and degradation rates. Such equations are automatically generated by computer from information contained in the functional scheme, after verification of schema coherence.

In a second step, one has to express the reaction rates as functions of components concentrations and kinetical parameters. That requires knowledge on implied reactions kinetics, on possible mathematical formulations... Therefore, that cannot be done automatically by computer; only an expert system can help the user in defining the most suitable mathematical expressions. Our computer aided modelling software is developped with such a goal.

IV.- SOFTWARE REALIZATION AND TEST

This software is written in Pascal language and implemented on a DN320 Apollo computer connected with a high resolution display which offers facilities generally used in CAD. Its use does not need knowledge in computer science or engineering.

Since two months,this software is under test on bioprocesses studied by others laboratories of our research group (photosynthetical bacteries process, lactic fermentation ,methanization process...). It has been presented to several bioengineers from industry (Rhone-Poulenc,Sanofi Elf biorecherche) and from university (UCB Lyon,LSGC Nancy ,LAAS Toulouse) who consider that this software answers the real requirements of their field , and who are interested to use it .

CONCLUSION AND PERSPECTIVES

The computer aided modelling software, we propose, is based on a mathematical modelling methodology issued from our experience in analysis and control of biotechnological processes. It allows the user easily and interactively to design functional scheme of bioprocesses exhibiting all the relevant variables and reactions. From such a scheme, it automatically provides a balance equation for every component. The aid in mathematical formulation is now in progress; in the future, it will be under the control of an expert system. Then, tools for simulation, identification and control will be added in order to get a working station useful for analysis and control of bioprocesses.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1

A. CHERUY, B. CHARPE, J.P. FLANDROIS
"Conception et utilisation de modèles pour l'analyse et le contrôle de procédés biologiques". Symposium : Modélisation et Optimisation de Processus biologiques, 24 et 25 Septembre 1986.

A. CHERUY, R. MONTELLANO, M.P. BERNIER
"Computer aided modelling of biotechnological processes". BAP Meeting, Capri, 2-6 May 1987.

A. CHERUY, R. MONTELLANO
"Computer aided design in modelling of bioprocesses". European Congress of Biotechnology, Amsterdam, June 1987.

A. CHERUY, M. LAKRORI
"Estimation en ligne des paramètres d'une fermentation : possibilités et limites en vue d'un contrôle de procédé". 23 Mars 1987. Colloque Informatique et Biologie à Paris.

IV.2

R. MONTELLANO
"Système d'aide à la modelisation de procédés biotechnologiques". Internal report 86/173. Laboratoire d'Automatique de Grenoble - E.N.S.I.E.G. - I.N.P.G. (1986)

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	No
Exchange of staff	No
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

- Joint experiment for testing the computer aided modelling system with INRA (march-july 87) with UCL (january-march 87).

- General meeting of the four contractors
  Grenoble 7-8/04/86
  Louvain La Neuve 7-8-9/12/86
  Bari 7-8/05/87

- Bilateral meetings
  . with INRA
  Grenoble 12/03/87
  Grenoble 9-10/07/87

  . with UCL
  Grenoble 9/01/87
  Grenoble 20/03/87

  . with IRSA
  Grenoble expected next october

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: U. C. L., Louvain-la-Neuve

Contract no.: BAP - 0032 - B

Project leader: G. BASTIN

Scientific staff:

Address:
Université Catholique de Louvain
Bâtiment Maxwell
Place du Levant 3
B - 1348 LOUVAIN-LA-NEUVE

Telephone no.: +32.10.432591

Telex no.: 59315 TELHY B

Other contractual partners in the joint project:

A. Cheruy, I. N. P. G. (St. Martin d'Hères)
J.P. Bovée, I. N. R. A. (Thiverval-Grignon)
A.G. Rozzi, IRSA/CNR (Bari)

Title of the research activity:

Advanced monitoring and computer control of biotechnological processes.

Key words:

Adaptive control, Bio-informatics, Bioreactors, Software sensors, Real time monitoring

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

To develop and to provide "bio-informatics" tools (algorithms, software and hardware) for automatic monitoring and computer control of bioreactors and biotechnological processes with a view to yield optimization and products quality improvement. These tools will be rationalized so as to accomodate a broad class of biological models and industrial processes. They will be validated by simulation and on pilot plants.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Design, analysis and experimental validation of **software sensors** and **adaptive controllers** for bioreactors. (Part 1).

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

ABSTRACT

During the first year of the BAP, the following new results have been obtained :

1. Design and analysis of **software sensors** for
1.1. on line estimation of biomass, substrate and specific growth rate from oxygen data in aerobic fermentations (including an experimental validation with the support of the SOLVAY company).
1.2. on line estimation of substrate, liquid product and specific growth rate from optical density measurements.

2. Design and analysis of **adaptive controllers** for
2.1. the regulation of volatile acids in anaerobic digestion processes (including a pilot-scale experimental validation in our laboratory).
2.2. the optimal operation of fed-batch fermentation processes.

1. SOFTWARE SENSORS

1.1. On line estimation of biomass concentration, substrate concentration and specific growth rate from oxygen measurements, with known yield coefficients.

Methodology : A single cell population - single limiting substrate aerobic fermentation in a perfectly stirred fed batch bioreactor is considered. The following data are available on line (either by measurement or by a user's choice) : (a) dissolved oxygen concentration C; (b) difference between inlet and outlet oxygen gas flow rates Q_0; (c) dilution rate D; (d) influent substrate concentration S_1; (e) the volume of the culture V. From these data, the following software sensor (= observer = estimation algorithm) can be shown to accurately perform the on line estimation of : (a) the biomass concentration in the reactor X; (b) the substrate concentration in the reactor S; (c) the specific growth rate μ.

$$
\begin{aligned}
dZ_1/dt &= -DZ_1 + V^{-1}Q_0 & \hat{X} &= Y_{X/C}^{-1}(Z_1 - C)\\
dZ_2/dt &= -DZ_2 + DS_1 & \hat{S} &= Z_2 - Y_{X/S}\,Y_{X/C}^{-1}(Z_1 - C)\\
dZ_3/dt &= -\lambda_1 Z_3 + X & & \\
dZ_4/dt &= -\lambda_1 Z_4 - DX & Z_1(0) &\text{ to } Z_5(0) > 0 \text{ arbitrary}\\
dZ_5/dt &= [\lambda_2 + Z_3^2 Z_5]^{-1} & & \\
d\hat{\mu}/dt &= Z_3 Z_5 (X - \mu Z_3 + Z_4) & &
\end{aligned}
$$

with $Y_{X/C}$, $Y_{X/S}$, known yield coefficients; λ_1, λ_2, design parameters at the user's disposal; Z_1 to Z_5 auxiliary state variables.

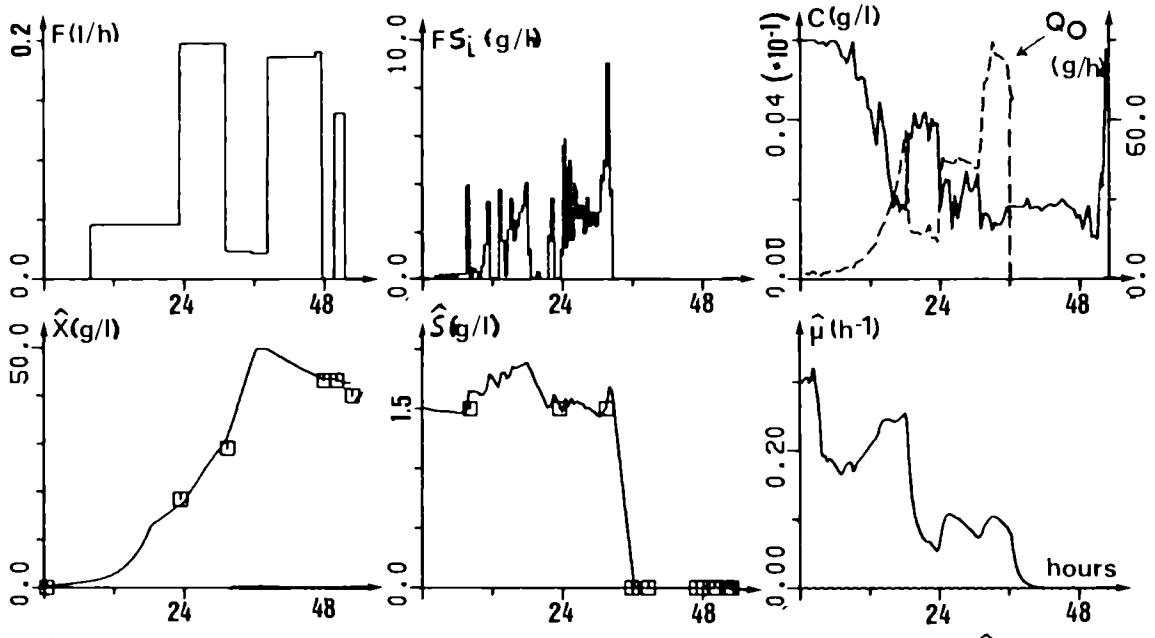


Fig.1. Example of software sensor: On line estimation of biomass ($\hat{X}$), substrate ($\hat{S}$) and spec. growth rate ($\hat{\mu}$) from on line measurements of diss. and gas oxygen(C and Q_0), flow rate (F) and substrate feed rate (FS_i)

Results and Discussion : A theoretical motivation and a complete stability analysis of this algorithm can be found in [8]. An experimental validation has been performed with the support of the SOLVAY company. Typical results are shown in fig. 1. where a very good agreement between on line estimates $\hat{X}$, $\hat{S}$ and off line data (chemical analysis) can be observed.

An application of the same methodology to mixed anaerobic cultures (lactic fermentation) is under investigation with one of our BAP co-contrators (INRA).

1.2. On line estimation of substrate concentration and specific growth rate from optical density measurements, without the prior knowledge of the yield coefficient.

We now suppose that the yield coefficients are unknown and that the following data are available on line : (a) biomass concentration X (through optical density measurements); (b) dilution rate D; (c) influent substrate concentation S_f. From these data, using the theory of adaptive observers (see [5]), a software sensor has been designed for on line estimation of : (a) the substrate concentration S; (b) the specific growth rate μ; (c) the yield coefficient Yx/s. This software sensor is described and analyzed in [5], [6]. A critical issue of such software sensors is that of parameter identifiability which is related to the persistence of excitation of a so-called "regressor". This issue is specifically addressed in [6] where sufficient experimental conditions for the estimation convergence are emphasized. This software sensor will be experimented on a new pilot plant set up, with the BAP support, in our laboratory (see [7]).

2. ADAPTIVE CONTROLLERS

2.1. Adaptive regulation of the volatile acids concentration in anaerobic digestion processes.

Motivation and Methodology : The intrinsic instability of anaerobic digestion processes is well known to be due to the accumulation of volatile acids (which have a growth inhibiting action). It is therefore of evident interest to investigate the possibility of controlling the process through the regulation of volatile acids (VA). The control objective is to regulate the VA concentration S at a prespecified desired value S^*, despite the fluctuation of the influent substrate concentration S_i, by using the dilution rate D as the control action. The following discrete time "adaptive external linearization feedback" control law, with a standard RLS parametric adaption has been designed :

$$D(t) = [S^* - S(t) + Y_{q/s}^{-1} Q(t)][\alpha(t)S_i(t) - S(t)]^{-1}$$

$$\alpha(t+1) = \alpha(t) + D(t)\, S_i(t)G(t)[S(t+1) - S(t) + D(t)(S(t) - \alpha(t)S_i(t))]$$

$$G(t) = G(t-1)[\lambda + G(t-1)\, S_i(t)D(t)]^{-1}$$

with t the current time index; Q(t) the methane gas flow rate; $\alpha(t)$ the adaptation parameter; G(t) and λ respectively the gain and the forgetting factor of the RLS algorithm; $Y_{q/s}$ the known yield coefficient.

Results and Discussion : A theoretical motivation and a validation by simulation of this control law will be reported in [8]. A successful pilot scale experimentation has also been performed. The results are shown in fig. 2 where it can be seen how the controller copes with two successive steps of load (which would otherwise have destabilised the reactor). See [11] for further details. A similar controller is under investigation for the regulation of bicarbonates in anaerobic digestion processes, in cooperation with one of our BAP co-contractors (IRSA).

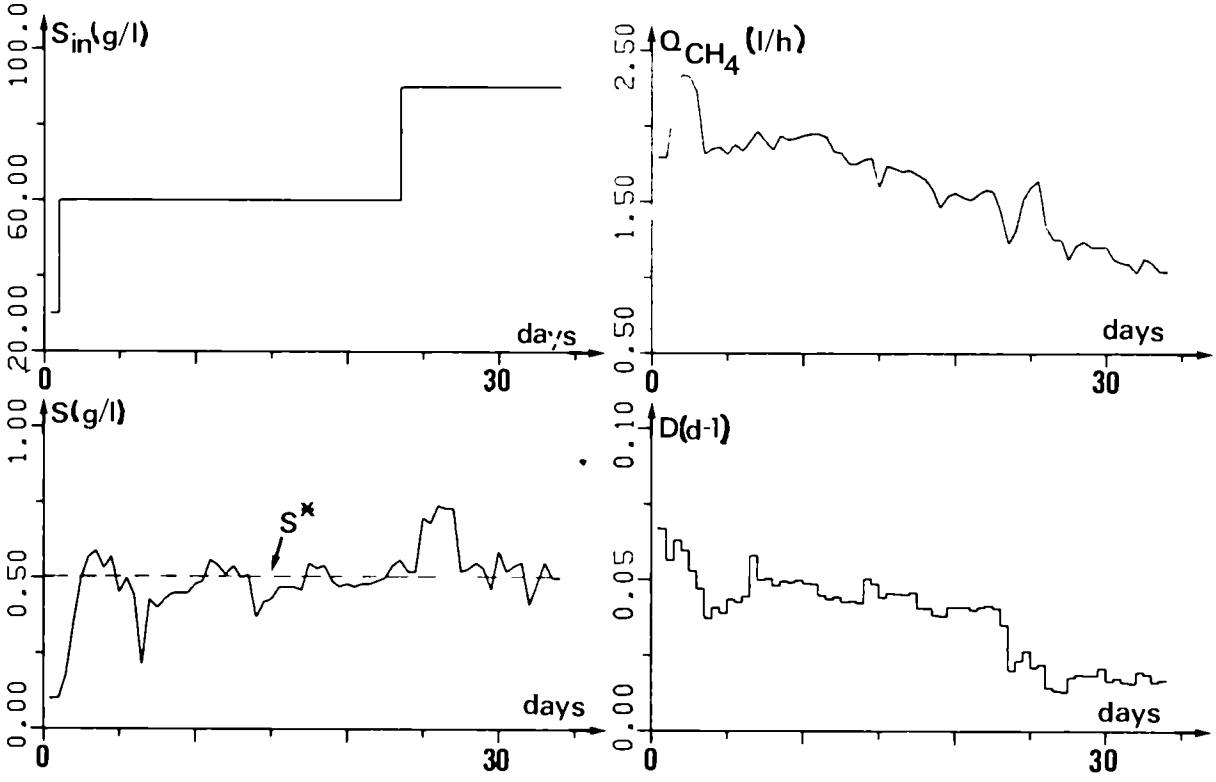


Fig.2. Adaptive regulation of the volatile acids concentration.

2.2. Adaptive control of fed batch bioreactors

A number of theoretical research works have focused, from the early 70's, on the application of optimal control theory to fed batch bioreactors in order to optimize the productivity in case of substrate inhibition. However, this approach leads to very complex control laws which are very difficult to implement in practice because they require a precise knowledge of the process kinetics (and especially of the analytical structure of the specific growth and production rates). Our contribution has been to show that adaptive control algorithms can constitute a valuable alternative to optimal control of fed-batch processes. The main advantages are : (i) their implementation is much simpler; (ii) they do not require any prior analytical models of the specific growth and production rates; (iii) their behavior is nearly optimal (and even completely optimal in some instances). In [4], two applications are discussed : optimization of the biomass production and of the production of a synthesis product in liquid phase. In each case, the effectiveness of the adaptive controller is illustrated through realistic simulation studies and the robustness against model inaccuracies is analyzed.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

Publications in scientific journals

[1] G. Bastin and D. Dochain (1986), "On line estimation of microbial specific growth rates", Automatica, vol. 22(6), pp. 705-710.

[2] P. Renard, D. Dochain, G. Bastin, H. Naveau, E.J. Nyns (1987), "Adaptive control of an anaerobic digestion process : a pilot-scale application", Biotechnology and Bioengineering, in press.

[3] G. Bastin and M. Gevers (1987), "Stable adaptive observers for timevarying non linear systems", IEEE Transactions Automatic Control, in press.

Short communications and Internal reports

[4] D. Dochain and G. Bastin (1987), "Adaptive control of fed-batch reactors", Proceedings 4th European Congress on Biotechnology, Amsterdam, June 87, pp. 127-130.

[5] I. mareels, M. Gevers, G. Bastin, D. Dochain (1987), "Exponential convergence of a new error system arising from adaptive observers" to be presented at the IEEE conference on Decision and Control, Los Angeles, December 1987.

[6] G. Bastin, I. Mareels, D. Dochain, M. Gevers (1988), "Sufficient experimental conditions for the convergence of an adaptive observer for non linear biochemical processes", to be presented at the 8th IFAC Symposium on Identification and System Parameter Estimation, Beijing, August 1988.

[7] P.Renard (1987), Détermination expérimentale de règles de conduite et validation d'observeurs adaptatifs en génie microbiologique. Premier rapport intermédiaire IRSIA, Unité de Génie Biologique, Univ. Cath. Louvain.

[8] D. Dochain and G. Bastin (1987), "On line estimation and adaptive control of bioreactors", Book in preparation, Int. Report, Laboratoire d'Automatique, Dynamique et Analyse des Systèmes, University of Louvain.

Theses

[9] P. Renard (1986), "Régulation par ordinateur d'un réacteur continu infiniment mélangé sans recyclage de biomasse active", Degree Thesis, Faculté des Sciences Agronomiques, Univ. Cath. Louvain, sept.86.

[10] D. Dochain (1986), "On line parameter estimation, adaptive state estimation and adaptive control of fermentation processes", Ph. D. Thesis, Unité AUTO, Cath. University of Louvain.

[11] S. Dou (1987), Degree Thesis in preparation.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)	Yes	
Joint meeting(s)	Yes	

Descriptive information for the above data.

1. Joint experiments:

- Design and experimentation of software sensors for lactic fermentations (mixed cultures) with INRA; exchange of softwares and data.
- Design and experimentation of an adaptive regulator of the bicar bonate concentration in anaerobic digestion processes with IRSA; Exchanges of softwares and sensors.

2. General meetings of the four contractors.

- Grenoble: 7-8 april 1986
- Louvain-la-Neuve: 7-8-9 december 1986
- Bari: 7-8 may 1987

3. Bilateral meetings.

- with INRA: Lille, 23/9/86; Grignon, 18/3/87 and 26/6/87
- with IRSA: Louvain-la-Neuve, 24-25/10/86 and 29/5 to 1/6/87; Rome, 6/5/87.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: I. N. R. A., Thiverval-Grignon
Contract no.: BAP - 0031 - F

Project leader: B. PERRET
Scientific staff: J. Mosse, G. Corrieu, D. Picque

Address: INRA - Centre de Biotechnologie
Agro-Industrielle - L.G.P.B.A.
F - 78850 THIVERVAL-GRIGNON

Telephone no.: +33.1.30.54.45.10

Telex no.: 697.388 F

Other contractual partners in the joint project:

A.G. Rozzi, IRSA/CNR (Bari)
A. Cheruy, I. N. P. G. (St. Martin d'Hères)
G. Bastin, U. C. L. (Louvain-la-Neuve)

Title of the research activity:
Advanced monitoring and computer control of biotechnological processes.

Key words:
Hardware interface, Computer control, Bioreactor, Sensors, Automation

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

General objectives of the project are to develop and to provide "bio-informatics" tools (algorithms, software and hardware) for automatic monitoring and computer control of bioreactors and biotechnological processes with a view to yield optimization and products quality improvements. These tools will be rationalized so as to accomodate a broad class of biological models and industrial processes. They will be validated by simulation and on pilot plants.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

To elaborate a local hardware and software digital interface between intrument-equipped bioreactors and a supervising minicomputer. This interface allows :

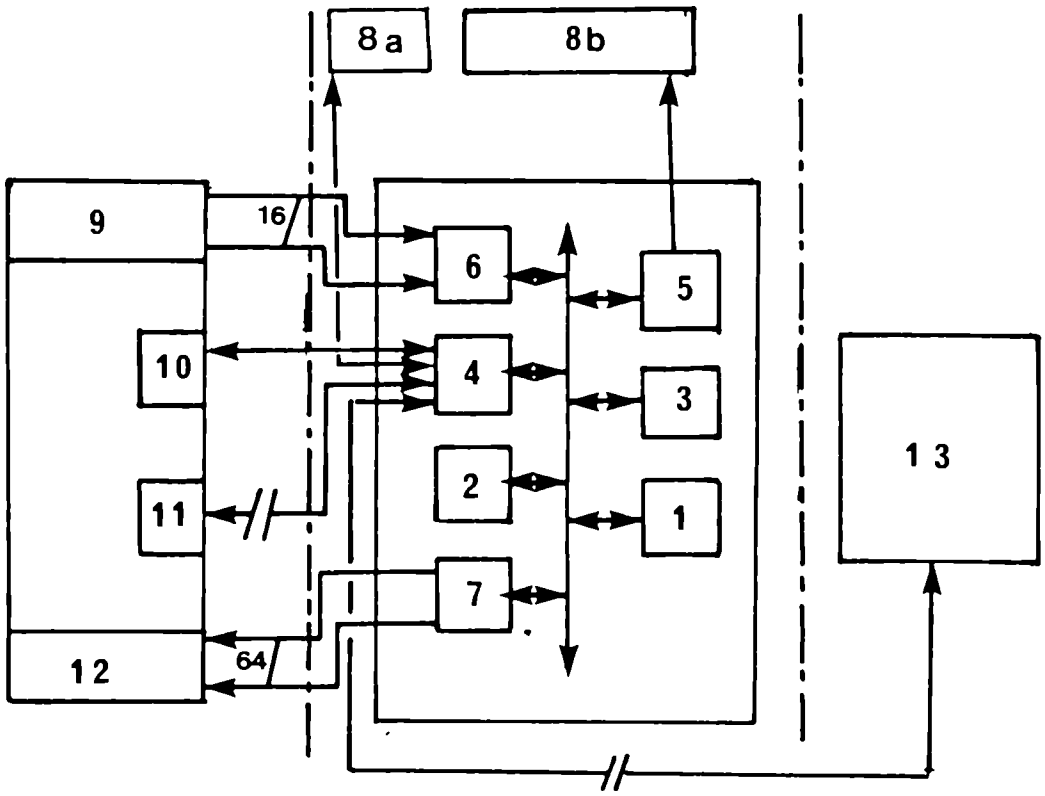
- data acquisition,
- local controls,
- tasks schedulling,
- dialogues.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY

1.1 HARDWARE CONSIDERATIONS : Each bioreactor is controlled by a decentralized stand-alone computer (STD cards, see figure 1-B).
Each STD card combines a 8-bits CPU Zilog Z80(1), an EPROM (8 kb to 56 kb) (2), a battery-backed RAM (2 kb to 8 kb) (3), peripheral I/O processors (4), four CDA (5), and, if needed, sixteen CAD (6) and sixty-four TTL input/output lines (7). Each card is connected to a terminal display(8a) and a recorder (8b). The analog-to-digital conversion is performed over the internal BUS (6) for sensors without CAD (9) or by serial lines RS 232C when the bioreactor is CAD-equipped (10). The actuators are controlled either through RS 232C when the bioreactor is TTL-equipped (11) or directly from the BUS (7) in other cases (12).
The central minicomputer (13) is a PDP 11/73 which provides 1 Mb RAM, 30 Mb fixed disk and two floppy disks. It is

connected to four terminal displays and to each STD card by RS 232C.



A : process B : Interface C : Central level

Figure 1 : General organization scheme

1.2 SOFTWARE CONSIDERATIONS : A standard analysis was made from the above specifications. It defined the software organization in languages- and hardware-independant modules.
The whole software is developped on the central computer. The low-level modules, written in Pascal (7 modules) and Z80 assembler (5 modules) are separately converted to Z80 absolute hexadecimal code by a PARAGON cross-compilator package (compilator, assembler, linker, loader, TEKTRONIC format converter) and brought to each STD card by an EPROM programmer. Each routine is tested and debugged before integration in its module. To operate the system all local functions have been written : input/outputs, interrupts, counter and timers.

2. RESULTS

Local software for the first three bioreactors (BIOLAFITTE, 2 x 15 l, 1 x 75 l, with CADs incorporated system) is now developped. It allows :

- general functions as :
  . **data acquisition and control** as listed hereafter

Measurements	Control	Actuators
temperature		
.sterilisation	Yes	steam
.fermentation	Yes	cold water/heat resistor
agitation speed	Yes	variator
pH	Yes	pumps
pO2	Yes	agitation speed/pressure/ air flow
pressure	Yes	motor valves
air flow rate	Yes	motor valve
foam level	Yes	pump
pCO2	No	
weight		
.bioreactor	No	
.acid and alkali	No	

  . **data storage**
  . **outputs on local displays and recorder**
  . **dialogues with operator and central computer**

- specific fermentations functions :
  . **calibrations of sensors** (before or during experiments)
  . **sterilisations** without and with fermentation media
  . **fermentation experiments**
  . **harvesting**
  . **ultrafiltration** (on process)

3. DISCUSSION

An unique software is able to control any bioreactor. It is quite reliable and fault-tolerant. The control parameters of the PID algorithm are experimentally identified for each controlling loop.
We must now develop minicomputer software for on-line and off-line data processing, model identification with feedback capabilities toward local levels; install automation of prelevement and feeding techniques (Ultrafiltration, Fed-batch, ...); take into account the addition of sensors and analytical data (HPLC, GPC, ...).
Further work will be performed for testing parameters estimation methods and new software sensors (Universite de Louvain, Laboratoire d'automatique de Grenoble).

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.2 SHORT COMMUNICATIONS, INTERNAL REPORTS

- Advanced monitoring and computer control of biotechnological plants.
  B. PERRET, J. MOSSE, G. CORRIEU.
  Capri, Italy, 2-6 May 1987

- On-line computer monitoring of pilot plants for starch hydrolysis, fermentation and distillation with an expert system.
  B. PERRET, D. GHOZLAN, H.J. VASQUEZ, A. RAMIREZ
  Proceedings 4th European Congress on Biotechnology
  Volume 3 : Abstracts/extended abstracts, edited by O.M. Neijssel, R.R. Van der Meer, K.Ch. A. M. Luyben.
  Amsterdam, June 14-19, 1987.

- Computer-bioreactor interfacing; control and data treatment.
  G. CORRIEU
  Workshop "Modelling and control of bioreactors" - Vaalsbroek, July 15-17, 1987.

IV.4 DEGREE THESIS AWARDED DURING THE PERIOD OF CONTRACT

- Informatisation et automatisation de bioreacteurs.
  M. DACLIN
  Stage de fin d'etudes - Ecole des Mines de St-Etienne.
  Juillet 1987.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

- Exchange of softwares and experimental data with the University of Louvain.

- General meetings of the four contractors :

  * Grenoble, 7-8 April 1986
  * Louvain-la-Neuve, 7-9 December 1986
  * Bari, 7-8 May 1987

- Bilateral meetings :

  with the University of Louvain :

  * Lille, 29 October 1986
  * Grignon, 18 March 1987
  * Grignon, 26 June 1987
  * Louvain-la-Neuve, 20-22 July 1987

  with the Laboratoire d'Automatique de Grenoble :

  * Grenoble, 12 March 1987
  * Grenoble, 9-10 July 1987

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: IRSA - CNR, Bari
Contract no.: BAP - 0162 - I

Project leader: A.G. ROZZI
Scientific staff: A.C. di Pinto, N. Limoni, C. Longobardi, S. Menegatti, V.N. Palmisano, M.C. Tomei

Address: IRSA - CNR
Via de Blasio 5
I - 70123 BARI

Telephone no.: +39.80.372015 or 372099

Telex no.: 810350 IRSA BA

Other contractual partners in the joint project:

A. Cheruy, I. N. P. G. (St. Martin d'Hères)
G. Bastin, U. C. L. (Louvain-la-Neuve)
J.P. Bovée, I. N. R. A. (Thiverval-Grignon)

Title of the research activity:
Advanced monitoring and computer control of biotechnological processes.

Key words:
Process control, Bicarbonate, Anaerobic digestion, Automatic analysers, Biotechnological processes

Reporting period: December 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The general objectives of the project are to develop and to provide "bio-informatic" tools (algorithms, software and hardware) for automatic monitoring and computer control of bioreactors and biotechnological processes with a view to yield optimization and products quality improvement. These tools will be rationalized so as to accomodate a broad class of biological models and industrial processes. They will be validated by simulation and on pilot plants.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Design, construction and preliminary testing of the prototype automatic instrument ("sensor") for bicarbonate determination in complex organic solutions produced by biotechnological processes,in particular by anaerobic digesters.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY

Inorganic carbon (IC) species, i.e. bicarbonate and CO_2 in solution and CO_2 in the gas are important operation parameters in a number of biotechnological processes. In particular, bicarbonate can be used to evaluate the buffer of the system and/or to evaluate variations of acid species produced by the reactor. No instruments are presently available for direct bicarbonate monitoring in complex organic solutions where acid base couples other than the inorganic carbon ones do not allow direct titration.

The automatic instrument to be developed within the present contract is simple and only requires pressure and temperature measurements as inputs. The principle of operation is the following: a fixed volume of bicarbonate solution is introduced in a vessel, where it takes a fraction (1/4 to 1/2) of the total volume. Pure carbon dioxide is bubbled at atmospheric pressure in the vessel until equilibrium (saturation) conditions are reached, then the vessel is sealed.m Strong acid solution is added in excess until pH is lower than 4 and bicarbonate is decomposed according to the equation:

$$NaHCO_3 + HCl \text{ --- } NaCl + H_2O + CO_2$$

Evolved CO_2 is partitioned between the liquid and the gasous phases according to Henry's law and consequently the vessel internal pressure increases. After new equilibrium conditions are reached, bicarbonate concentration is calculated as a function of the overpressure P. If CO_2 is assumed to behave as an ideal gas, the equation which gives bicarbonate concentration BA is:

$$BA = P \cdot (K_g \cdot V_g / V_l + K_H)$$

where V_g and V_l are the gas and liquid volumes respectively, $K_g = 1/(R \cdot T)$ and K_H Henry's constant. Both parameters K_g and K_H depend on the temperature T and can be easily interpolated by simple functions.

A lay-out of the prototype analyser BA1 is shown in Fig. 1. Valves are indicated as V, ricycle and acid pumps as PR and PA. In Table I the working cycle of the instrument is reported.

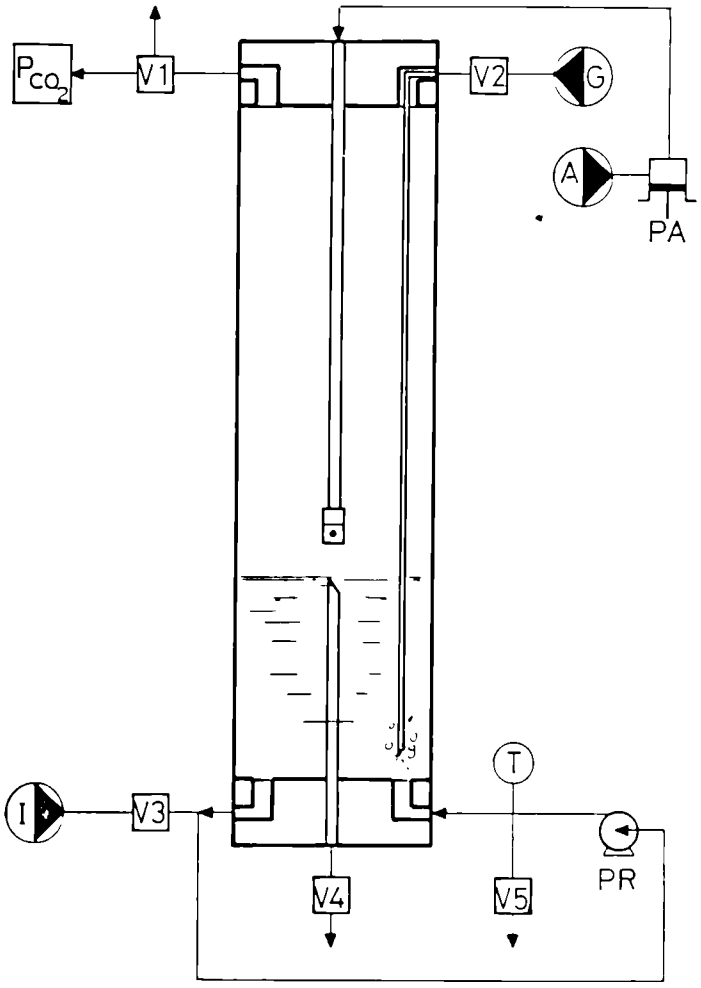


Fig. 1

PHASE	TIME (min)	WASH & LOAD SAMP.	SATUR WITH CO_2	ACID ADD	EQUILI-BRATION	P_{CO_2} READING	DISCHARGE SAMPLE
		1	2	3	4	5	6
		2	10	2	2	1	3
V A L V E	V1						
	V2						
	V3						
	V4						
	V5						
P_{CO_2}	PA						
	PR						

Table I

The analyser operates discontinuously (one determination each 20 minutes). In the prototype the various phases of the cycle are determined by a cam shaft and the pressure signal from a transducer is converted to a digital output on a led display.

In subsequent instruments, pressure and temperature signals will directly converted to bicarbonate concentration by a microprocessor which also controls the phases of the cycle.

2. RESULTS.

The BA1 prototype has been tested using pure bicarbonate solutions prepared with analysis grade chemicals in distilled water and using diluted organic solutions (effluents of anaerobic digesters fed on olive oil mill effluents). The range of bicarbonate concentration was 25 to 100 mM. Operating temperature in the range 20-25 ^{o}C.
During the first trials the determinations were less accurate than epected (10-20% error) mainly because of problems related to the variable volume of the liquid sample, depending on the feed flow rate which altered the level of the overflow tube (the axial tube in fig. 1). This problem has been overcome by using a constant head feed system.
Subsequent results are plotted in Fig. 2 and show a good correlation between bicarbonate concentration and overpressure P.

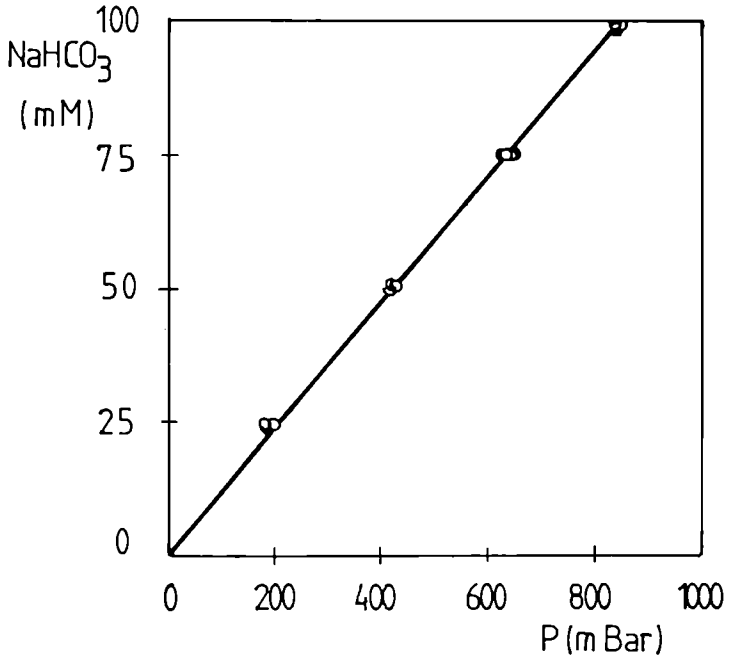


Fig. 2

3. DISCUSSION

Bicarbonate recoveries of the order of 90-95% have been obtained assuming carbon dioxide to be an ideal gas. The reasons of this incomplete recovery depend on the non ideal behaviour of carbon dioxide (at ambient temperature and pressure) and on minor technical problems related to the experimental set-up (recycle pump operation, CO_2 bubbling during the saturation phase, temperature control). The volume of added strong acid also affects the determinations. If the non-ideal behaviour of CO_2 is taken into account, e.g. by using Van der Vaals equation, recoveries higher than 95% have been obtained.

This first report describes relatively few experiments because the contract has been in fact signed at the end of 1986 and therefore the research activity has been only started afterwards.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

NO PUBLICATIONS ISSUED

TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff	Yes	
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

Exchange of software with the University of Louvain

Visit of Mr. V.N. Palmisano to the University of Louvain
(24-25 October 1986)

General meetings of the four contractors
* Grenoble 7-8 April 1986
* Louvain-la-Neuve 7-9 December 1986
* Bari 7-8 Mai 1987

Bilateral meetings (University of Louvain)
* 24-25 October 1986
* 29 May and 1st June 1987

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: The Wellcome Foundat. Ltd, Beckenham

Contract no.: BAP - 0150 - UK

Project leader:
Scientific staff: R. BOMFORD

Address: The Wellcome Research Laboratories
UK - BECKENHAM, KENT BR3 3BS

Telephone no.: +44.1.6582211 EXT.218

Telex no.: 23937 WELLAB BECKENHM

Other contractual partners in the joint project:

B. Robson, University of Manchester
J. Garnier, Université de Paris-Sud

Title of the research activity:
Computer-aided peptide and protein engineering software development.

Key words:
Secondary structure prediction, Protein folding, Protein engineering, Synthetic vaccine, Energy calculation

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The development of the software for protein secondary and tertiary structure prediction and for identification of antigenic sites is proceeding in the laboratories of my partners Robson (Manchester) and Garnier (Orsay). The contribution of this laboratory is to validate the programmes by empirical testing of prediced peptides for their ability to induce antibodies to the parent protein.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

To compare the programme PRED 85 from Manchester with other existing methods of predicting antigenic sites.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

METHODOLOGY

The methodology of the software development has been described in the reports of my partners Robson and Garnier, and this report will concentrate on the criteria that will be applied to the validation process.

The objective is to develop a programme that will maximise the chances of raising high-affinity antibodies to proteins, for purposes of vaccination, diagnostic reagents, or other experimental work, by immunizing with synthetic peptides.

Ideally, the programme should have the following properties:

(i) It should select the antigenic sites on proteins that have already been defined empirically by immunizing with synthetic peptides.

(ii) It should provide predictions which extend those already available by existing methods such as hydrophilicity.

(iii) The peptides predicted by the programme, both those which resemble those commonly predicted by existing methods and those which are different, should have a high probability of raising antibodies against the parental protein.

RESULTS

The efficacy of the current programme, PRED 85, in predicting already known antigenic sites has been tested on the VPI antigen of the Foot-and-Mouth Disease Virus. It completely predicted one of the two sites, which, being hydrophobic, was missed by hydrophilicity analysis and partically predicted the second. This drew our attention to the importance of sites which would be missed by existing methods of prediction.

A more extensive comparison of PRED 85 with existing methods of prediction has been carried out on the cytokine Interleukin-1 (human IL-1β). The three major current methods of predicting external sites based on hydrophilicity, accessibility and mobility all give highly concurrent results. PRED 85 also predicts these consensus regions, but in addition picks out an additional peptide.

Peptides from three of the consensus regions of IL-1β predicted by PRED 85 raised antibodies in rabbits which immunoprecipitated radiolabelled IL-1β.

DISCUSSION

Our preliminary results are encouraging in that they indicate that PRED 85 will pick up prospective antigenic sites which are too hydrophobic for detection by conventional procedures. Future experimental work will concentrate on such sites in viral and mediator proteins.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

Bomford, R., Abdulla, E., Hughes-Jenkins, C., Simpkin, D. and Schmidt, J. Antibodies to Interleukin-1 raised with synthetic peptides. Identification of external sites and analysis of Interleukin-1 synthesis in stimulated human peripheral blood monocytes. Immunology (in press).

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)	Yes	
Joint meeting(s)	Yes	

Descriptive information for the above data.

The programme PRED 85 has been supplied by Manchester to Beckenham, and selected amino acid sequences of proteins suitable for validation are being provided by Beckenham for Manchester and Orsay.

Meetings between the three contractors are held about every four months.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Victoria Univ. of Manchester

Contract no.: BAP - 0149 - UK

Project leader: B. ROBSON

Scientific staff:

Address: Epsitron Protein & Peptide Engineering
Research Unit - Dept. of Biochemistry
University of Manchester
Stopford House
UK - MANCHESTER M13 9PT

Telephone no.: TEL +44.61.2738241 EXT.97

Telex no.: 668932 MCHRUL G

Other contractual partners in the joint project:

R. Bomford, The Wellcome Research Lab. (Beckenham)
J. Garnier, Université de Paris-Sud

Title of the research activity:
Computer-aided peptide and protein engineering software development.

Key words:
Secondary structure prediction, Protein folding, Protein Engineering, Synthetic vaccine, Energy calculation

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Refinement of parameters, methodologies, and concepts for computer- aided protein engineering, including aspects of prediction at the secondary, supersecondary, and tertiary structure levels. To develop a pragmatic approach with practical applications, particular emphasis is given to those procedures which aid in the design of artificial peptide vaccines.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Software tools for interactive molecular graphics, energy minimization, and prediction of epitopic sites as a basis for vaccine design.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Methodology.

Design of artificial peptide vaccines depends (1) on recognising surface features of proteins which are capable of raising antibodies, (2) on the synthesis of analogues or copies of these surface regions, (3) on their attachment to a carrier which confers immunogenicity, and (4) on their parenteral presentation to raise antibodies active against the original protein. The surface features may be continuous epitopes consisting of a continuous segment of sequence, or discontinuous epitopes, consisting of residues brought together from different parts of the sequence by the folding of the protein chain.

Prediction of those sites which form the basis of synthesis is a sound practical framework on which to hang much of the development of protein engineering software. Improved vaccine design is possible by a progressively more detailed picture of the protein at secondary, supersecondary and tertiary structure levels, providing those predictions are adequate. The supersecondary and tertiary structure levels are appropriate to the design based on discontinuous determinant and in practice depend on the detection of portions of protein structure

"domains" which are related and of known conformation; once the general fold motif is established, molecular modelling including global energy minimization and molecular dynamics can be used to tidy the details. In contrast, secondary structure predictions represent a refinement of existing methods for continuous epitopes: the classical technique of searching for a segment of above average polarity implies most often a prediction of loop, and this is here to be extended to consider not only loop but also helix and sheet regions. This extension implies the innovation that epitopes may be recognised which are not necessarily polar on average. In brief the methods being developed are thus as follows:-

(a)Improved secondary structure prediction

(b)Validation of secondary structure prediction by consistency with the $2^6=64$ patterns of polar and non-polar residues which can occur in a six residue segment of sequence. Recognition on this basis of a polar facet of helix and sheet which forms an epitope, even though the polarity of the segment overall may be low (since non-polar residues are present to interface with the protein core).

(c) Ranking of epitopes in order according to their most likely efficacy. This depends on a rule- based system which takes account of the experimental results. For example, that helix ends might or might not be highly suitable for vaccines will depend on the results after synthesis and testing and the program is progressively calibrated on this basis.

(d)Recognition of distantly related sequences. Conformationally related domains are of a limited number of types, and relation of at least part of a protein of interest to one of known conformation has a high probability. The sequence relationships, which must be recognised in order to discover such conformational relationships, are not generally obvious to the eye.

(e) Interactive graphics and energy modelling to refine predictions based on related domains and to assemble the domains into a whole protein structure, .i.e. to produce tertiary prediction.

These techniques are currently advanced and imply extensive detail which can be made available on request.

2. Results

The procedure gives a good account of the epitopic sites in the proteins for which they are known and published. Indeed, this data is used in part in calibration of the method. However, such data does not include functional but untested regions, while those which were identified are to some extent dependent on test method and test animal. To this end we have employed the pragmatic approach of our own synthesis and testing programme combined with that of Wellcome and Cooper's Animal Health. The latter commercial bodies report an acceptable degree of success apparently leading to patent applications. In our own hands, 7 epitopes were proposed in Nature for HIV virus envelope protein. Six of these interacted with antibody from AIDS patients, while two (inlcuding the 7th lacking the above action) raised anti-HIV antibodies in rabbits. While this satisfies the scientific aspects, there has been no reported success from these peptides in man where

the HIV virus remains maliciously uncompromising. A number of other tests on other protein systems await the experimental results.

3.Discussion

One benefit of this emphasis on vaccine design is that success is much more tolerant of imperfects or lack of atomic detail in predictions, compared with, for example, design of artificial enzymes. In this sense we are obtaining an important application area which will be progressively tractable within the developing state of the art.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

"Studies on rationales for an expert system approach to the interpretation of protein sequence data. Preliminary analysis of the human epidermal growth factor receptor"
By R.V.Fishleigh, B.Robson, J.Garnier and P.W.Finn, FEBS Letters, 214, 219-225 (1987).

"Further Development of Protein Secondary Structure Prediction using Information Theory. New Parameters and Consideration of Residue Pairs"
J.F.Gibrat, J.Garnier and B.Robson. J. Mol. Biol., in press.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

A major development which may hopefully serve as a more general pattern in EEC joint contracts has been the establishment of the BIOMAT Laboratory. This is a single but geographically split laboratory partly located in the University of Manchester and partly in the laboratories of Institut National de la Recherche Agronomique. It is formally under the jurisdiction of both these bodies, who encourage exchange of staff and sharing of equipment. To facilitate administration, Dr. Garnier has been granted an honorary professorship at the University of Manchester, attached to the research unit, of which Dr. Robson is a director. In this scenario staff exchange with joint studies, and meetings are frequent and encouraged by contractual format.

"Materials", signifying in our case, magnetic tapes and diskettes of computer software and data, are equally frequently exchanged. Establishment of an electronic link has been less successful because of incompatabilities between the two stations. We have had, on the other hand, no problem in establishing links with British and German installations, and can at least transmit card images to our French collaborators. Nonetheless, this difficulty has encouraged a face-to-face contact which will hopefully not diminish as the difficulties in electronic contact are overcome.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: I.N.R.A., Orsay

Contract no.: BAP - 0148 - F

Project leader: J. GARNIER

Scientific staff:

Address: Université de Paris-Sud
Lab. de Biochimie Physique
Bâtiment 433
F - 91405 ORSAY CEDEX

Telephone no.: +33.1.69416429

Telex no.: 692166 FACORS

Other contractual partners in the joint project:

R. Bomford, The Wellcome Research Lab. (Beckenham)
B. Robson, University of Manchester

Title of the research activity:
Computer-aided peptide and protein engineering software development.

Key words:
Secondary structure prediction, Protein folding, Protein engineering, Synthetic vaccine, Energy calculation

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

To develop and improve software, methodologies and concepts for computer-aided protein engineering, emphasising those features which also have more general implications. Specific aspects which are inter-related or often used in unison are (1) secondary structure prediction, (2) prediction of epitopic protein surface sites as a basis for vaccine design, (3) supersecondary structure analysis and prediction, (4) energy calculation and interactive molecular graphics. In most cases these will be tested and refined in a practical, pragmatic way, to emphasise the factors more important in vaccine design, by synthesis and testing of peptide vaccines by collaborators.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Both laboratories are to work on particular problems associated with molecular graphics. However this has been postponed to some extent by the delay in obtaining the requisite hardware on site. The collaborative work with our english partners has been divided into two parts. We are developing basic, generally applicable algorithms, which our english collaborators are modifying and integrating into the vaccine design software. Already we have made very significant advances in secondary structure prediction and in the next phase of the project we will be tackling the problem of tertiary structure prediction by molecular dynamics, energy minimization and data base analysis.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1 - METHODOLOGY

For improvement of the prediction of secondary structures two methods were considered : the homologue method of Levin et al., (FEBS Letters, 1986, 205, 303-308) and the GOR method of Garnier et al., (J. Mol. Biol., 1978, 120, 97-120). These methods have been improved by :

1 - Developing a new data base from the Brookhaven protein data bank in selecting the well resolved and refined proteins : resolution greater than 2.8 A with a cristallographic R factor better than 0.25.

2 - Optimization of both the length of the peptides to be compared and the cutoff scores of the homologue method. The consideration of the family of proteins (Alpha rich, Beta rich, low secondary structure and mixed proteins) together with the establishment of the new data base leads also to a very significant improvement of the prediction accuracy.

3 - Addition of information using residues pairs to the GOR method and the use of the new data base. The use of the information theory has been further developed and this should lead to further improvements in accuracy with an increase of the data base.

4 - An attempt to develop a combination of these two programs with a bit pattern recognition method is under way, the results of which will be given in a later report.

2 - RESULTS

The homologue method gave a 59 % prediction accuracy for three states (Helix, Beta strand and Coil), when the protein to be predicted and all proteins with a percentage identity greater than 22 % were excluded from the calculation. The new data base provided a 1 % improvement in the results from 59 % to 60 % correctly predicted residues. An exhaustive series of tests was carried out to determine the optimal values for the window length and cutoff. This led to an improvement in the prediction accuracy of 3 % to 63 %. A scale developed to show the degrees of confidence in the results gave much the same results as the GOR method (see table I). The homology based method shows itself very powerful when homologous proteins are involved. When explicitly using the information that a homologous protein exists in the data base the accuracy of prediction increases very significantly. All proteins with a percentage identify greater than 30 % are predicted at more than 75 % correctly assigned residues. A prediction of the protein folding type based on the global amino acid content has been developed. This is 80 % accurate for four folding types (Alpha rich, Beta rich, Low secondary structure, and Mixed type proteins). Using this prediction, the secondary structure prediction increased to 66 %.

We have re-evaluated the information values used in the GOR method of secondary structure prediction with the new data base. The framework of information theory provides a means to formulate the influence of the local sequence upon the conformation of a given residue, in a rigorous manner. It is shown that the first level approximation, involving single residue parameters, is only marginally improved by an increase of the data base from 56 to 58 % of correctly predicted residues for three states. The second level approximation involving pairs of residues provides a better model. We have determined the significant pairs and the number of dummy observations necessary to obtain the best result for the prediction. This new version of the GOR method increases the accuracy of prediction by 7 % bringing the percentage of residues correctly predicted up to 63 % for three states when the protein to be predicted is removed from the data base to calculate the parameters (see table I).

Table 1

ACCURACY OF THE PREDICTIONS

Scale[1]	1	2	3	4	5
GOR III Pair Information					
Number of residues	11237	8200	5710	2531	900
% of residues	100	78	51	23	8
% correctly predicted	63	68.7	74.6	83.9	91.7
Homologue program					
Number of residues	12120	9407	6757	2404	979
% of residues	100	78	56	20	8
% correctly predicted	63	67.1	72.5	82.2	87.8

1 - The scale refers to the difference (information values or scores) between the predicted conformation and the immediate next conformation. Greater is the scale numbergreater is this difference. The results are expressed as percent of correctly predicted residues in three states : helix, Beta structure and aperiodic.

A program to combine the GOR and Homologue methods is currently under development. Preliminary results gave a 65 % prediction accuracy.

3 - CONCLUSION

These results shows that secondary structure prediction methods, or combination thereof are probably limited to 65 - 68 % correctly assigned residues, without a large increase in the data base. However the predictions can be improved by the addition of further information, for example as shown, the secondary structure prediction for a protein homologous with a protein in the data base is greater than 75 %, and there is a global 3 % improvement with the addition of the protein folding type prediction. These results provide an optimistic outlook for computer aided conformational determination.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1 Publications in Scientific journals :

Further Developments of Protein Secondary Structure Prediction using Information Theory. New parameters and Consideration of Residue Pairs. Gibrat J.F., GARNIER J. and Robson B. J. Mol. Biol. (in press).

IV.2 Technical note

Loops in proteins. Levin J.M., Jenkins J.A., Garnier J. (submitted to Science).

IV.3 Review letter.

Studies on rationales for an expert system approach to the interpretation of protein sequence data.

Preliminary analysis of the human epidermal growth factor receptor.

R.V. Fishleigh, B. Robson, J. Garnier and P.W. Finn.

FEBS Letters, 214,219-225, 1987, (This work is discussed in Dr. B. Robson's report).

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Our transnational cooperation has been very active with Dr. B. Robson's group in Manchester and Drs. Bomford and Aston from Wellcome. We exchanged computer programs with the Manchester group : energy calculation program LUCIFER from E. Platt and B. Robson, secondary structure prediction programs (GOR and homologue) from ORSAY. We had joint experiments and meetings with Dr. Bomford to test the vaccine program (see Dr. B. Robson report), and Dr. Robson and I meet regularly in Orsay or in Manchester. Inspired by the EEC contract, our two organizations, University of Manchester and INRA, decided to create a joint laboratory at both locations. This joint laboratory is named BIOMAT. There have, however, been some technical problems regarding a convenient routine file transfer link resulting from unexpected incompatibilities between the communicating systems. As a result we are exploring the facilities offered by Eurokom which are under test.

On a more general basis, the lack of technical assistance greatly slows our ability to distribute the improved versions of the secondary structure programs to other academic laboratories. One may note that there is also a commercial demand from private companies which is being taken into consideration with our british colleagues.

CULTURE COLLECTIONS

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Public Health Laboratory Service Board, London
Contract no.: BAP - 0002 - UK

Project leader: L.R. HILL
Scientific staff: V.M. Hughes (PHLS), E. Ward (CEC), C.S. Jones (PHLS)

Address: National Collection of Type Cultures
Central Public Health Laboratory
61 Colindale Avenue
UK - LONDON NW9 5HT

Telephone no.: +44.1.200.4400 EXT.3707/8

Telex no.: 8953942 DEFEND G

Other contractual partners in the joint project:

D. Claus, D. S. M. (Göttingen)

Title of the research activity:

European resource centres for plasmid-bearing bacterial strains.

Key words:

Plasmid collection, Genetic stability, Plasmid database, Host strains, Transposons

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The establishment of collections of plasmid bearing bacterial strains of medical (NCTC) and biotechnological (DSM) importance. Prior to this contract, there was no such provision within Europe. The project envisiges-

the setting up of plasmid laboratories
the collection of relevant strains from the scientific community
the establishment of methods and quality control procedures for both collected strains and patent deposits.
determination of optimum preservation and distribution methods
cataloguing of strains, construction of microcomputer databases.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Continued accessioning of strains from the Datta Collection of Gram negative clinical isolates and their plasmid transconjugants, supplemented with strains from other sources. Transfer from agar slope to freeze dried ampoule storage and sampling after one years storage where possible. Establishment of documentation and quality control routines for host strains, plasmids, transposons and vectors. Construction of the microcomputer database, data entry and compiling of the catalogue. Continuation of the supply of strains and information on request and catalogue distribution.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

METHODOLOGY and RESULTS

THE PLASMID COLLECTION

The collection available for distribution now comprises some 280 strains, and includes antibiotic resistant clinical isolates; reference plasmids for Inc grouping, molecular weight determination, resistance gene exemplars, and metabolic properties; transposons in plasmids and bacterial chromosomes; recipient strains; and vectors of historical interest.

Recent accessions include a colicin reference set, Pseudomonas Inc exemplars, transposon derivatives, E.coli K12 hosts for genetic manipulation, mini Inc gal plasmids and Streptococcal plasmids and recipients.

All the strains are examined on accession for the viability and purity of the culture, and the host strain checked for identity, auxotrophy, resistances, metabolic markers, recA deficiencies and phage resistance. Plasmid, vector and transposon encoded properties are confirmed usually by tests for antibacterial resistances, colicinogeny, metabolic markers and determination of correct plasmid DNA molecular weight. Vector plasmid structure and certain transposon inserts are confirmed by restriction endonuclease digestion and mapping. Selected plasmids are

tested for incompatibility properties or transposability of certain genes. Plasmid DNA received, is first transformed into a suitable recipient strain, and the verified host plasmid combination then given an accession number.

PRESERVATION OF GENOTYPIC AND PHENOTYPIC CHARACTERS BY FREEZE DRYING. Newly accessioned strains are stored in vials containing soft agar, and initially strains were distributed in this form. However such storage is bulky and some vials have been found to desiccate. A freeze drying programme was begun in January 1986 following the protocol in Figure 1 and applying the relevant quality control proceedures described above. Cultures were examined before dry (BD *), after dry (AD **) and after one years (1YR) ampoule storage, and selective and non-selective counts were made to estimate the % plasmid retaining cells (Table 1).

FIGURE 1

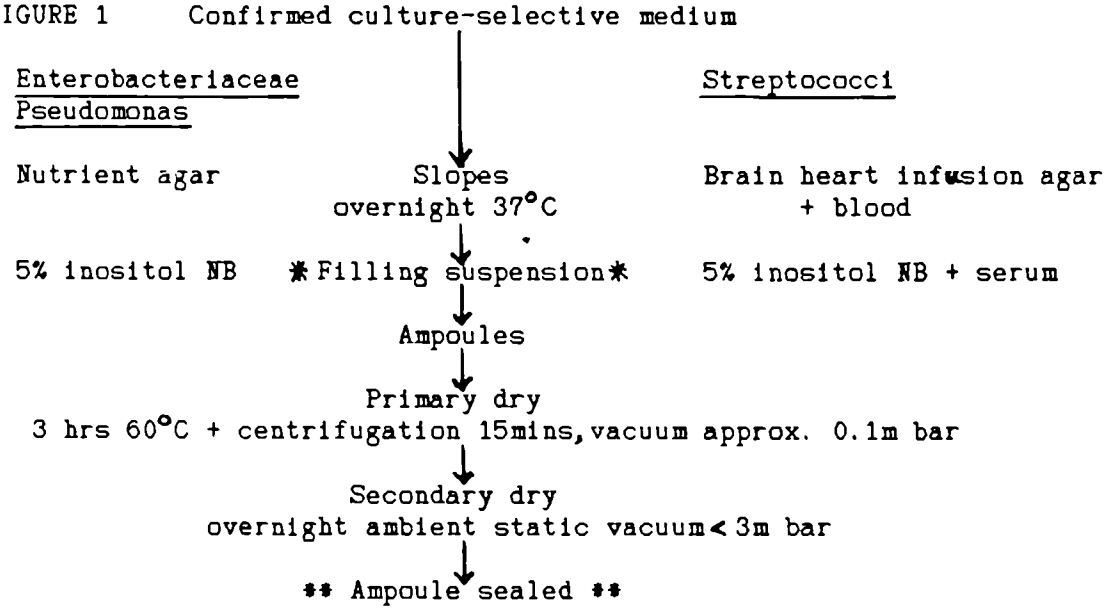


TABLE 1	TOTAL DRIED BD=AD	STABLE BD=AD=1YR	FAILED AD
PLASMID FREE STRAINS			
E. coli (includes recA and chromosomal transposons)	32	11	--
Enterobacteriaceae	5	2	--
Pseudomonas	4	1	--
Streptococci	3	--	--
PLASMID BEARING STRAINS			
E. coli R+	117	64	2
recombinant	36	8	1
others, (incl. multiplasmid)	26	8	--
Pseudomonas R+	15	--	--
Proteus, Klebsiella, Streptococci	7	--	--
	245	94	3

Many of the plasmid free strains were enfeebled, such as recA or with multiple auxotrophies, and one Pseudomonas strain had a tendancy to revert; these strains have been dried successfully and retained stability and viability.
Vectors, transposon insert plasmids and naturally occuring plasmids in a variety of host strains have, with 3 exceptions, retained all their tested characters and have proved stable. Unsuccessful dryings were a plasmid of IncD, known to be unstable in E.coli K12, and pHH502 and R144 known to delete DNA and markers; optimum preservation conditions have yet to be determined.

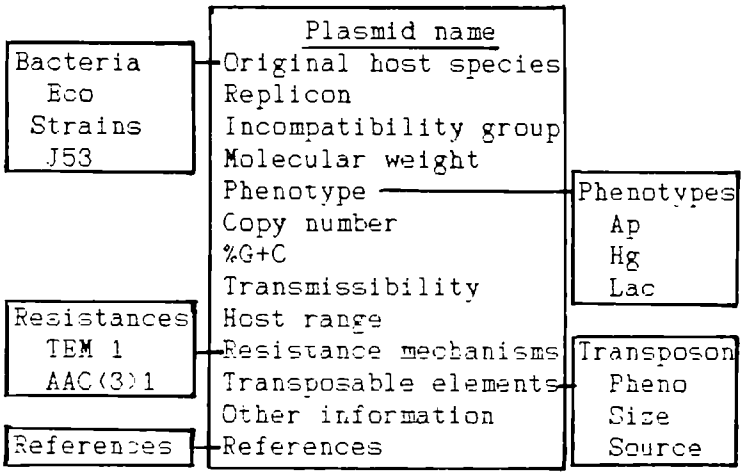
PLASMID SECTION DATABASE.
The database was constructed and developed using purchased database management software (ASPECT- Microft Technology), run on an Apricot Xen microcomputer with a 20Mb hard internal disc. It is organised into Plasmid, Strain, Transposon and Accession files and their supporting "look-up" files. An example of the fields within the Plasmid file and the relevant "look-up" files is shown in Figure 2. The catalogue is compiled by merging fields from the Plasmid and Accession files and generating new files for catalogue references and accession lists. The database incorporates powerful search facilities permitting a rapid selection of plasmids with particular properties in response to a request.

STRAINS SUPPLIED

Strains supplied	85/85	86/87
PHLS	129	58
UK	66	95
EEC	101	76
Overseas	32	80
	328	309

Categories	85/86	86/87
Inc	155	107
Transposon	20	30
Colicin	42	30
Mol Wt	25	51
Recipient	18	24
Pseudomonas	14	13
Specific	54	54
	328	309

FIGURE 2 - PLASMID FILE



DISCUSSION
Europe is now provided with an initial collection of plasmids of medical importance, available to scientists, industrialists and clinicians. Freeze drying ensures the stability of most genotypic and phenotypic properties. The database is an information resource suitable for searching and good use has been made of the collection in its first two years. We hope to increase our Gram positive holdings and to lay down seed stocks of catalogue strains in liquid nitrogen.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

Hughes, V.M., Jones, C.S. and Ward, E. (1986) Preliminary Catalogue of Plasmids, Phages, Strains and Transposons. PHLS London.

Hughes, V.M., Jones, C.S. and Ward, E. (1987) NCTC Plasmid Collection - New Accessions.

Hughes, V.M., Jones, C.S., Ward, E. and Hill, L.R. European Resource Centres for Plasmid Bearing Bacterial Strains - The NCTC's Plasmid Collection. BAP Contractors Meeting, Ioannina Greece, April 1987, Poster.

Hughes, V.M. European Resource Centres for Plasmid Bearing Bacterial Strains- The NCTC and DSM Joint Project. BAP Contractors Meeting, Ioannina Greece, April 1987, Talk.

Hughes, V.M., Jones, C.S. and Ward, E. From Sex Factors to Jumping Genes- The NCTC's Plasmid Collection. Poster presented at:-

European Culture Collections Organisation meeting, London UK, Sept. 1986.
XIV International Congress for Microbiology, Manchester UK, Sept. 1986
3rd European Congress of Clinical Microbiology, The Hague Holland, May 1987

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff	Yes	
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

EXCHANGE OF MATERIALS

Several strains were sent from the NCTC collection for inclusion in DSM's molecular weight standards kit.
Plasmid and strain stability results have been exchanged and compared for the different types of strain in each collection.

EXCHANGE OF STAFF

Dr Christine Rohde (DSM) visited in August 1986 and discussed methodology, collection content and saw the NCTC database in operation. Dr Elaine Ward (NCTC) visited DSM in April 1987 taking with her NCTC protocols, examples of our documentation and the specifications for the NCTC database. She returned with DSM protocols and forms and had discussions on the development of the DSM database.

JOINT MEETINGS

Dr L.R.Hill and Dr Victoria Hughes (NCTC), and Dr Christine Rohde (DSM) had further discussions at the BAP Contractors meeting in Ioannina, Greece in April 1987 and Dr Hughes was able to discuss with other BAP contractors and invited speakers the development of the plasmid database and the accessioning of further strains.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Ges. für Biotechn. Forschung mbH, Braunschweig
Contract no.: BAP - 0007 - D
Project leader: D. CLAUS
Scientific staff: C. Rohde

Address: Deutsche Sammlung von Mikroorganismen - DSM
Gesellschaft für Biotechnologische Forschung mbH
Grisebachstrasse 8
D - 3400 GÖTTINGEN

Telephone no.: +49.551.393822

Telex no.: 952667 GEBIO D

Other contractual partners in the joint project:

L.R. Hill, National Collection of Type Cultures (London)

Title of the research activity:
European resource centres for plasmid-bearing bacterial strains.

Key words:
Patent deposits, Plasmid collection, Plasmid stability, Preservation methods, Recombinant DNA

Reporting period: June 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The plasmid project of the DSM together with its partner, the NCTC, has the aim of establishing specialized bacterial plasmid collections, exchanging expertise and collaborating in research projects. NCTC has concentrated on medically relevant plasmids, DSM on biotechnologically interesting plasmids. Important tasks of both collections are: cataloguing plasmid bearing strains, performing quality control procedures, investigating plasmid stability after different long term storage preservation techniques. The latter is especially important since the genetic stability of patent deposits has to be ensured over a period of 30 years and since a fast growing percentage of patent deposits is plasmid bearing.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

DSM is expected to become a resource centre of plasmids with industrial relevance. After application of different preservation techniques the stability and "viability" of plasmids has to be checked comparatively. Fast routine procedures of plasmid isolation have to be worked out. "Viability testing" of free plasmids by transformation and the availability of suitable hosts is the precondition for accepting plasmid DNA as patent deposits under the Budapest Treaty. For this treaty issue of a viability statement for all biological material is mandatory. The facility to deposit pure plasmids is expected to be widely used. Except patent deposits, all plasmids and hosts are made freely available worldwide.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY

Culture preservation: At DSM plasmid bearing bacterial strains are preserved for long term storage in liquid nitrogen with glycerol as cryoprotectant. All strains are also lyophilized. Each plasmid bearing strain is grown on selective medium before preservation in order to maintain the plasmid(s). Before and after preservation viability of the cultures is tested and survival is compared.

Plasmid isolation: Plasmids of *Escherichia coli* strains are routinely isolated using small culture volumes. The cells are lysed according to the method of J.H. Crosa and S. Falkow (Manual of Methods for General Bacteriology, ASM, 1981). This fast isolation procedure is always used in case of relatively small plasmids. Large plasmids of Gram-negative bacteria are isolated according to F. Casse et. al., J. Gen. Microbiol. 113, 229 - 242 (1979). Molecular weights of plasmids are estimated in agrose gels.

2. RESULTS

Service activities: DSM now offers different groups of plasmids within hosts: i) a molecular weight reference "kit", containing 15 plasmids in the range of 2,0 kb-93,6 kb, ii) a collection of 20 degradative plasmids in different strains of *E. coli*, *Pseudomonas putida*, *Alcaligenes eutrophus*, and *A. paradoxus*, coding for degradation of different pesticides, iii) some cloning vectors in strains of *E. coli*. Plasmid-free host strains of *E. coli* for transformation are also available.

Patent deposits: DSM accepts not only microorganisms for deposit under Budapest Treaty, but also pure plasmid DNA. A reliable transformation system had to be established. Plasmid DNA is stored at -20 C and in liquid nitrogen, and transformants are preserved in liquid nitrogen. During these procedures loss of plasmid markers seems to be extremly unlikely. The percentage of plasmid bearing patent strains compared to the number of all patent strains at DSM is growing enormously : 1986 37%, 1987 (first quarter) 67%. All of the plasmid bearing patent strains of *E. coli* have been checked for their plasmid maintenance after lyophilization of the cultures, and the results are satifying: 97,3% of the strains maintained their plasmids stably. However, the plasmids were detectable in each case after preservation in liquid nitrogen. In 2,9% of all deposits the plasmids could hardly be detected in agarose gels, but the strains grew well on media selective for the plasmids.

Plasmid screening in strains of *Ancylobacter* (former "*Microcyclus*") are Gram-negative, vibroid to ring-like organisms, capable of growing with hydrogen as the energy source at low oxygen concentrations. Over 20 strains of *Ancylobacter* have been characterized physiologically, morhologically and genetically at DSM (by C. Herbst). Comparison of the strains lead to new taxonomic classifications within this genus: *Ancylobacter* consists of at least 3 different species. In a screening progamme 27 strains of the genus were checked for plasmid content, because it was already known that 2 of them carried large plasmids of more than 100 kb. We could show that large plasmids of similar sizes are widespread among these *Ancylobacter* strains. Only 2 strains were free of plasmid DNA.

3. DISCUSSION

Patent deposits: The stable maintenance of plasmid DNA after lyophilizing the bacterial hosts shows that this is most cases a suitable preservation method. Strains which contain recombinant plasmid DNA seem to be maintain their plasmids normally.

Nevertheless in 2,7% of the *E. coli* strains their plasmids are not stable after lyophilization. It was already described by other authors that storage in liquid nitrogen is the preservation method of choice for plasmid bearing bacteria. Absence of bands in agarose gels, however, does not necessarily mean that plasmids are lost completely. In these cases decreasing copy numbers must be considered.

Plasmids in *Ancylobacter*: The relatedness and grade of identity of the large cryptic plasmids of *Ancylobacter* strains has to be shown by comparing restriction patterns using different restriction enzymes. The plasmids of this genus should be the subject of a more intense research progamme, because in *Alcaligenes eutrophus* the ability of hydrogen oxidation is coded by a megaplasmid, which was intensively studied.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

C. Rohde and D. Claus: The German Collection of Microorganisms (DSM), a European resource centre for plasmid bearing bacterial strains of biotechnological importance, Biotechnology Action Programme, Meeting of contractors, Ioannina (Greece), April 1987 (Poster).

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

Several plasmid bearing strains were received from the NCTC plasmid collection. It is planned to join experimental research projects with the NCTC, especially projects which are related to collection specific subjects. Incompatibility grouping of plasmids will be such an object. During a visit at the NCTC in London, August 1986, experimental experience was exchanged. Specialization of the two plasmid collections was one of the most important discussion topics, as well as the experimental procedures during handling of deposits of plasmidbearing strains. In April 1987 a scientist of the NCTC's plasmid laboratory visited DSM. The establishment of a plasmid databank at DSM which is planned for the first half of 1988, was most urgent topic of this meeting. Scientists of both plasmid laboratories attended the meeting of contractors at Ioannina, Greece, in April 1987.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: P.H.L.S., Salisbury

Contract no.: BAP - 0001 - UK

Project leader: A. DOYLE

Scientific staff: R. Allen, R. Smith, S. Hunt, A. Walkland

Address:
ECACC - PHLS
Centre for Applied Microbiology
& Research
Porton Down
UK - SALISBURY, WILTS. SP4 OJG

Telephone no.: +44.980.610391 EXT. 511/12

Telex no.: 47683 PHCAMR G

Other contractual partners in the joint project:

H. Galjaard, Erasmus University (Rotterdam)

Title of the research activity:
European human genetic mutant cell bank.

Key words:
Genetic, Human, Cell bank, Lymphoblastoid

Reporting period: April 1986 - July 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

A collaborative project to establish a new Biotechnological Resource. This will comprise a cell bank derived from patients (and their families) with known genetic disorders. The Salisbury bank will lay emphasis on a Collection of EBV-transformed B-lymphoblastoid cell lines and the Rotterdam bank will lay emphasis on a collection of fibroblasts.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

To initiate Collection, set up Database between Collaborating Centres, allowing exchange of computer data. Produce Accession forms and literature. Establish Molecular Biology Laboratory within ECACC and to become familiar with necessary techniques. To initiate Collection of Human Genomic Screening Probes. To establish a network of Collaborating Centres within UK.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY

Following extensive discussion with collaborating centres the parameters have been set for the Collection of data within the European Human Genetic Mutant Cell Bank. The necessary Deposition forms have been agreed and computer data bases are established. The final format for exchange of data between the Contracting laboratories is now being pursued. It is the intention to produce a joint catalogue within the European Collection for Biomedical Research.

The Epstein-Barr Virus transformation laboratory is operational and receiving peripheral blood samples for establishment of B.lymphoblastoid cell lines. Some adaptation of techniques has been required due to the small sample volume submitted in many cases. Culture of some already existing cell lines has necessitated mycoplasma eradication to be carried out. The Molecular Biology aspects of the project is now set up to analyse DNA using RFLP's and DNA/RNA probes.

2. RESULTS

A network of collaborating centres has been established within the UK involving prominent Clinical Genetic Departments. These centres have already submitted numerous cell lines to the Bank. Informal meetings are arranged to discuss progress and priorities. The centres include: Dept. of Genetics, University of Oxford; Institute of Child Health, London; John Radcliffe Infirmary, Oxford; Guy's Hospital, London; Duncan Guthrie Institute, Glasgow; Wessex Regional Cytogenetics Unit, Salisbury. In addition a local regional advisor has been appointed, Dr. Dennis, Southampton General Hospital.

3. DISCUSSION

From these sources, over 1,000 cell lines have been submitted to the Collection since commencement of the contract. A network of Collaborating centres within the UK is established to supplement and enhance the work of the Contracting laboratories. Work is continuing to establish a distribution stock of cell lines and to make data on the material more widely available.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

1V2. INTERNAL REPORT. Appendix A

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff	Yes	
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

Exchange of samples has taken place between the Collaborating Centres and a member of ECACC staff has visited the Rotterdam laboratory to facilitate co-ordination of the computerisation of data within the banks. In addition, a member of the Rotterdam group attended a collaborative meeting with UK Clinical Geneticists held at Oxford.

A spontaneous meeting was held between the Salisbury and Rotterdam groups and two other BAP Contractors - Prof. G.B. Ferrara, Genoa and Prof. H. Grosse-Wilde, Essen. This meeting was held in Genoa in February 1987. Amongst the results of the meeting was a decision to co-ordinate our activities in the future as the European Collection for Biomedical Research. An important step as regards the Salisbury group was the decision to support the proposal that ECACC should become the major centre for testing the HIV status of human cell lines held by all the Cell Banking groups.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Stichting Klinische Genetica Regio Rotterdam

Contract no.: BAP - 0006 - NL

Project leader: H. GALJAARD

Scientific staff: M.F. Niermeijer, W.J. Kleijer

Address:
Erasmus University
Dept. Cell Biology & Genetics
P.O. BOX 1738
NL - 3000 DR ROTTERDAM

Telephone no.: +31.10.4087215

Telex no.:

Other contractual partners in the joint project:

A. Doyle, N.C.A.C.C. - P.H.L.S. (Salisbury)

Title of the research activity:
European human genetic mutant cell bank.

Key words:
Genetic disease, Human cultured fibroblasts, Cell bank, Family studies, DNA-makers

Reporting period: January 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Storage of cell material (mainly cultured fibroblasts) from patients and relatives with genetic Mendelian disorders and chromosomal variants and -aberrations. Availability of this material (in collaboration with other cell banks) is essential for development of new genetic diagnostic tests (DNA, enzyme analysis), also to improve genetic counseling to future relatives of patients affected with genetic disorders.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

1. Continuing collection of cell strains from patients/relatives (±700/year).
2. Development of computerised registry and -catalogue.
3. Collaboration with other cell banks (Salisbury, Genua, Essen).

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

General outline

There are ±4000 human genetic disorders with Mendelian inheritance, associated with a high risk of recurrence in siblings or offspring of patients. Enzyme or protein defects have been identified in ±200-300 of these disorders. The rapidly increasing number of polymorphic DNA markers has added a completely new dimension to (presymptomatic and prenatal) diagnosis and carrier testing of genetic disease. They are available now for all human chromosomes and may serve to make a diagnosis of diseases even in the absence of knowledge of the pathogenetic molecule (like in cystic fibrosis). At the same time, chorionic villus biopsy (CVS) added a new dimension to monitor pregnancies at an early stage (10th week) for certain genetic disorders.

The benefit of these developments will be dependent upon the continued availability of DNA material from patients with a genetic disease and their relatives, including from deceased index cases. Centers for

genetic research and diagnosis will increasingly need cell material from properly classified families (including unaffected relatives) wherein genetic Mendelian disorders segregate.
Sets of cultures of certain groups of diseases will be developped (if possible simultaneously in both centers);
- diploid human fibroblasts, in the Rotterdam laboratory
- long-term lymphocyte cultures, in the Salisbury laboratory.

The Rotterdam laboratory already has a collection of ±2200 cell strains of patients and carriers of metabolic and other genetic diseases and 1400 strains of chromosomal disorders. The Salisbury collection contains a.o. human tumour cells and a collection of animal cell strains. Both centers (will) have an electronic catalogue facility.

Specific activities in 1986-7

1. Larger informative cell strain sets were collected from families with important autosomal dominant,-recessive, and X-linked diseases: tuberous sclerosis, neurofibromatosis, von Hippel-Lindau disease, Polyposis coli, cystic fibrosis, Duchenne muscular dystrophy, X-L mental retardation with fragile site, adrenoleukodystrophy, etc.
2. Computerised registry and -catalogue
   For a registration system, the configuration available in the clinical center of Rotterdam was used. This consists of a VAX computer, with a relational data basis system (Oracle) and DQL.
   Of special importance in such a system (having many aspects of a genetic registry) are reliability of data, confidentiality (privacy) and possibility to make links between various persons from one family. A coding system of genetic conditions must fullfill requirements for precision not available in the ICD (international classification of diseases). For this purpose, we choose to use wherever possible - Mc Kusick's catalogue listings of genetic diseases, supplemented by the ICD-BPA, the British Paediatric Association Adaption to the ICD. The latter allows sharper description of congenital malformations, and enables unique syndromes to be catalogued as combinations of symptoms.

   Families are linked by family numbers.

   All cell strains collected previously and during this program are reviewed for completeness of diagnostic information, etc.

3. Collaboration between cell banks
Exchange of information, planning of coordinated activities was discussed at various meetings (see V).
Storage of data in compatible form (by adherence to identical diagnostic system (see III,2) was strongly advocated.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV,2 Niermeijer MF, Kleijer WJ, Galjaard H, Doyle A (1987) The European Human Genetic Cell Bank CEC Meeting on "New Methods in Animal Cell Cultures" Seillac, May 24-7 1987.

Niermeijer,MF (1987)
Report to Biotechnology Action Program section 2:1.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff	Yes	
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

09.10.86 Meeting at Dept of Genetics, Oxford (6B) (prof. Edwards). Introduction of program to British Genetic Services, represented by Prof. Edwards, Prof. Ferguson-Smith (Glasgow), Prof. Bobrow (London), prof. Patrick (London), by Dr. A.Doyle (Salisbury) and M.F.Niermeijer (Rotterdam). An advisory board was proposed for Great Britain.

24.02.87 Meeting at National Institute Cancer Research (Genua, prof. Ferrara); subjects:
- AIDS-testing in cell culture (to be developped in Salisbury).
- collaboration between cell banks in Salisbury (long-term lymphocyte cultures, tumour tissues/ cells, quality control), Essen (long-term lymphocyte cultures, HLA typing collection, HLA typing cells, long-term lymphocyte cultures of families with genetic cancer syndromes) and Rotterdam (fibroblasts from monogenic and chromosome disorders).
  This collaboration might be extended in the future and include gene probes, monoclonal antibodies and tumour tissues. In this way, the option arises of a European Collection for biomedical research.
- Compatibility of diagnostic listings (see III,2) was discussed.

24-27.05.87 C.E.C. Meeting on "New Methods in Animal Cell Culture" (organiser: Prof. Paraf) Seillac (France).
Presentation of various programs in BAP action program.
For the cell culture collections, the general approach and division of activities was seen as complementary. Further arrangements for coordinated activities were made.

- Other exchanges
Dr. Doyle (Salisbury) visited Rotterdam in 1986.
In May, 87, a co-worker of the Genua Cell Bank visited Rotterdam to study the organisation and computer registration.
In June '87, a co-worker of the Salisbury program visited Rotterdam to study the set-up of the computer-registry.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: The Agricultural University of Athens
Contract no.: BAP - 0143 - GR

Project leader: G. KALATZOPOULOS
Scientific staff: E. Tsakalidou, E. Manolopoulou, E. Kabaraki

Address: Agricultural College of Athens
Dept. of Postgraduate Studies
Dairy Section
Votanikos
GR - ATHENS 118 55

Telephone no.: +30.301.3465333

Telex no.: 225018 AGSA GR

Other contractual partners in the joint project:

G. Corrieu, I.N.R.A. (Thiverval-Grignon)

Title of the research activity:

Creation of a lactic acid cultures collection. Modelling and control techniques of thermophilic mixed culture.

Key words:

Collection of lactic acid bacteria, Thermophilic bacteria, Modelling and control techniques of thermophilic mixed culture

Reporting period: December 1986 - July 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Organisation of a lactic acid bacteria collection isolated from: a) natural fermented dairy products
b) offers of European laboratories
Study of biochemical and technological characteristics of the isolated cultures in order to isolate new strains with technological interesting aspects.
Preparation and maintenance of isolated cultures in order to provide cultures gratis to greek factories.
In collaboration with the French laboratories we will verify the results from the modelisation of selected thermophilic bacteria by the production of different kinds of yoghurt.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

We have selected from different regions of Greece traditional dairy products like yoghurt and fresh cheese of good quality made from sheep's and goat's milk. From these products we have isolated 153 lactic acid bacteria, which we try to identify according to the classical methods. At the same time we have started to choise the most convenient methods for the study of the proteolytic acid lipolytic systems of the isolated bacteria,according to the facilities of our laboratory, in order to determine their technological properties.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Methodology

Isolation and identification of bacteria

Bacteria isolated in selected media were identified using morphological and physiological tests,the kits of API-CH50, API-ZYM and the evolution of acidity during incubation at $37^{o}/42\ ^{o}C$.

Study of the enzymatic systems of bacteria

Bacterial strains

The Lactobacillus strain used for this study was *L. bulgaricus* 398CNRZ. Cells were stored at $-20^{o}C$ in sterile Litmus Milk.

Growth of cells

The milk media and the method described by Ezzat et al (1)were adopted for growing the cells. Growth was assessed by optical density measurment at 480 nm (2).

Release of cell-wall-associated (CWE) proteinases and preparation of intracellular extract (IE)

A modification of the method described by El Soda et al.(3) was used. The release of lactate dehydrogenase (LDH) was used to estimate cell lysis (4).

Protein determination

The method of Lowry et al. (5) was used with bovine albumin serum as a standard.

Electrophoretic fractionation of aminopeptidase (AP) and esterase (ES) activities

These enzymes were detected after electrophoresis of the cellular extracts (6). AP were detected on gels with L-Leucine -β-naphthylamid (7). ES with α-naphthyl-acetate(8).

Spectrophotometrical detection of aminopeptidase (AP), endopeptidase (EP) and esterase (ES) activities.

AP, EP and ES activities were assayed at 410 nm by assessing the degree of hydrolysis of L-leucine-4-nitroanilide (LNA), N-acetyl-L-alanine-4-nitroanilide (NAANA) and 2-, 4-Nitrophenyl-butyrate (NpB) respectively (9), (10).

Proteolysis of casein

The method described by Exterkate (9) was used for no labeled casein.

2. Results

I. Selection of lactic acid bacteria for the collection

a) from traditional milk products

isolated (not completely identified)	153
Lactobacillus	42
Streptococcus	111

b) from foreign cultures

[illegible]	9	[illegible]	16
[illegible]	14	[illegible]	3

L. acidophilus	7	*S. diacetilactis*	8
L. brevis	1	*S. cremoris*	3
L. casei	4		

II. Biochemical results of *L. bulgaricus* 398 CNRZ

Post-electrophoretic tests			Spectrophotometrical detection			
Enzyme / Fraction	ES(Rf)	AP(Rf)	AP	EP	ES(2NpB)	ES(4NpB)
CWE	nr	nr	nr	nr	nr	nr
IE	0.32 0.40 0.48	0.33	1600(a)	nr	nr	5.4(b)

nr : no reaction

a : moles nitroaniline.10^{-10}/ml.h

b : mμmole 4-NpB/min

3. Discussion

The identification of the isolated strains is in progress.We are also planning the study of the genetical material of these strains in order to confirm their intraspecies differentiation The propagation of the microorganisms will begin after we'll have received the ordered equipment.

The first biochemical results are indicative for the behaviour of the *L. bulgaricus* 398 CNRZ. Further experiments with other substrates and under miscellaneus conditions especially with temperature and pH will complete the study of biochemical profil. The electrophoretic profil will contribute to this aspect too. The study of its proteolytic activity with casein as substrate is now in progress.

REFERENCES

1. N. Ezzat, M. El Soda, C. Bouillanne, C. Zevaco and P.Blanchard, Milchwissenschaft 40(3), 140-143 (1985)
2. T.D. Thomas and K.W. Turner, N.Z. Jl. Dairy Sci. Technol. 12, 15-21 (1977)
3. M.El Soda, M.J. Desmazeaud, D. Le Bars and C. Zevaco, J.of Food Prot. 49 (5), 361-365 (1986)
4. Biochemica Information (Boehringer)
5. O. Lowry, N. Rosebrough, A. Farr and J. Randall,J. Biol.

Chem. 193, 265-275 (19751)

6. G.C. Cooper, Biochemische Arbeitsmethoden, Berlin, New York de Gruyter, 1980
7. C.G. Miller and K. Mc Kinnon, J. of Bacteriol. 120,355-363 (1974)
8. W.J. Harper, A. Carmona de Catrie and J.C. Chen, Milchwissenschaft 35 (3), 129-132 (1980)
9. F.A. Exterkate, Neth. Milk Dairy J. 29, 303-319 (1975)
10. E. Brandland, T. Zizer, Öster. Milchwirtschaft, wiss.Beil. 28, 15-24 (1973)

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.2. Short Communication, internal reports

G. Kalatzopoulos

Biotechnological Contribution for the progress of the fermented dairy products in the mediterranean region.

Report for the workshop of COST 91 bis sub-group Food Biotechnology, 13-14 April 1987, Lisbon-Portugal

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	No
Exchange of staff	No
Joint experiment(s)	No
Joint meeting(s)	Yes

Descriptive information for the above data.

Culture Collection and Genetic Engineering of Microorganisms
Ioannina, Greece, 23-25 April 1987.
(Biotechnology Action Programme, Meeting of Contractors)

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: I. N. R. A., Thiverval-Grignon
Contract no.: BAP - 0144 - F

Project leader: G. CORRIEU
Scientific staff: M. Desmazeaud, E. Spinnler, J. Richard, C. Beal, A. Zouari, V. Julliard

Address: INRA - L.G.P.B.A.
Centre de Biotechnologie Agro-Industrielles
F - 78850 THIVERVAL-GRIGNON

Telephone no.: +33.1.30.54.45.10

Telex no.: 697.388 F

Other contractual partners in the joint project:

G. Kalatzopoulos, Agricultural College of Athens

Title of the research activity:

Creation of a lactic acid cultures collection. Modelling and control techniques of thermophilic mixed culture.

Key words:

Lactic acid bacteria, Collection culture, Mixed cultures, Modelling, Control

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

1* Creation of a lactic acid thermophilic bacteria culture collection.

This collection will include industrial strains selected on the basis of technological properties and strains isolated from Greek's traditional yogurts and cheeses.

2* Improvement of knowledge and control of mixed cultures of thermophilic lactic acid bacteria.

Widely used in dairy industries, mixed cultures are poorly known on microbiological, physiological and technological aspects. We intend to quantify the cooperation phenomenon and to improve the starter's and yogurt's production processes using modelling and technics of on-line automatic control.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

- to compare technological parameters of several strains of thermophilic lactic acid bacteria in order to be able to create a strain collection of industrial interest.

- to characterize the effect of cooperation between strains on growth parameters.

- to find on-line measurements of the different strains cultivated together to be able to control mixed culture growth.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

CREATION OF A LACTIC ACID CULTURE COLLECTION

1 - Methodology:

Samples of yogurt made in 10 small manufactures in Cretis were stored at 0°C until their bacterial analysis 2 weeks later. After that time streptococci and lactobacilli were counted using conventional methods (i.e. MRS incubated in anaerobic condition and M17). An attempt was also done to improve the recovery of bacteria stressed by a long storage under anaerobic conditions at low temperature and low pH (use of another medium clamed to be less selective than MRS or M17, enumeration procedure under nitrogen atmosphere using deaerated diluent and pre-reduced culture media, addition of catalase to the culture media). Viability of the microflora of yogurt samples during storage was also observed.

At each stage of the study, colonies differing in morphology or pigmentation were isolated from the different media used and classified in the usual way. Streptococci and lactobacilli were then submitted to the classical procedure for identification of the species. 20 strains of each were studied using additionnal biochemical and physiological tests, to select criteria for intraspecies differenciation.

2 - Results

Maximum care to reduce the influence of oxygene on bacteria during enumeration gave slightly higher counts than the ordinary procedure. A small improvement was also observed when using media containing catalase. On the other hand, there was a slight decrease of the lactobacilli during the storage of the yogurts and a relative stability of the streptococci.

All the colonies growing on MRS were lactobacilli and those growing on M17 streptococci whatever their appearance. About 200 strains of each group were isolated during the study. All the streptococci were identified as S. thermophilus and the lactobacilli as L. bulgaricus. Additionnal tests failed to reveal intraspecies differences in both group.

3 - Conclusions

A collection of about 200 strains of S. thermophilus and 200 strains of L. bulgaricus isolated from traditionally made yogurts is now available. Intraspecies differenciation by technological criteria (growth and acidification rates, post-acidification, aroma production...) and by other biochemical means (e.g. protein electrophoresis) are planned in order to select a few (6-10 ?) strains of both groups for a more detailed study.

CONTROL OF MIXED CULTURES

1 - Methodology

Cultures were made in 10 l fermentor with pH and temperature automatic control. Dissolved CO2 was measured with an Ingold probe. Gaseous CO2 was estimated with a volumeter. Urease activity was measured by NH4+ estimation. Cell counts were made using Breed's method. Lactose and galactose were measured by HPLC. Enzymatic methods were used to quantify Glucose, D Lactate and L Lactate. The growth rates and the different yields were calculated by an appropriate data analysis software. Especially as it was impossible to differenciate the substrate of each population growth yields were calculated in regard of the fermentation products (L. bulgaricus producing D. Lactate and S. thermophilus L. lactate).

2 - Results

a) On line estimation of populations concentrations

To control a mixed culture of several populations it is necessary to quantify the concentrations of each population. This estimation is of first importance to compare this value to a set point (percentage of each population...) and if necessary to act on the system.

In the case of mixed cultures of Streptococcus thermophilus and Lactobacillus bulgaricus we looked for methods permitting on-line measurement of these populations.
In the litterature it has been demonstrated that a strain of S. thermophilus could produce urease. We showed that many strains of S. thermophilus own this property. Moreover we found that if this urease is not constitutive, a low level of urea in the medium permits a maximum expression of this character. In that case, the urease activity is proportional to the S. thermophilus concentration. This enzyme produces CO2 and NH4+ from urea.If urea is in excess, rate of CO2 production is proportional to enzyme concentration. As a consequence the Streptococcus concentration is proportionnal to rate of CO2 production. We verified this result in pure and mixed cultures on two different media and with different pH and temperature set points.

Dissolved CO2 measurement are usable for Streptococcus populations of 10 7 to 10 9 cells/ 1. (figure 1)

The measurement of CO2 volumes produced can be used for bigger populations.

We are studying how using the neutralizer amount needed to maintain a constant pH. Moreover on-line D and L Lactic measurement may allow us to estimate total population or Lactobacilli population.

b) Study of the protocooperation between strains of S. thermophilus and L. bulgaricus.

Our results were obtained in pure and mixed culture on milk based medium. Our objectives were to quantify growth rate and growth yield in mixed cultures containing 0 to 100 % of S. thermophilus. These results permitted to show an improvement of growth yield of L. bulgaricus in the presence of S. Thermophilus. However on that medium the mixed cultures do not show any improvement of growth rate. Assays are made on poorer medium (enriched whey), close to industrial media, to see if the results are the same.

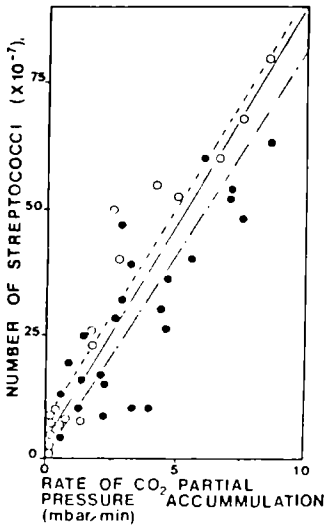


Fig. Correlation between rate of dissolved CO_2 accumulation and the number of streptococci in the culture. These results were obtained in pure culture of *S. thermophilus* (O--- O) and in mixed cultures with *L. bulgaricus* (●- - - ●). The straight line corresponds to the points obtained in pure and mixed cultures together. This is the result of 10 assays where the pH was regulated at defined values between 5.5 and 6.75, temperature between 38°C and 44°C, the initial concentration of urea was between 1 and 2.5 g/l

3 - Conclusions

At the moment one of the two strains can be followed by on-line measurement, work is done to obtain the same result with the other.

The characterization of growth yield and growth rate show an improvement of growth yield of L. bulgaricus. It is not the case of the growth parameters of S. thermophilus in mixed cultures on the medium choosed.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

1 - Publications in Scientific journals, monographs.

- H.E. SPINNLER, C. BOUILLANNE, M. J. DESMAZEAUD G.CORRIEU.
  Measurement of the partial pressure of dissolued C02 for estimating the concentration of St. thermophilus in coculture with Lb. bulgaricus.
  Appl. Microb. Biotechnol. (1987) 25, 464-470

- V. JUILLARD, H.E. SPINNLER, M.J. DESMAZEAUD, C.Y. BOQUIEN.
  Phénomènes de coopération et d'inhibition entre les bactéries lactiques utilisées en industrie laitière.
  Le lait (1987) 67, 149-172

2 - Short Communications, Internal reports.

- H.E. SPINNLER, C. BOUILLANNE, M.J. DESMAZEAUD
  Réunion Société Française de Microbiologie (Groupe des chercheurs travaillant sur les bactéries lactiques). Communication orale. Février 1987, Caen.

- G. CORRIEU
  Réunion Société Française de Microbiologie (Conservation des microorganismes). Communication orale. Mars 1987, Lille.

- ZOURARI A. Compte rendu de fin de stage. Bourse CEE. Avril 1987

3 Doctorate Thesis, Degree Thesis.

- JUILLARD V.
  Mise en évidence d'une activité uréasique chez Streptococcus thermophilus (Septembre, 1986). D.E.A. de Sciences Alimentaires, Université de Paris VII.

- COLOMBAN C.
  Etude de la coopération entre Streptococcus thermophilus et Lactobacilus bulgaricus (Octobre, 1986). DEST de Nutrition. U.E.R. de Pharmacie. Chatenay Malabry.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff	Yes	
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

Exchange of material:

- strains exchanges
- Critis's cheese

Exchange of staff:

- A. Zurari from E.S.A.(Athens) to INRA(Jouy-en-Josas)

Joint meeting:

- Jouy-en-Josas May 14th 1986
- Grignon January 27th 1987
- Athens April 22nd 1987

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Universitätsklinikum Essen
Contract no.: BAP - 0037 - D

Project leader: H. GROSSE-WILDE
Scientific staff:

Address:
Institut für Immungenetik
Universitätsklinikum Essen - GHS
Virchowstrasse 171
D - 4300 ESSEN 1

Telephone no.: +49.201.7232526

Telex no.: 8579573

Other contractual partners in the joint project:

G.B. Ferrara, I. N. R. C. (Genova)

Title of the research activity:

Bank of immunogenetically defined human B-lymphoblastoid cell lines.

Key words:

Epstein-Barr-Virus, HLA homozygous lines, HLA linked diseases, Leukemia, HLA associated diseases

Reporting period: June 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The general aim is to set up a collection of EBV transformed B-cell lines from individuals known to be homozygous for HLA (A,B,C,DR,DQ,DP,D) and other linked markers (Bf, C2, C4A, C4B). The collection is expanded to patients with HLA linked or associated diseases and their families. One major goal of this project is to compile and update a catalogue with all immunogenetical relevant data generated. This catalogue and the respective lines will be made available to interested groups and relevant companies for recombinant DNA work as well as for new drug development.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The specific aims during the last year were:

1. Selection, HLA-typing, complotyping, EBV-transformation, expansion and freezing of immunogenetical important B-cell lines from homozygous individuals and patients with HLA linked or associated diseases and their families.
2. Development and establishment of control steps for sterility (mycoplasma) and identity of the lines incorporated into the bank.
3. Generation of a computer data base for storage of relevant informations of the lines, and data exchange through EUROKOM.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

In cooperation with Dr. Ferrara (IST Genova, Italy) and under the patronate of the European Community we have started since 07/1986 to collect and stock EBV-transformed human lines. The aim of this bank is to provide stable and continuously growing lines for seroligical, cellular, biochemical, and molecular biological research. Source of these lines are families with HLA-linked or associated diseases, hematological disorders, and families with unique immunogenetical constelations as HLA homozygosity, gene duplication(s), or recombination within the HLA-complex. The more than 300 established lines comprise the following HLA-linked or associated diseases: psoriasis vulgaris, SLE, CDLE, IDDM, HLA-B27 positive or negative spondylitis ankylosans, CAH, and narkolepsy. In addition we collect tumor cells and EBV-transformed lines from leukemic patients and their relatives (AML, CML, ALL, etc.).

"Finger printing" of individual lines: Before freezing and storage the lines are analysed for HLA Class I & II and compared to original typing data. This check involves serological and biochemical characterisation.

Microbial contamination: Mycoplasma infection of human lines is often detectable. We test every line before freezing and after regrowing for mycoplasma infection (Hoechst dye). Positive lines undergo treatment with antibiotics and/or mouse macrophages. The treatment is indexed in the catalogue.

Protection of privacy: All lines of the bank are labelled with individual code numbers to provide protection of private data from patients and their relatives. Only family relationship, diagnosis, and HLA-type is incorporated in the official banking catalogue.

Future perspectives: Several laboratories has been asked for and offered lines or blood specimens to the bank. The received material will be analysed carefully to avoid duplications. The collection of leukemic cells offers the possibility to establish continuously growing lines provided that in the future specific vectors are available. Furthermore immunogenetically relevant cDNA probes and oligonucleotide probes will be included in this European Collection for Biomedical Research (ECBR).

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

None

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	No
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

ad 1: We have exchanged material, i.e. cells and established cell lines with the Instituto
Nationale per la Riserca sur Cancro, Genova, Italy.

ad 2: dito

ad 3: Establishment of human cell lines from donors producing HLA specific antibodies. The experiments are in progress, and the results will be given in the next report.

ad 4: During the report period three meetings with participants of both laboratories have been organized. Two of them took place in Genova/Italia and one in Essen/Bundesrepublik Deutschland.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: I.N.R.C., Genova

Contract no.: BAP - 0072 - I

Project leader: G.B. FERRARA

Scientific staff: B. Parodi, M. Talio, G. Angelini, S. Ravazza, L. Ugozzoli, A. Longo

Address: Istituto Nazionale per la Ricerca sul Cancro
Viale Benedetto-XV 10
I - 16132 GENOVA

Telephone no.: 0039.10.352896

Telex no.: 216353 ISTEX I

Other contractual partners in the joint project:

H. Grosse-Wilde, Universitätsklinikum Essen

Title of the research activity:

Bank of immunogenetically defined human B-lymphoblastoid cell lines.

Key words:

Epstein Barr Virus, HLA-homozygous lines, Gluten enteropaty, Juvenile diabetes mellitus, Psoriasis vulgaris

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The aim of this project is to set up a bank of human B lymphoblastoid cell lines from individuals known to be homozygous with respect to HLA specificities, from patients affected with diseases proved or supposed to be of genetic origin, and from their families.
These lines can be used for better define, both at the cell surface level and at the gene level, the role of the HLA molecules in the immune response.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

1) Selection, extensive HLA typing and production of EBV lines from:
   a. consanguineous individuals, to be used as healthy reference panel in studies of polymorphism of HLA;
   b. families significant for HLA-linked/associated diseases.
2) Expansion, sterility assays and storage of B cell lines and PBL of each individual.
3) Creation of a suitable computerized data bank, with clinical, biochemical, molecular data from each patient.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1) Methodology

Transformation of human B cells is performed by Epstein Barr Virus infection. As infecting virus, the supernatant of a Marmoset EBV-secreting B cell line (B95-8) is used.

HLA typing is performed by microlymphocytotoxicity assay (A, B, Cw, DR, DRw, DQw antigens), by Primed Lymphocyte Test (DP antigens) and by Southern Blot Hybridization (alpha and beta DR, alpha and beta DQ, alpha and beta DP, beta DO and alpha DZ).

Because micoplasma infection is frequent in cultured human cell lines, every line is tested for micoplasma contamination by a culture isolation assay, before freezing and after regrowing.

2) Results

At the moment in Genova are stored about 400 B cell lines, including a large panel of HLA-homozygous lines (180 lines), a number of HLA-typed healthy families (48 lines), and significant families with HLA-linked/associated diseases, as IDDM (56 lines), gluten enteropathy (130 lines), etc.
To confirm the identity of the lines stored, the serological HLA typing is controlled before each shipment.

The lines stored in the bank are used in our laboratory for different purposes: the HLA-homozygous lines are tested by classical serological methods for new HLA specificities (QATL-like antigens, serologically detectable DP antigens). Cellular studies are performed by human alloreactive T cell clones, to map the function of Class I and Class II molecules in the immune response.
The lines are used also for DNA studies with genomic probes and oligonucleotides, for the detection of old and new polymorphisms.

A computerized data bank for the cataloguing of the lines has been set up, and a catalogue common to the two institution collaborating in this project will be soon published.

3) Discussion

The possibility of indefinitely maintainig in culture human B cell lines by EBV transformation opens great perspectives to the biological research.
Such cells represent a virtually endless source of homogeneous cellular material for studies on molecular biology, biochemistry and function of B lymphocytes.
The availability of a large panel of well characterized B cell lines from families representative for HLA-linked/associated diseases allows, thanks to new molecular biology techniques as oligonucleotide probes and gene amplification, a further comprehension of the role of HLA antigens in immune response and diseases.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

Continuous exchange of material (EBV-producing cell line and supernatant, EBV-transformed B cell lines), as well as of informations on new technical approaches to transformation, typing and micoplasma contamination assays, takes place between the two laboratories collaborating in this project.

Two joint meetings have been held, up to now.
The first in Genova on February 24th.This was in common with the other contractorsof Biotechnology Action Program section 2.1: Culture Collections, and the participants were: Prof. Dr. M.F. Niermeijer (Rotterdam, the Nederlands), Dr. A. Doyle (Salisbury, G.B.), Prof. Dr. H. Grosse-Wilde (Essen, GFR) and Prof. G.B. Ferrara and coworkers (Genova, Italy).
The second was in Essen on July 6-8 1987, between the two collaborating contractors in the joint project, with as participants Prof. Dr. H. Grosse-Wilde and Dr. I. Doxiadis (Essen), Prof. G.B. Ferrara, Dr. A. Cauvin and Dr. B. Parodi Genova).

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Museum d'Histoire Naturelle, Paris

Contract no.: BAP - 0028 - UK

Project leader: M.-F. ROQUEBERT

Scientific staff:

Address: Museum National d'Histoire Naturelle
Laboratoire de Cryptogamie
12, rue Buffon
F - 75005 PARIS

Telephone no.: +33.1.43313521

Telex no.:

Other contractual partners in the joint project:

D. Smith, C. A. B. (Kew)
N.M. Nolard-Tintigner, Inst. d'Hygiène et d'Epidém. (Bruxelles)
G.L. Hennebert, U. C. L. (Louvain-la-Neuve)
C. de Bièvre, Institut Pasteur

Title of the research activity:

Development of improvement techniques for the preservation of fungal strains of biotechnological importance.

Key words:

Fungal strains, Preservation methods, Stability, Metabolite production growth

Reporting period: December 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Culture collection of fungi provide a vital genetic resource for biotechnology. They provide essential services for the conservation of living organisms and are a necessary adjunct to the patent system. In this view it is important to preserve for a long term the integrality of physiological and biochemical features of the strains. The aims of the project is to improve methods of preservation of fungi of biotechnological importance by development of optimal procedures for freezing and freeze-drying and cheking the characteristics of the strains after treatment and during some years of storage.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

LCP participates to this project by providing strains of biotechnological interest and by checking stability of fungi after cryopreservation and freeze drying.

•LCP looks at Aw and temperature tolerances and tests secondary metabolites (for example, patulin production is checked for *Penicillium expansum*).

Two fungi are actually studied: *Penicillium expansum (LCP 3384) and Mucor racemosus (CBS 260 68)*.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1 METHODOLOGY

Metabolite production: after treatment, CBSsend us, for each strain, 7 slants inoculated with the 6 treated spores suspensions and one untreated. From them, new spores suspensions are prepared, streaked on Cz agar substrate (in 90mm Petri dishes), and incubated at 22°C during 10 days. Sporulating cultures and their substrates are removed from Petri dishes and mixed with 20ml chloroform-methanol (1:1, v/v) for extraction of metabolites. After 15mn of sheaking and slight heating, the mixture is filtered and available for TLC. Regular spots are deposited at the origin of TLC plates against a patulin standard (for *P.expansum*); after drying, plates are developped in two different systems, under saturated conditions.

System 1:	TEF	toluen	System 2:	chloroform
		ethylacetat		methanol
		90% formic acid		(98/2: v/v)
		(70/40/4: v/v/v)		

Patulin is detected under short-wave UV light (254nm).

Aw tolerance: Aw tolerance is investigated by measuring fungal growth on a range of Aw controlled solid medium, determinated after preliminary tests. Suitable concentrations of glycerol are incorporated to a 2% malt agar

medium to obtain Aw from 0,90 to 0,995. Medium are inoculated by spots of spores suspensions and incubated at 25°C.

Temperature tolerance: the principle of this test is similar to the Aw tolerance one. 2% malt agar medium or Cz medium are inoculated and incubated at 5, 25, 30 or 32°C.

The 8 preservation technics are refered as: 1-subculturing maintenance, 2-freeze-drying, 3-out of -20°C, 4-quick freezing out of -196°C, 5-quick freezing out of -135°C, 6-slow freezing out of -196°C, 7-slow freezing out of -135°C, 8-storage at -80°C.

2 RESULTS

Patulin production by *P.expansum*

As shown on the photograph (figure 1), patulin production is maintained in each sample.

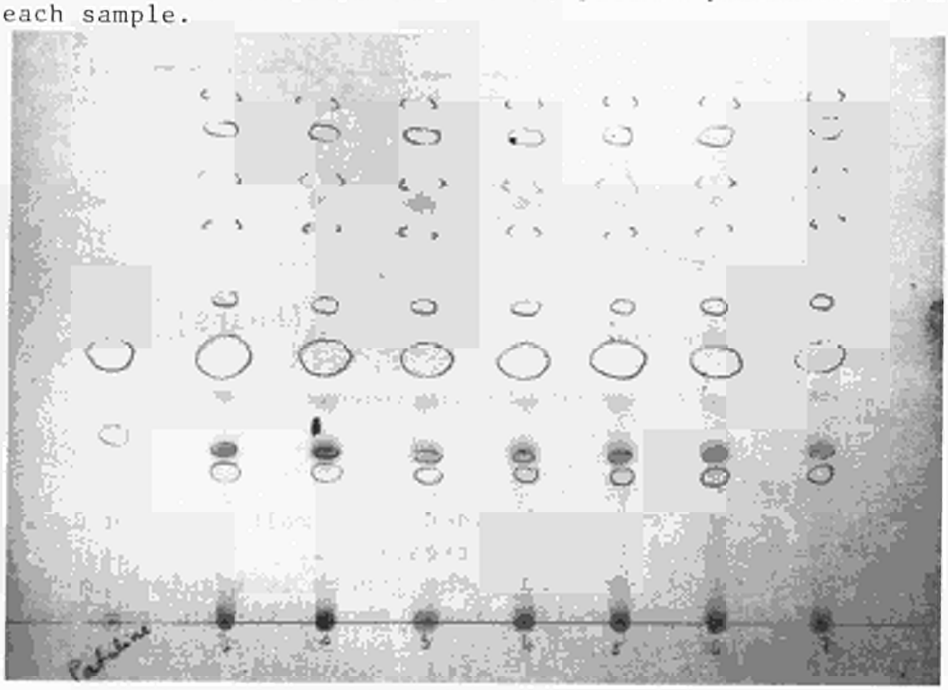


Figure 1: patulin production by *P. expansum* after preservation treatment (solvant system 1).

AW tolerance:

Penicillium expansum: Figure 2 shows the growth of the fungus on 0,99 Aw medium (8 preservation methods). They are very closed one from other and this grouping is similar on other Aw medium tested: 0,90-0,92-0,94-0,98 and 0,995. So, linear growth of *P.expansum* seems not to be altered, on various Aw medium, by the used treatments.

Mucor racemosus: Figure 3 is a representative exemple (Aw 0,98) of the divergence of the 8 growth curves. The worse growth is obtained after storage at - 80°C (8) and the best after freezing and storage at -196°C and -135°C (4,6 and 7). Theese results indicate that the type of preservation may have an incidence on the fungal growth of *M.racemosus*.

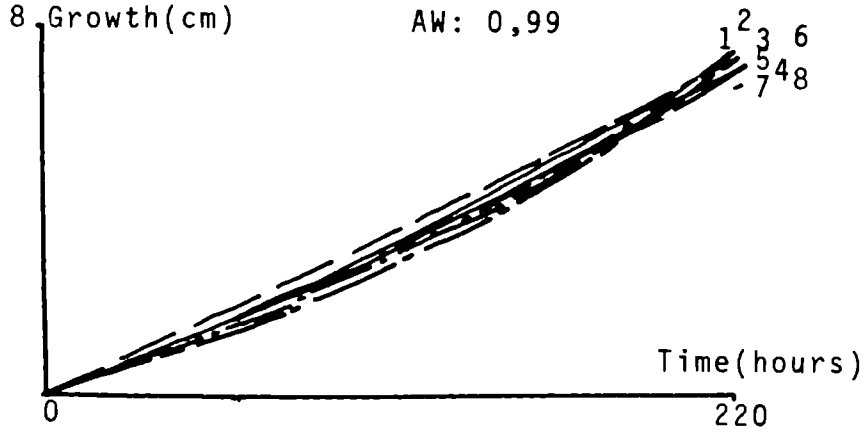


Figure 2: growth curves of *P. expansum* differently preserved (treatment 1 to 8, see methodology) on Aw 0,99 medium.

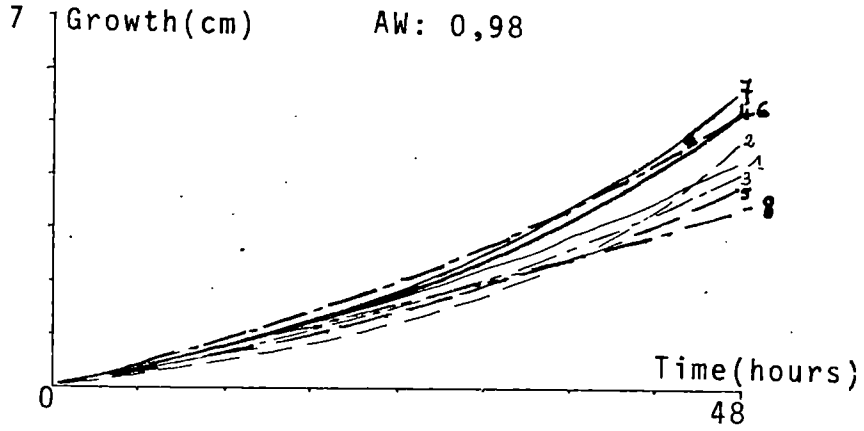


Figure 3: growth curves of *M. racemosus* differently preserved on Aw 0,98 medium.

Temperature tolerance:

Penicillium expansum: growth curves obtained on 2% malt agar and Cz at 5, 25 and 30°C are very closed for the 8 treatments.

Mucor racemosus: at 5°C on 2% malt agar, the growth is regular for the 8 treatments. At 25 and 32°C, we obtain a lower growth for the treatment 1 (subcultures) and 3 (out of -20°C).

3 DISCUSSION

The effect of preservation treatment on stability of fungal strains seems differ with the species tested.

The *Penicillium expansum* strain shows no difference for metabolite production nor Aw and temperature sensibility with the preservation treatment used.

Mucor racemosus appears more sensible. Storage at -196°C after quick or slow freezing (4 and 6) are the best conditions of preservation for stability of growth. In some case freezing speed before storage is important as shown by the results obtained after storage at -135°C.Slow freezing (7) is more efficient than quick freezing (5).Cryomicroscopy will be an help to understand this phenomenon. Traditional subculturing preservation appears not to be optimal method of preservation since growth is affected when incubated at 25 and 32°C. Development on Aw 0,98 medium is better after storage at -196°C than after subculturing.

Nevertheless, the growth of *M. racemosus* is usually very fast (9cm by 48 H.) and mesuring of colony diameter may be a method not sensible enough to determine true comportemental differences. An evaluation of the germination rate of spores on different conditions should complement those preliminary results.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	No
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

EXCHANGE OF MATERIALS AND JOINT EXPERIMENTS

Fungal strains with known biological activities are exchanged with other contractors in view of constitute a pool of strains of biotechnological importance. For the moment, *Penicillium expansum* (LCP 3334) is the support of complementary experimentsfrom all the contractors for testing methods of preservation and stability.

JOINT MEETINGS

Two meetings have been held between contractors. The first (Bruxelles 14 Nov. 1986) for choosing strains and methods, planning the experiments and dispaching the responsibilities. During the second (Louvain 24 Fev. 1987) first results were exchanged and discussed and planning modified.

Ioannina (25 Avril 1987) presentation and discussion about the project.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Inst. d'Hygiène et d'Epidémiologie, Brussels
Contract no: BAP - 0028 - UK

Project leader: N.M. NOLARD-TINTIGNER
Scientific staff: V. Baldewijns, Rouma
H. Bequin

Address: Institut d'Hygiène et d'Epidémiologie
Section Mycologie
14, rue Juliette Wytsman
B - 1050 BRUXELLES

Telephone no.: +32.2.6425630

Telex no.: 21034 IHEBRU

Other contractual partners in the joint project.

M.F. Roquebert, Museum National d'Histoire Naturelle (Paris)
G.L. Hennebert, U. C. L. (Louvain-la-Neuve)
C. de Bièvre, Institut Pasteur (Paris)
D. Smith, C. A. B. (Kew)

Title of the research activity

Development of improvement techniques for the preservation of fungal strains of biotechnological importance.

Key words:
Microorganisms, Fungi, Yeast stability, Lyophilisation, Cryopreservation

Reporting period: January 1987 - June 1987

I GENERAL OBJECTIVES OF THE JOINT PROJECT:

The specific objective of the project is to improve methods of preservation of fungi of biotechnological importance by development of optimal procedures for freezing and freeze-drying of strains from a range of groups with different attributes. At the end of the project recommendations for procedures for both long term storage methods will be published, bearing in mind the need to recommend low-cost methods whetever appropriate. All preservation and stability tests must be carried out on parralel cultures from the same source material.

II SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Considerable immunological and biochemical differences are observed between molds extracts from the same species. IHEM will examine the effects of storage on antigenic stability of reference strains commonly used in allergology and of freshly isolated strains .
Preservation techniques affect pathogenicity of some strains by means of transformations which are frequently associated to irreversible morphological modifications. Pathogenicity of dermatophytes and yeasts will be evaluated after liquid nitrogen storage and freeze-drying on freshly isolated strains and collection strains.

III SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

The initial aim of this 6 months research period was to improve methods for evaluation of fungal extraction methods and protein stability of a selected strain of *Penicillium expansum* after a range of 7 preservation procedures.

1.Methodology

* Preparation of fungal antigenic extract
A synthetic medium was selected, which doesn't contain any high molecular weight compound. Thus, all the polymeres detected in the extracts can be ascribed to the fungus.
* Estimation of proteins:
proteins were estimated by the Lowry method, with Precinorm S as standard
standard regression output: n° of observations: 10 in duplicate
R squarred: 0,991,constant 0, x coefficient (as concentration). 0.007774

* Enzymology

The 7 extracts (dilution1/3) were screened on the API ZYM system (Bio-Mérieux), a semi-quantitative micromethod for the research of enzymatic activities. Each cupule of the Api gallery was inoculated with 65 microliters of dilute extract, incubated 4 hours at 37°and the revealed with Fast Blue BB salt. The colours developed were compared with those of the colour chart.

* Isoelectric focalisation in polyacrylamide gel.

The IEF were performed on the PAGEPLATE 3,5-9,5 (LKB 1804-101). The fixed conditions were, for one plate: 1500 V, 50 mA, 30 W. The t° of the cooling plate was 10°C. The plates were stained with Coomasie Brillant Blue R250 and the pH values mesured on the plate with LKB surface pH electrode (LKB 2117-111)

* Rast

Our reference extract A045 was coupled to CNBr-actived cellulose discs and integrated in a RAST screening for moulds
IgE positive human sera was selected.

2. Results

Fungal antigenic extracts

The test set includes P expansum untreated strain (LCP3384) and 6 subcultures after preservation .
No marked difference was observed during growth of the tested strains. Mycelium was abundantly produced during the 2 first days, sporulation became visible after the 3th day and gave, after 11 days a final mycelium rind with abundant sporulation
Estimation of proteins is summarized in the next table

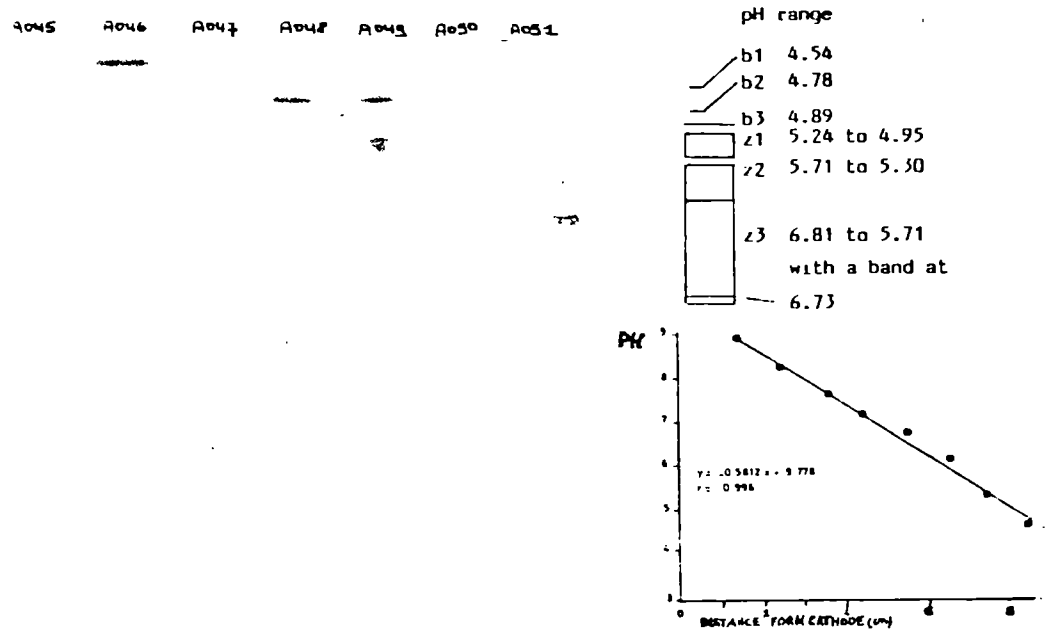
extract n°	preservation	mg/100ml extract	protein (mg/g mould)
A045	untreated	86,05	1,12
A046	-135°/rapid	94,28	1,12
A047	-135°/slow	52,39	1,07
A048	-196°/slow	63,92	1,21
A049	-196°/rapid	49,33	0,59
A050	-20°/slow	55,66	0,64
A051	out of lyophilisation	47,59	0,51

Enzymology (in duplicate)

19 enzymes were tested 2=phosphatase alkaline , 3=esterase , 4=esterase lipase , 5=lipase , 6=leucine arylamidase , 7=valine arylamidase 8=cystine arylamidase 9=trypsin-like , 10=α-chymotrypsine-like 11=phosphatase acid , 12=phosphoamidase , 13=αgalactosidase , 14=βgalactosidase , 15=βglucuronidase , 16=αglucosidase , 17=βglucosidase 18=Nacetylβ glucosaminidase , 19=αmannosidase 20=αfucosidase 13 enzymes gave a positive response (2,3,4,6,7,11,12,13,14,16,17,18 et 19). For the 7 extracts αmannosidase shows quantitative variability

Isoelectric focusing.

The protein pattern of each fungal extract was studied several times by isoelectric focusing and compared to 2 referencve extracts (untreated strain). As can be seen on the next figure, there are no visible differences between the 7 patterns. The more concentrated focused bands of A046 pattern may be explained by his higher protein content. In the future, we shall try a pH range 5,5-8,5 for a better vizualization of z2 and z3 zones.



Rast

In order to find human positive sera, mould positive sera were screened for *Penicillium spp.* Our reference extract showed cross-antigenicity with *P. notatum* extracts. A pool of positive sera is now available. Therefore, it will be possible in the future to test the antigenic stability of our extracts by the RAST inhibition method.

CONCLUSIONS:

Electrophoretic and enzymatic studies of fungal extracts prepared in IHEM laboratory with the tested strain ICP3384 of *P. expansum* showed very little differences in soluble protein production after 11 days of standardized culture wathever preservation method used. Enzymatic activity of the fungal extract, expressed as protein content, proved only quantitative variability for one enzyme (α manosidase).

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

Short communication:

Standardization of fungal allergenic extracts, importance of culture preservation methods. Book of abstracts p. 110 of the meeting of BAP-contractors on "Culture Collections and Genetic Engineering of Microorganisms", Ionnina, 23-25 april 1987. (CEC edition).

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	No
Joint experiment(s)	No
Joint meeting(s)	Yes

Descriptive information for the above data.

A. In the frame of the Preservation project.

a. Exchange of material:
exchange of strains, after preservation procedures, with CBS.

b. joint meetings:

* Preservation meetings
  - 14 november 1986 (preparative meeting), BRUSSELS.
  - 24 februari 1987, LOUVAIN-LA-NEUVE.

* BAP-contracter meetings
  -23-25 april 1987, IONNINA.

B. Out the scope of the Preservation-project

* participation at the contract BAP-0134-B on "A European network of microbial culture collections: integrated catalogue".

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: U. C. L., Louvain-la-Neuve
Contract no.: BAP - 0028 - UK

Project leader: G.L. HENNEBERT
Scientific staff: Lic. F. Dumas (Nov. 1986 - March 1987)
Ing. J.F. Berny (July 1987 -)

Address: Université Catholique de Louvain
Lab. de Mycologie Systématique et Appliquée
3, Place Croix du Sud, Bte 8
B - 1348 LOUVAIN-LA-NEUVE

Telephone no.: TEL +32.10.433742

Telex no.: 59037 UCL B

Other contractual partners in the joint project:

D. Smith, C. A. B. (Kew)
M.F. Roquebert, Museum National d'Histoire Naturelle (Paris)
N.M. Nolard-Tintigner, Inst. d'Hygiène & d'Epidém. (Bruxelles)
C. de Bièvre, Institut Pasteur (Paris)

Title of the research activity:
Development of improvement techniques for the preservation of fungal strains of biotechnological importance.

Key words:
Microorganisms, Fungi, Yeast Stability, Lyophilisation, Cryopreservation

Reporting period: May 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

In search of the optimal preservation method, test strains of fungi are preserved by freeze-drying and by freezing and storage at low temperature, according to 6 and 10 distinct procedures respectively, four of them in dupplicates. The procedures are a combination of a slow and a fast cooling rate, a slow and a fast warming rate at drying and four low temperatures of storage. They are evaluated depending on the viability and the stability of properties of both filamentous and yeast fungi of biotechnological importance. The properties investigated for stability after preservation are morphology, growth response assimilation and fermentation ability, brewing properties, protein serology, mycotoxin production, membrane lipids and fatty acids, protein composition, pathogenicity and allergenicity.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

This Laboratory will evaluate the stability of the morphology and of the growth response of some strains of filamentous fungi (*Penicillium expansum*, particularly) and the stability of morphology, carbon and nitrogen assimilation, carbon fermentation, growth factors requirements, osmotolerance, thermotolerance, enzymatic reactions, acid production and brewing properties on three strains of industrial beer yeast. The brewing properties are the fermentative profile at low and high temperature, the final density, the final attenuation, the alcohol and ester production, the amino acid production, the diacetyl concentration, the flocculation ability, the fermentative rate and the rate of respiratory mutations.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Methodology

The methodology differs greatly depending on the kind of organisms tested. In a first period of time, investigations have been carried out on filamentous fungi only, at the exclusion of the yeasts. In a preliminary state of work, the proposed methodology of testing the morphology and the growth response of filamentous fungi has been applied on 3 different strains : LCP 3384 *Penicillium expansum* (MUCL 29300), IPM 1666-86 *Trichophyton rubrum* (MUCL 29301) and CBS 260.68 *Mucor racemosus* (MUCL 29302), before preservation treatment and after preservation by one-step freeze-drying and cryopreservation at - 20°C, - 135°C and -196°C after slow cooling (rate 1° min^{-1}) and at -135°C and - 196°C after fast cooling (rate 50° min^{-1}).

The strains have been cultivated on the recommended medium for micro morphology i.e. Czapek Agar, Malt Agar 2 % and 4% at 24°C respectively. The growth rate and colony morphology have been evaluated on a series of media including three glucose concentrations 0,1, 1 and 2 % combined with three different nitrogen sources at 0,1 % (KNO_3, NH_4NO_3 and peptone) and an adjusted 2 % Malt Agar at pH 4,8, 6 and 7.

Inoculation of the culture plates has been carried out either by single conidium transfer (*Penicillium expansum* and *Trichophyton rubrum*) or by polyconidial transfer by an equal volume of conidial suspension (*Penicillium expansum* again and *Mucor racemosus*). All conidium inoculum originated from the first culture after treatment. Three repetitions have been made and growth measurements averaged.

2. Results

None of the three strains have shown visible variation in their micro morphology before and after the treatments. None of the three strains have shown signiticative variation in growth rate among treated strains themselves and between treated and untreated strains, neither on standard media nor on different C/N ratios and different nitrogen sources, nor at different pH values. Some ditferences in growth rate at different sugar concentration or at different pH were observed, depending on the strain response to such factors, not on the treatments.

Growth rate of *Penicillum expansum* LCP 3384 is slightly lower on 0,1% sugar than on 1 % and 2 % sugar on which it is the same, particularly on 0,1 % KNO_3 rather than on 0,1 % NH_4NO_3 or peptone on which it is similar.

In *Penicillium expansum* LCP 3384 typical abundantly sporulating colonies with zonation of coremia develop either on media with different nitrogen sources at 1 % and 2 % sugar concentration, or on Malt Agar 2 % adjusted at pH 6 and 7. Conidiation is scanty but still coremial at 0,1 % sugar concentration and is abundant but velvety (not coremial) at pH 4,8 or less. On Czapek Agar the colonies are rich of ochraceous-white mycelium with concentric zones of aggregated conidiophores and some definite coremia at sorne distance from the centre.

The colony pattern is after most preservation treatments the sarne as before treatment. *Penicillium expansum* LCP 3384 has shown, however, variations in the colony pattern after some treatments. After lyophilization, the colony shape originated from polyconidial inoculum showed a more lobate margin, although the sporulating surface appeared homogeneous and normally coremiform. After freezing at 1° min^{-1} and storage at - 20°C *Penicillium expansum* showed a more striking difference. In all polyconidial colonies developed after that treatment, differentiated sectors appeared. They are either well sporulating but velvety, forming no coremia, or very poorly sporulating, showing predominant mycelium and a slightly higher growth rate.

Correlatively sorne of the monoconidial colonies developed after preservation after slow cooling rate and storage at -20 C differ from the typical one in some way.

Another culture experiment based on 100 monoconidial cultures of *Penicillium expansum* before and after treatments has shown a rate of sectorization of 2 % after storage at - 20°C, but as well, in the untreated strains. The variants are differing from the wild type in their color and coremial development but are classifiable as the same species *P. expansum* (MUCL 29413 and

29414). A single conidium isolate of the untreated strain representative of the wild type is maintained (MUCL 29412) for further studies.

No significant variation in growth rate has been observed between treatments neither for *Trichophyton rubrum* nor *Mucor racemosus* in single conidium culture and in polyconidial culture respectively. It appeared, however, in *Mucor racemosus* and in some conditions on Malt Agar 4 % and at pH 6 and 7 but not at pH 4,8 that all treated strains have a growth rate very slightly superior as the untreated strains, but no particular treatment being better than the others.

No variations in colony pattern have been observed in those strains of *Trichophyton rubrum* and *Mucor racemosus*. Both the untreated and the treated strains of *Trichophyton rubrum* showed a wide range of sectorization on the different culture media.

3. Discussion

The sectorization might be an expression of the instability of a strain through the preservation procedures, for so far that it does not appear spontaneously in the untreated strain. The fact that a sectorization appeared in the untreated strain of *Penicillium expansum* cultivated from single conidia does not allow any interpretation of the sectorization observed after treatment. The experiment should be carried out from single conidium culture again.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

2.Short communications

- Development of improved techniques for the preservation of fungal strains of biotechnological importance. Oral Communication presented by G.L. Hennebert at the Meeting of BAP Contractors, CEC, Ioannina, 23-25 April 1987.

- Temperature variation in a one-step freeze-drying procedure for the long term preservation of fungi. By G.L. Hennebert. Poster presented at the Meeting of BAP Contractors, CEC, Ioannina, 23-25 April 1987. Abstracts p. 124, 1987.

- Sectors in *Penicillium expansum* after preservation at - 20°C. By G.L. Hennebert. Poster presented at the Meeting of BAP Contractors, CEC, Ioannina, 23-25 April 1987.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

1. Exchange of materials

1) Contribution of four of the project partners by distributing fungal strains for treatment and investigation by the other partners.

LCP 3384 *Penicillium expansum* from M.F. Roquebert, Paris

IPM 1666-86 *Trichophyton rubrum* from C. De Bièvre, Paris

CBS 260.68 *Mucor racemosus* from J. Stalpers, Baarn

CBS 118.80 *Pythium aphanidermatum* from J. Stalpers,Baarn

CBS 398.63 *Sordaria fimicola* from J. Stalpers, Baarn

CBS 333.85 *Schizophyllum commune* from J. Stalpers, Baarn

MUCL 28286 *Saccharomyces carlsbergensis* from G.L. Hennebert, Louvain-la-Neuve

2) Contribution of one of the other partners by treating fungal strains for investigation of their stability after treatment by other partners.

CBS applied the preservation procedures on the distributed strains.

2. Joint Meetings

1) At Institut d'Hygiène et d'Epidémiologie, Brussels, on November 14, 1986, organized by Dr. N. Nolard. Report prepared by D. Smith (CMI).

2) At the MUCL Culture Collection, Catholic University of Louvain, Louvain-la-Neuve, Belgium, on February 24, 1987, organized by Prof. G.L. Hennebert. Report prepared by D. Smith (CMI).

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Institut Pasteur, Paris Contract no.: BAP - 0028 - UK

Project leader: C. DE BIEVRE
Scientific staff: B. Dennetiere

Address: Institut Pasteur
Unité de Mycologie
25, rue du Dr. Roux
F - 75015 PARIS

Telephone no.: +33.1.45688357

Telex no.: 250609 PASTEUR F

Other contractual partners in the joint project:

M.F. Roquebert, Museum National d'Histoire Naturelle (Paris)
N.M. Nolard-Tintigner, Inst. d'Hygiène & d'Epidém. (Bruxelles)
G.L. Hennebert, U. C. L. (Louvain-la-Neuve)
D. Smith, C. A. B. (Kew)

Title of the research activity:

Development of improvement techniques for the preservation of fungal strains of biotechnological importance.

Key words:

Trichophyton rubrum, Lipids, Phospholipids, Sterols, Biopreservation

Reporting period: May 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The preservation of the microorganisms is an important problem. Many proceedings have been used in the different laboratories : some of them are simple and give good results. Many strains survive when they are preserved by freeze drying and the whole when cryoprotected. But it has been demonstrated that changes in morphology or physiology may occured. So it is important to improve the proceedings. For this purpose it is important to know the mecanism of the cellular change.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Since several years it has been speculated that the cellular membrans could be affected during preservation in particular during the thawing or freezing processes. The phospholipids and sterol, are important constituants of the membrans and so it is interesting to study the composition in these constituants of non preserved strains and of preserved strains according to several proceedings.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY

Extraction of lipids was performed on lyophilised mycelium by a mixture of chloroform-methanol (2:1,v:v).

The extract was purified by Folch's method, with aqueous NaCl 0,65 %.

The different classes of lipids were separated on silicic acid column according to Rovser, and Dittmer and Wells.

Quantification of phospholipids were done by weighing and dosage of phosphorus by Allen's method.

Phospholipids were saponified by 0.5N NaOH in methanol and the resulting fatty acids were methylated by boron trifluoride in methanol.

Composition of methylated fatty acids was determined by GLC on a 15% DEGS column.

Qualitative analysis of phospholipids was performed by TLC on silicagel. The composition of the solvent used was chloroforme-methanol-acetone-acetic acid-water (6:2:8:2:1, v/v).

Chromatographs were revealed by Dittmer's reactif, specific of phospholipids, Munier and Macheboeuf's reactif specific of phosphatidylcholine and ninhydrine specific of phosphatidylethanolamine and phosphatidylserine.

Commercial standards were used too for the identification of the different phospholipids.

The quantification of the different classes of phospholipids was performed after TLC and scrapping of the spots, by dosage of the phosphorus according to Bartlett.

2. RESULTS

Despite some morphological or physiological alterations observed, there is no significative changes in the amount of phospholipids before and after treatments of conservation. (Table 1).

Table 1 : Concentration in phospholipids of unpreserved or preserved mycelium, expressed as ug of phosphorus per g of material.

Temperature of conservation	rate of cooling	Average of two observations	deviation
1 untreated strain	-	101	28
2 - 20°c	-	77	25
3 -135°c	1°c/min	77	9
4 -135°c	50°c/min	122	12
5 -196°c	1°c/min	123	51
6 -196°c	50°c/min	106	10
7 lyophilised	-	116	37

In the same way, these treatments have no effect on the qualitative composition in phospholipids..

The major classes were identified as phosphatidylethanolamine (45%), phosphatidylcholine (40%), phosphatidylinositol (13%) and cardiolipids (3%).

On the contrary, some modifications were detected in the fatty acids composition. These modifications are : a decrease in the amount of linoleic acid (C18:2) and in some cases the apparition of arachidic acid (C20) which was not detected before treatments of conservation as shown in table 2.

Table 2: Fatty acids composition before or after treatments of conservation. (average of two observations and deviation).

*	C14:0	C14:1	C16	C16:1	C18	C18:1	C18:2	C20
1	0,11 (0,09)	0,19 (0,13)	22,34 (1,86)	0,46 (0,32)	2,02 (1,18)	13,44 (2,83)	61,13 (1,71)	0
2	0,33 (0,11)	0,78 (0,08)	34,93 (1,5)	0,18 (0,08)	1,04 (0,4)	12,61 (1,2)	49,98 (0,39)	0
3	0,32 (0,09)	0,65 (0,53)	26,66 (0,58)	0,80 (0,57)	3,44 (1,00)	14,43 (1,57)	52,54 (3,5)	0,29 (0,40)
4	0,22 (0,04)	0,74 (0,03)	26,92 (0,92)	0,65 (0,50)	1,54 (0,72)	11,56 (1,30)	57,14 (4,99)	0,75 (1,05)
5	0,16 (0,10)	0,66 (0,23)	26,71 (1,58)	0,28 (0,01)	1,32 (0,38)	12,51 (3,66)	58,07 (2,52)	0
6	0,32 (0,07)	0,78 (0,45)	30,06 (2,18)	0,46 (0,15)	3,04 (1,75)	11,57 (1,12)	52,89 (4,35	0,67 (0,67)
7	0,22 (0,09)	0,71 (0,14)	25,04 (0,52)	0,47 (0,71)	2,09 (0,40)	14,66 (1,80)	56,61 (2,89)	0

* These figures refer to conditions listed table 1

C14:0 myristic acid
C14:1 myristoleic acid
C16:0 palmitic acid
C16:1 palmitoleic acid
C18:0 stearic acid
C18:1 oleic acid
C18:2 linoleic acid
C20 arachidic acid

In parallel to the modifications detected in the fatty acids composition, two modifications in physiological and morphological characteristics were observed :

- the growth in liquid medium which was more important after cryopreservation or lyophilisation (table 3).
- some procedures of preservation (-20°C, -80°C and -196°C at the rate of 1°c/min or 50°c/min, -135°c at the rate of 50°c/min and lyophilisation) induce the formation of macroaleuriospores witch were not observed in the untreated strain.

Table 3 : Mass of lyophilised mycelium after 4 days of culture in liquid medium.

*	Average of two observations	Deviation
1	5,5	0,9
2	9,2	1,3
3	7,5	1,7
4	8,2	1,0
5	12,3	2,8
6	13,1	1,8
7	9,5	1,1

* These figures refer to conditions listed table 1.

3. DISCUSSION

The effects of six proceedings of preservation have been studied. Some results are noteworthy. The sporulation of test strain has been modified after preservation and the rate of the growth was increased. The composition in the fatty acids of phospholipids is diversely modified according to the initial treatment. The different values obtained have been examined by the use of statistical methods in particular, factor analysis. The representation of the complex system of values in a plane (after mathematical treament) which represents about 80% of the total variance shows : that the values are separated in several groups according to the treament. Taking as reference the non-preserved group it is noted that the closest group correspond to the freeze dried strains and the two farthest correspond to a short storage at -20°c and a short storage at -196°c after a cooling rate of -50°c mn^{-1}.

New experiments will be performed after different periods of storage and in parallel way we shall precise the constituants implicated and the site of the changes. On the base of these first results, it seems that the amount of palmitic acid and linoleic acid is modified and that there is a neosynthesis of arachidic acid (not observed before).

These changes seem stable over a period of five months. The composition in lipids is probably related to permeability of the membranes so to the vital functions of the cells , and implicated into the growth, the sporulation and others physiological changes leading to the production of metabolites.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

The present work or a part of which will be included in the thesis of Mister Dennetière in charge of the experiments in the laboratory.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	No
Joint experiment(s)	No
Joint meeting(s)	Yes

Descriptive information for the above data.

Many conditions of preservation have been retained for this project. The preservation has been performed in several laboratories and the strains sent to other participants. Many strains were prepared by the CBS and regularly distributed (freeze drying-cryoprotection).

The Institute of Hygiene and epidemiology and the Pasteur Institute are in charge of the preservation at -80°c.

Until now several meetings have been organised in Bruxels. The main results obtained until now were presented or discussed in Ionina during the meeting consecreated to the biotechnology.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: C. A. B., Kew Contract no.: BAP - 0028 UK

Project leader: D. SMITH
Scientific staff: S.M. Ward
J. Stalpers & S. Tan (co-contractor, CBS)
G.J. Morris & G.E. Coulson (sub-contractor, Cell Systems Ltd)

Address: CAB - International Mycological Institute
Ferry Lane
UK - KEW, SURREY TW9 3AF

Telephone no.: +44.1.9404086

Telex no.: 847964 COMAGG G (mark "ATTN CMI")

Other contractual partners in the joint project:

M.F. Roquebert, Museum National d'Histoire Naturelle (Paris)
N.M. Nolard-Tintigner, Inst. d'Hygiène & d'Epidém. (Bruxelles)
G.L. Hennebert, U. C. L. (Louvain-la-Neuve)
C. de Bièvre, Institut Pasteur (Paris)

Title of the research activity:

Development of improvement techniques for the preservation of fungal strains of biotechnological importance.

Key words:

Cryomicroscopy, Freeze-drying, Cryopreservation, Fungi, Stability

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Optimization of techniques of freezing and freeze-drying for the preservation of the viability and genetic and biotechnologically significant properties of fungi. This will be attained by determining the effects of freezing and thawing on selected strains and employing the findings to optimize methods of cryopreservation and freeze drying. The viability, colony morphology, physiology, pathogenicity and other aspects of genetic stability will be monitored before preservation, immediately after, and during storage under a variety of conditions.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

a. Establishment of sub-contracts with Cell Systems Ltd for a study cryomicroscopy, and with the Centraalbureau voor Schimmelcultures (Baarn) to carry out part of the preservation.
b. Devise and carry out experiments to determine the effect of freezing and thawing on selected strains of fungi.
c. Devise new procedures for the preservation of selected fungal strains by the techniques developed and monitor viability and colony morphology after their application.
d. Preserve sufficient replicants of strains for use by co-contractors in stability testing.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

METHODS

i. Cryomicroscopy At Cell Systems Ltd: A light microscope fitted with a temperature-controlled stage was used to observe 19 fungal strains representing different groups of fungi including those that are difficult to preserve during freezing and thawing (Smith et al, 1986).

ii. Preservation At CMI: The established techniques of centrifugal freeze drying, shelf freeze drying and liquid nitrogen storage (Smith, 1986) were employed for the preservation of test strains and comparison with techniques developed using the findings from the cryomicroscopy study. Five freeze drying techniques and storage at -20, -40 and -196 using different cooling protocol are being applied.

iii. Preservation At CBS: The standard CBS freeze drying method and cooling at different rates to -20, -135 and -196°C have been applied to some of the test strains.

iv. Stability of metabolite production after preservation at CBS: The metabolite production of strains was tested before and after preservation using TLC methods.

v. Viability and morphological examination at CMI and CBS: After resusitation viability of the strain is checked by counting the number of grown propagules, vital staining (CBS only) and, if possible, performing spore/conidia germination tests. The morphology is observed by specialist taxonomists.

RESULTS

i. Cryomicroscopy: Three main responses of fungal hyphae to cooling have been observed.

a. Extensive shrinkage at slow cooling rates with intracellular ice forming at fast rates of cooling, (in Phytophthora humicola 50% of hyphae froze at 53°C min^{-1} ; no ice was seen at rates up to 100°C min^{-1} in Aschersonia, Phytophthora nicotianae, the young small diameter hyphae of Serpula lacrymans, Lentinus edodes, Volvariella volacea and the yeast cells of Sporobolomyces).

b. Shrinkage at slow cooling rates and ice nucleation in 50% of hyphae at cooling rates between 4 and 18°C min^{-1} depending on the fungus (Achlya, 6; Alternaria, 12.5; Mucor, 10.5; Penicillium, 15; Saprolegnia, 4; Schizophyllum, 9; Trichoderma, 5; Trichophyton, 18).

c. No shrinkage at cooling rates below 1°C min^{-1}, shrinkage at faster rates up to 50°C min^{-1} and less shrinkage at faster rates. In these hyphae viability was highest when no shrinkage occurred and intracellular ice formation usually killed the hyphae. This occurred in the young hyphae of the dry rot fungus Serpula lacrymans.

Shrinkage by 30% or more and formation of intracellular ice usually killed the hyphae. The only exception in the 19 fungi tested so far has been Penicillium expansum which survived intracellular ice formation.

ii. Preservation: Twelve of the sixteen strains preserved so far have survived all the preservation techniques. The strains of Pythium, Volvariella, Lentinus and Schizophyllum failed all freeze drying methods employed. Pythium also failed to recover from -20 and slow cooling to -196°C and Volvariella failed to recover from -20, and either cooling rate to -196. Trichophyton recovered well from all techniques but showed sectoring after recovery from slow cooling to -196.

iii. Stability: No difference has been seen in the secondary metabolite production of Penicillium expansum after preservation by all the techniques. However, Schizophyllum has shown some differences. A currently unidentified extra spot has been seen on TLC plates from cultures recovered from slow cooling to -135 and -196°C. Irregularities have also been noticed with this strain recovered from -20 and fast cooling to -196.

DISCUSSION

The three different responses observed by cryomicroscopy during cooling can be linked to taxonomic groups. Hyphomycetes show the classical response to cooling, shrinkage, at slow cooling due to exposure to hypertonic solutions, and freezing, at faster cooling rates. Most of basidiomycetes studied behave differently; small diameter hyphae shrink without intracellular ice formation (with the exception of Schizophyllum) and wide hyphae freeze at all cooling rates. In the Mastigomycotina the species of Phytophthora require fast cooling to induce intracellular ice formation; the remaining representatives behaved similarly to the hyphomycetes.

Extensive shrinkage and intracellular ice formation (with the exception of Penicillium expansum) were the lethal events during cooling. Methods of cryopreservation can therefore be improved by avoiding these stresses. Modifications to techniques has already improved the survival of the basidiomycete Serpula lacrymans which has double the survival rate at 0.5°C min^{-1} cooling than at 1° C min^{-1} cooling.

References: Smith, D. (1986). PhD thesis, London University.
Smith, D.; Morris G.J.; Coulson, G.E. (1986). Journal of General Microbiology 132, 2012-2021.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

Internal reports

CCIS Newsletter, Issue 6, Autumn 1986
CAB International Mycological Institute Services & Publications 1987.
Intercom, The Newsletter of the CAB International Mycological Institute, 5 December 1986.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)	Yes	
Joint meeting(s)	Yes	

Descriptive information for the above data.

The laboratories participating in the research project are CAB International Mycological Institute (CMI) and the Centraalbureau voor Schimmelcultures (CBS) Netherlands who are primarily responsible for preservation and morphological tests; Institut d'Hygiene et d'Epidemiologie (IHE), Brussels, Universite Catholique de Louvain (MUCL), Lab. de Mycologie Systematique et Appliquee, Louvain-la-Neuve, Institut Pasteur, Unite de Mycologie (IPM), Paris and Museum National d'Histoire Naturelle, (LCP) Paris who are all primarily responsible for testing particular properties of the test fungi before and after preservation, IHE and IPM with the additional responsibility of storage at -80°C.

Two meetings of representatives of the participating laboratories have been held at the Institut d'Hygiene et d'Epidemiologie (1HE), Brussels on 14 November 1986 and at MUCL on 24 February 1987 to establish a joint stratergy to carry out the work shown in Table 1 with selected test strains (up to 74).

The initial aim was to preserve 50 strains by different freezing and freeze-drying procedures and each laboratory was to apply their techniques to each to discover if preservation or storage had any effect on the strain's properties. However, after initial experiments with 7 strains this approach be impractical in the time available and proved to close co-operation of LCP and MUCL with the preservation laboratory at CBS has been analysed to ensure that the strains they have established tests for are preserved in numbers they are able to deal with and at suitable times. Such links have also been established for IPM and IHE with CMI.

CMI and CBS have selected 12 strains to preserve in duplicate carrying out viability and stability of secondary metabolite production before preservation, after preservation and during storage.

Table 1 Distribution of work between the collaborating laboratories

	CMI	CBS	MUCL	LCP	IPM	IHE
(a) Supply of strains	+	+	+	+	+	+
(b) Preservation						
Controlled FD	+					
One stage FD		+				
Two stage FD	+					
-20	+	+				
-40	+					
-80					+	+
-135	+	+				
-196	+	+				
(c) Cryomicroscopy	+					
(d) Stability						
DNA (Mitochondrial)					+	
Morphology		+	+			
SEM/TEM				+		
* Growth parameters			+	+		
Physiology						
(i) Fermentation			+			
(ii) Brewing properties			+			
(iii) Serology						+
Biochemistry						
(i) Secondary metabolites		+		+		
(ii) Lipids and sterols					+	
(iii) Spectrophotometry					+	
Pathogenicity						
(i) Inoculation						+
(ii) Toxin (quantification)						+
(iii) Allergy (ELISA, IEF, RAST, CUT-TEST)						+

BIOREACTORS

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: De Landbouwhogeschool Wageningen
Contract no.: BAP - 0043 - NL

Project leader: J.A.M. DE BONT
Scientific staff:

Address: Agricultural University
Dept. of Microbiology
Hesselink van Suchtelenweg 4
NL - 6703 CT WAGENINGEN

Telephone no.: +31.8370.83754

Telex no.:

Other contractual partners in the joint project:

A. Maule, Microbial Technology Lab. (Salisbury)

Title of the research activity:
Microbial production of commercially important hydroxylated compounds from halo-aromatics.

Key words:
4-chlorobenzoate, 4-hydroxybenzoate, Dehalogenase, Hydroxylated aromatic compounds, Haloaromatics

Reporting period: August 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The overall project aims at making use of enzymes capable of converting halogen (chlorine, bromine) substituted aromatic compounds into the corresponding hydroxylated compounds. Such hydroxylated aromatics are of great importance in the pharmaceutical and petrochemical industries.

The research strategy is to investigate the dehalogenating/hydroxylating enzymes in terms of natural occurence, substrate specificity and product formation.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Various conditions have been tested to obtain anaerobic 4-hydroxybenzoate formation from 4-chlorobenzoate. It was demonstrated that an almost stoichiometric conversion was possible when cells were precultivated under special conditions and when nitrate was available to the producing cells. Enrichment cultures containing other halogenated aromatic compounds Have been set up, and four new microorganisms have been isolated. These organisms will be screened for the presence of a dehalogenase similar to the enzyme present in the 4-chlorobenzoate degrading organism.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Methodology

Alcaligenes denitrificans NTB-1 isolated previously (ref. 1) was routinely grown in a chemostat, volume 1 liter, dilution rate 0.015 h^{-1} , 15 mM 4-chlorobenzoate, mineral salts medium and KNO_3 6 gl^{-1}, under oxygen limited conditions. Incubation experiments under anaerobic conditions were performed in 30 ml serum shake flasks, and oxygen concentrations were measured during the incubations with a gas chromatograph. The concentrations of the different aromatic compounds were quantitatively analysed by means of reverse-phase high-performance liquid chromatography. For enrichments mineral salts medium with 2 mM haloaromatic was used, and various soil and sewage

samples were added. After growth in the liquid medium material was streaked onto plates and individual colonies grown at the expense of the haloaromatic compound were picked and checked for purity.

Results and discussion

Recently, bioformation of 4-hydroxybenzoate (4-HBA) from 4-CBA by Alcaligenes denitrificans NTB-1 was obtained when 4-CBA grown cells were incubated with 4 CBA under conditions of low and controlled oxygen concentrations. However, at an oxygen concentration of almost zero a significant decrease in 4-HBA formation was observed, and no production occurred under fully anaerobic conditions. (ref. 1,2) These observations were unexpected because of the hydrolytic nature of the dehalogenation (ref. 3). The lack of activity may be caused by the need for an active 4-CBA transport system which needs energy and thus will only function in the presence of an electron acceptor. This hypothesis is supported by experiments in which electronacceptors other than molecular oxygen were tested.

Washed cells of NTB-1, precultivated in a chemostat under oxygen-limited conditions on 4-CBA and nitrate, were able to convert 4-CBA anaerobicelly to 4-HBA in the presence of nitrate, whereas in the absence of nitrate no 4-HBA was formed (fig. 1,2). Cells not adapted to denitrifying conditions showed no activity with or without the addition of nitrate under anaerobic conditions. However, the cells producing 4-HBA in the presence of nitrate were only active for a few hours whereas cells kept under aerobic conditions continued to convert 4-CBA for a longer period of time. At present this inhibition of the prolonged anaerobic 4-HBA formation with nitrate is investigated. The formation of nitrite during the incubation or the absence of an additional energy source can not explain this inhibition.

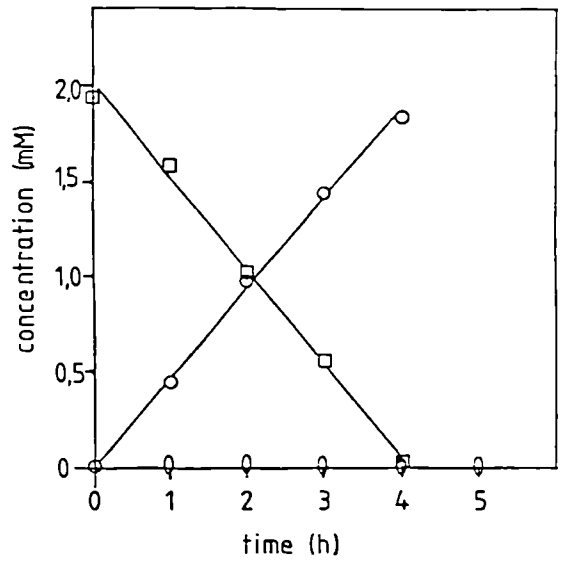


Fig.(1) Aerobic conversion of 4-chlorobenzoate with NTB-1 cells. (4-CBA □ , 4-HBA 0 , chloride O).

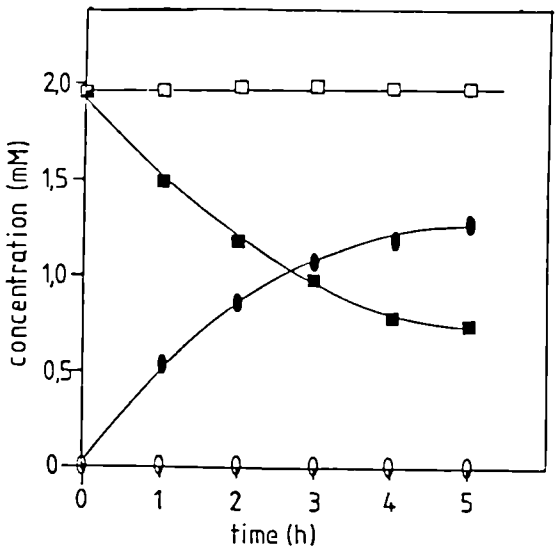


Fig.(2) Anaerobic 4-hydroxybenzoate formation from 4-chlorobenzoate with NTB-1 cells, in the presence (4-CBA ■ , 4-HBA ●), and absence of nitrate (4-CBA □ ,4-HBA 0).

Until now the hydrolytic enzyme has been detected in only few organisms and appeared to be active with only a few para-substituted haloaromatics. Therefore, enrichment cultures on more than thirty halogenated aromatic compounds have been set up with various inoculum. Isolation of organisms using the haloaromatic compound as sole carbon and energy source has been succesful so far with 4-chloromandelic acid, 4-fluorophenylglycine, 2-fluorobenzoate, and 4-chlorobenzoate. The isolates will be screened for the presence of the dehalogenase.

References

(1) Tweel van den W.J.J. (1986), Appl. Microbiol. Biotechnol. 25, 289-294.

(2) Tweel van den W.J.J. (1987), Appl. Environ. Microbiol. 53, 810-815.

(3) Marks T.S. (1984), Biochem. Biophys. Res. Commun. 124, 178-181.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	No
Exchange of staff	No
Joint experiment(s)	No
Joint meeting(s)	Yes

Descriptive information for the above data.

Capri, Italy, 2 - 6 may, 1987.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: P.H.L.S., Salisbury

Contract no.: BAP - 0140 - UK

Project leader: A. MAULE

Scientific staff: M. Ruffett, N. Thurman, T. Marks, K. Mackintosh

Address: Microbial Technology Laboratory
PHLS Centre for Applied
Microbiology and Research
UK - PORTON DOWN, SALISBURY SP4 0JG

Telephone no.: +44.980.610391 EXT.395

Telex no.: 47683 PHCAMR G

Other contractual partners in the joint project:

J.A.M. de Bont, Agricultural University (Wageningen)

Title of the research activity:

Microbial production of commercially important hydroxylated compounds from halo-aromatics.

Key words:

Haloaromatics, Dehalogenation/hydroxylation, Microbial transformation

Reporting period: October 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Hydroxylated aromatic compounds are of great importance in the pharmaceutical and petrochemical industries. However, at present there are no readily available preparative chemical reactions for direct, specific hydroxylations on an industrial scale. Consequently, the hydroxyl group has to be introduced by an expensive series of reactions. Certain microorganisms, however, can specifically dehalogenate and then hydroxylate aromatic compounds. Thus, the aim is to develop a microbiologically-based process for the production of hydroxylated aromatic compounds using the corresponding halogenated aromatic substrate. Whole cells, cell-free extracts or purified enzymes will act as biocatalysts for the specific transformations.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

1. To select a range of chlorinated aromatic substrates whose corresponding hydroxylated derivatives have potential commercial value. Also to develop sensitive, reliable techniques for the analysis of the parent compounds and hydroxylated metabolites.

2. Set-up an enrichment/isolation programme to select for microorganisms from wide ranging environments (aerobic, anaerobic, freshwater, marine) with dehalogenation/hydroxylation activity.

3. Test isolates for dehalogenation/hydroxylation activity and determine the major pathways of transformation.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY

The compounds used in the enrichment studies were: 2,3 and 4 monochlorobenzoates, 2,3; 2,4; 2,5; 2,6; 3,4; and 3,5 dichlorobenzoates and 2,3,6-trichlorobenzoate; 2,3 and 4-monochlorphenols, 3,5-dichlorophenol and 1,3,5-trichlorbenzene. Aerobic, freshwater enrichments were in shake flasks (2L) with 200ml mineral salts medium (Marks, 1986), 1mM halogenated substrate, and sewerage sludge (20ml) inoculum. Anaerobic, freshwater enrichments (100ml) were in buty rubber capped serum bottles (125ml) with an H_2:CO_2:N_2 (10:10:80) atmosphere, and cysteine HCl (0.1g/100ml) reducing agent. For the corresponding marine enrichments a synthetic sea water medium (DIFCO) was added to the mineral salts medium. Chlorobenzoate and hydroxybenzoate analysis was by reverse phase HPLC using a μBondapack column (Waters Instruments). Acidified, ether extracts (4ml) of culture aliquots (4ml) were air-dried, resuspended in the mobile phase (methanol:

water:acetic acid; 60:40:1 and the u.v. absorbance measured at 254nm. Chlorophenols and chlorobenzenes were analysed by electron-capture GC (Pye-Unicam, 4500), using a glass column packed with 3% SE30 and injector, detector and column temperatures of 230°C, 250°C and 150°C, respectively. Chlorophenols were first derivatized using pyridine/acetic acid (Atuma & Oker, 1985). Enrichments showing loss of parent compound were sequentially subcultured in fresh-halo-aromatic-containing medium over a 3-4 week period. Pure cultures were then isolated on tryptone soya agar (Oxoid) and tested for growth on haloaromatics. All incubation was at 25°C. In oxygen-uptake studies a Rank oxygen-electrode (Rank Bros., Bottisham, Cambs) was used.

2. RESULTS

Chlorobenzoate disappearance was noted in several aerobic freshwater enrichment cultures (3-chlorobenzoate, 4-chlorobenzoate, 2,4-dichloro-benzoate and 3,5-dichlorobenzoate). From these cultures three bacterial strains of 3-chlorobenzoate degraders and four bacterial strains of 4-chlorobenzoate degraders have been isolated. In all cases, these pure culture isolates transform 2mM quantities of the parent chlorobenzoate in 15-20h. Early results with oxygen-electrode studies suggest that the path-way of 3-chlorobenzoate transformation in at least one isolate is by way of the target compound 3-hydroxybenzoate.

The cultures metabolising 3,4-dichlorobenzoate and 3,5-dichlorobenzoate comprise several bacterial types, and thus have to be further purified.

Chlorobenzoates were stable in anaerobic enrichments, and chlorophenols were stable in both aerobic and anaerobic enrichments, even after two months incubation. Marine enrichments have only just been set-up, and thus have had little time to develop.

3. DISCUSSION

Several strains of aerobic, freshwater bacteria that utilise 3- or 4-chlorobenzoates as sole carbon sources have been isolated in pure culture. At least one of these isolates brings about the desired dehalogenation/hydroxylation reaction using 3-chlorobenzoate. This isolate will be studied in greater detail to determine optimum conditions for transformation, 3-hydroxybenzoate production, and also its degree of substrate specifi ity.

Chlorobenzoate transformation was not detected in anaerobic cultures. This may be because longer acclimatization periods are required, as suggested by Shelton & Tiedje (1984). Chlorophenol transformation was not evident either aerobically or anaerobically, possibly because of their toxic nature (Milner & Goulder, 1986). Attempts to overcome this by using a lower range of chlorophenol concentrations (0.01-0.1mM are currently underway.

REFERENCES

1. Atum, S.S. & Okor, D.I. (1985). Gas chromatographic determination of pentachlorophenol in human blood and urine. Bull. Environ. Contam. Toxicol. 35, 406-410.
2. Marks, T.S. (1986). The microbial degradation of chlorobenzoic acids. PhD Thesis, CNAA, Thames Polytechnic, UK.
3. Milner, C.R. & Goulder, R. (1986). Comparative toxicity of chlorophenols, nitrophenols, and phenoxyalkonoic acids to freshwater bacteria. Bull. Environ. Contam. Toxicol. 37, 714-718.
4. Shelton, D.R. & Tiedje, J.M. (1984). Isolation and partial characterization of bacteria in an anaerobic consortium that mineralizes 3-chlorobenzoic acid. Appl. Environ. Microbiol. 48, 840-848.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

1. Poster presentation "Microbiological transformation of chlorinated compounds" at Biotechnology Action Programme meeting, Capri, Italy, 2-6 May 1987.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

A. Exchange of Materials.

1. Arthrobacter sp. Strain TM-1 sent to Dr. De Bont for comparison with his isolate.
2. Dr. De Bont's isolate (Alcaligenes eutrophus) sent by De Bont to us.

B. Joint Meetings.

August 13/14 1986. Visit by Dr's. Woodrow, Maule and Marks to Dr. De Bont at the University of Wageningen, Holland, to discuss the roles played by the two groups in the project. References of common interest and cultures also exchanged.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Ges. für Biotechnol. Forschung, Braunschweig

Contract no.: BAP - 0060 - D

Project leader: A.F. BÜCKMANN

Scientific staff:

Address: Ges. für Biotechnologische Forschung mbH.
Abt. Enzymtechnologie
Mascheroder Weg 1
D - 3300 BRAUNSCHWEIG

Telephone no.: +49.531.6181306

Telex no.: 952667 GEBIO D

Other contractual partners in the joint project:

G. Carrea, C. N. R. (Milano)
K.D. Kulbe, Fraunhofer -Inst. für Grenzflächen- und Bioverfahrenstechnik, Stuttgart

Title of the research activity:

Continuous synthesis of fine chemicals by cofactor dependent enzymes with simultaneous cofactor regeneration.

Key words:

NAD(P) (H) dependent enzymes, Fine chemicals

Reporting period: July 1986 - June 1987

V. TRANSN[illegible]JECTIVES OF THE JOINT PROJECT:

[illegible]t economic methods for the continuous NAD(P)(H) dependent enzymatic [illegible]s of high value fine chemicals with emphasis on (1) 12-ketochenodeoxy [illegible]c acid, an expensive precursor for the chemical synthesis of chenodeoxy cholic acid, a drug to treat gallstone ailment non surgically (12α-hydroxy steroid dehydrogenase), (2) mannitol and sorbitol, compounds important in food technology (mannitol- and sorbitol dehydrogenase) and (3) ^{15}N-labeled L-phenylalanine and L-tyrosine (very expensive compounds of diagnostic potential) (L-phenylalanine dehydrogenase)*).
Further the enzymatic oxidoreduction of poorly water soluble substrates (neutral steroids, long chain alcohols) in non-conventional environment (organic solvents and biphasic organic-aqueous media) will be studied.
*) (3) is an additional objective

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

I Continuation of the development of a new synthesis of N^6-(2-aminoethyl)-adenine derivatives of cofactors with emphasis on NADP and FAD

II Study of the interaction of N^6-functionalized NAD and NADP derivatives with NAD(P) dependent glucose dehydrogenase and NADP dependent 12α-hydroxy steroid dehydrogenase

III Production and purification of L-phenylalanine dehydrogenase from Sporosarcina ureae (DSM 317)

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

I. METHODOLOGY

N^6-(2-aminoethyl)-NADP and -FAD were synthesized by a new two-step procedure. NADP and FAD were first alkylated with ethyleneimine to N(1)-(2-aminoethyl)-NADP and -FAD (resp. 46 % and 36 % yield). Subsequently N(1)-(2-aminoethyl)-NADP and -FAD were converted to N^6-(2-aminoethyl)-NADP and -FAD by Dimroth rearrangement under unusually mild aqueous conditions [resp. pH 6.0, 50°C, for 4 h (25 % transformation) and pH 6.5, 40°C, for 7 h (65 % transformation)]. Simultaneously tricyclic 1.N^6-ethanoadenine-NADP and -FAD arose in this second step (resp. 75 % and 35 % transformation). After purification by ion exchange chromatography the overall yields were 13.2 % for N^6-(2-aminoethyl)-NADP and 12.5 % for N^6-(2-aminoethyl)-FAD.

A detailed description of the procedures of so far can be found in a patent containing also details for the analogous synthesis of N^6-(2-aminoethyl)-NAD and the coupling of N^6-(2-aminoethyl)-NAD, -NADP and -FAD to macromolecules, e.g. carboxylated polyethylene glycol (PEG, M_r20,000) and CH-Sepharose-4B (Bückmann, 1987). N^6-(3-sulfopropyl)-NAD and N^6-(2-hydroxy-3-trimethylammoniumpropyl)-NAD were prepared by Carrea et al. (unpublished work).

K_M and V_{max} values for the various cofactor derivatives with respect to glucose dehydrogenase (Bacillus megaterium) and 12 α-hydroxy steroid dehydrogenase (Clostridium group P, strain C 48-50) were determined according to standard procedures at the laboratory of G. Carrea (Milano).
L-phenylalanine dehydrogenase, inducable by L-phenylalanine, was produced by aerobic fermentation of Sporosarcina ureae (DSM 317) at 50 L scale in a loop fermenter as described by Campagna and Bückmann (1987).

2. RESULTS AND DISCUSSION

The Dimroth rearrangement to convert N(1)-alkylated to N^6-alkylated adenine derivatives of cofactors is generally carried out under harsh alkaline aqueous conditions to achieve an acceptable conversion rate (e.g. pH 11, 70°C, for 2 h). For N(1)-(2-aminoethyl)-NAD, -NADP and -FAD a different reaction behaviour with respect to the Dimroth rearrangement has been discovered in that this rearrangement can be carried out under unusually mild conditions (e.g. pH 6.0-6.5, 40-50°C, for 4-7 h), still at a relatively fast rate. These conditions are favourable for the instable FAD and, consequently, the chemical reduction of the C-4 position of the nicotinamide, usually preceding the Dimroth rearrangement step under the extreme conditions indicated, can be omitted for N(1)-(2-aminoethyl)-NAD and -NADP - first example of a smooth conversion of N(1)-functionalized NAD(P) to N^6-functionalized NAD(P) in the oxidized form -.
Additionally, the simultaneously appearing tricyclization to 1.N^6-ethanoadenine NAD, -NADP and FAD is a new reaction in heterocyclic chemistry. By optimization of the chromatographic purification steps the relatively low overall yields, reported for N(1)-(2-aminoethyl)-NADP and -FAD, will be improved.

Glucose dehydrogenase and 12α-hydroxy steroid dehydrogenase were initially foreseen respectively as generally applicable NAD(P)H regenerating enzyme and as biocatalyst for the conversion of cholic acid to 12 α-ketochenodeoxy cholic acid in connection with the enzyme membrane reactor concept using cofactors attached to water-soluble macromolecules. For glucose dehydrogenase and 12α-hydroxy steroid dehydrogenase this concept is abandoned, since it could be demonstrated, that PEG (M_r20,000)-N^6-(2-aminoethyl)-NAD and -NADP are not functioning as cofactors for these enzymes at concentrations suitable for reactor conditions (<1 mM).

By testing N^6-(2-aminoethyl)-NAD and -NADP it could be verified, that a dramatic raise of the Michaelis constant K_M due to the introduction of a 2-aminoethyl group at the N^6-position of the adenine moiety had caused the unfavourable coenzyme properties of PEG(M_r20,000)-N^6-(2-aminoethyl)-NAD and -NADP (Table I). Changing the charge on the adenine [N^6-(3-sulfopropyl)-NAD]or introducing a positively charged and more bulky group [N^6-(2-hydroxy-3-trimethyl-ammoniumpropyl)-NAD] brougth no substantial alteration.

Formate dehydrogenase may replace glucose dehydrogenase as NADH regenerating enzyme. For 12 α-hydroxy steroid dehydrogenase the merits of coimmobilized enzyme systems with simultaneous NADP regeneration will be investigated.

After optimization of the fermentation conditions, e.g. by adding D(+)biotin and a defined mixture of trace elements, Sporosarcina ureae (DSM 317) turned out to be a good producer of L-phenylalanine dehydrogenase (approx. 4000 U/L fermentation solution). Results of preliminary studies point to a considerable thermal stability of this L-phenylalanine dehydrogenase, since respectively 95 % and 70 % of the activity remained after exposure of cell extract (25 % w/w) to 40°C and 50°C for 20 min.
The purification of L-phenylalanine from Sporosarcina ureae (DSM 317) is in progress with emphasis on the use of a new affinity method.

Table I: Comparison of the Michaelis constant (K_M) and relative velocity (V^R_{max}) of NAD(P), N^6-(3-sulfopropyl)-NAD, N^6-(2-hydroxy-3-trimethyl-ammonium-propyl)-NAD, N^6-(2-aminoethyl)-NAD(P) and PEG(M_r20,000)-N^6-(2-amino-ethyl)-NAD(P) for glucose dehydrogenase and 12 α-hydroxy steroid dehydrogenase

	Glu-DH		12α -HS-DH	
	K_M (μM)	V^K_{max} (%)	K_M (μM)	V^R_{max} (%)
NAD	64	100	-	-
NADP	57	100	13	100
N^6-(3-SP)-NAD	1000	6	-	-
N^6-(2H-3-TMAP)-NAD	2000	41	-	-
N^6-(2-AE)-NAD	4500	15	-	-
N^6-(2-AE)-NADP	n.d.	n.a.	n.d.	n.a.
PEG-[N^6-(2-AE)-NAD]	2000	3	-	-
PEG-[N^6-(2-AE)-NADP]	n.d.	n.a.	n.d.	n.a.

n.d. = not determined n.a. = negligible activity

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1 PUBLICATIONS IN SCIENTIFIC JOURNALS, MONOGRAPHS, ...

Campagna, R. and A. F. Bückmann (1987)
Comparison of the production of intracellular L-phenylalanine dehydrogenase by Rhodococcus species M4 and Sporosarcina ureae at 50 l-scale.
Appl. Microbiol. Biotechnol.: accepted for publication.

IV.3 PATENT DEPOSITED IN CONNECTION WITH THE RESEARCH PROGRAMME

Bückmann, A. F. (1987)
Process for the production of N^6-substituted NAD, NADP or FAD.
German Patent DP 36.17.535.8

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)	Yes	
Joint meeting(s)	Yes	

Descriptive information for the above data.

During the course of the first year of the contract an exchange of NAD and NADP derivatives took place with Dr. Carrea (Milano) and Dr. Kulbe (Stuttgart). With Dr. Carrea joint experiments were carried out with respect to the coenzymatic activity of these coenzyme derivatives.

Joint meetings took place discussing the preparation of poster presentations for the first BAP meeting on enzyme engineering in Capri, Italy, 2-6 May

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: C. N. R., Roma Contract no.: BAP - 0065 - I

Project leader: G. CARREA
Scientific staff: R. Bovara, G. Ottolina, P. Pasta, S. Riva

Address: Istituto di Chimica
degli Ormoni, CNR
Via Mario Bianco 9
I - 20131 MILANO

Telephone no.: +39.2.2847737 or +39.2.2853348

Telex no.:

Other contractual partners in the joint project:

K.D. Kulbe, Fraunhofer - Institut für Grenzflächen- und Bioverfahrenstechnik (Stuttgart)
A.F. Bückmann, Ges. für Biotechnologische Forschung mbH (Braunschweig)

Title of the research activity:
Continuous synthesis of fine chemicals by cofactor dependent enzymes with simultaneous cofactor regeneration.

Key words:
Dehydrogenases, cofactor regeneration, macromolecular cofactors, membrane reactors, fine chemicals.

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Working out echonomic methods for the continuous NAD(P)(H)-dependent enzymatic synthesis of high-value fine chemicals with emphasis on: (1) 12-Ketochenodeoxycholic acid, expensive precursor for the chemical synthesis of chenodeoxycholic acid to treat gallstone ailment non-surgically (12α-hydroxysteroid dehy drogenase). (2) Mannitol and sorbitol, compounds important in food technology (mannitol and sorbitol dehydrogenases). (3) ^{15}N-labelled L-phenylalanine and L-tyrosine, very expensive compounds of diagnostic potential (L-phenylalanine dehydrogenase). Furthermore, the enzymatic oxidoreduction of neutral steroids and long chain alcohols in organic solvents and biphasic aqueous-organic media will be studied.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Small-scale synthesis of water-soluble macromolecular derivatives of NAD. Characterization of the derivatives with ^1H NMR and FAB-MS. Study of the coenzymatic properties of the derivatives with selected dehydrogenase enzymes.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

METHODOLOGY

Functionalization of NAD. NAD (and also adenine and adenosine) was functionalized at different sites of the adenine ring using different reagents including ethyleneimine, iodoacetic acid, 1,3-propane sultone, (2,3-epoxypropyl) trimethylammonium chloride, and bromine plus diaminohexane.

Polymer preparation. Polyethylene glycol was carboxylated at the terminal ends and polyacrylic acid was obtained by alkaline hydrolysis of polymethylacrylate.

Synthesis of macromolecular NAD. The functionalized NAD derivatives were linked to the polymers using 1-(3-dimethylamino propyl)-3-ethyl-carbodiimide.

Spectrometric analyses. Functionalized NAD (and also adenine and adenosine) was characterized with ^1H NMR and FAB-MS.

RESULTS AND DISCUSSION

The effects of the position of coupling to NAD (N-1, N^6 or C-8 of the adenine ring) and the nature of the polymer (neutral polyethylene glycol, basic polyethylenemine and acidic polyacrilic acid) on the coenzymatic properties of water-soluble macromolecular NAD derivatives have been investigated systematically. The enzymes selected for the study were two hydroxysteroid dehydrogenases, used for the synthesis of bile acids of pharmaceutical interest, and glutamate dehydrogenase, formate dehydrogenase and glucose dehydrogenase, which most effectively regenerate NAD(P), NADH or NAD(P)(H), respectively.

It was found that the N^6 position of the adenine ring and neutral polyethylene glycol were the site and polymer giving the most satisfactory results for the majority of these enzymes. Furthermore, polyethylene glycol-N^6-(2-aminoethyl)-NAD was also the derivative with the highest-yield and the least laborious preparation. Glutamate dehydrogenase, formate dehydrogenase and 3α-hydroxysteroid dehydrogenase with polyethylene glycol-N^6-(2-amino-ethyl)-NAD had V_{max} values which were 39, 57 and 66% of those with NAD and K_m values 3.5, 5.5 and 17 times those with NAD. No derivatives had good activity with glucose dehydrogenase and 7α-hydroxysteroid dehydrogenase and therefore macromolecular cofactors with better coenzymatic properties still must be found for the exploitation of these enzymes in membrane reactors.

The usefulness of ^1H NMR and FAB-MS for the characterization of functionalized NAD derivatives has also been investigated. NAD, adenine and adenosine have been alkylated with 1,3-propane sultone and the derivatives examined by ^1H NMR and FAB-MS. The fragmentation pathway of NAD derivatives demonstrated that the alkylating molecule was introduced only in the adenine ring. Similar results were obtained with other alkylating agents such as (2,3-epoxypropyl) trimethylammonium chloride, ethylenemine and iodoacetic acid. On the other hand, ^1H NMR spectra made it possible to localize the alkylation site in the adenine ring. It should be emphasized that the derivatives obtained by alkylation of NAD with 1,3-propane sultone or (2,3-epoxypropyl) trimethylammonium chloride contain a strong acidic group (sulfonic) or a strong basic group (tetraalkylammonium) and therefore could be retained by oppositively charged membranes with no need for macromolecularization.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

1) Riva, S., Bovara, R., Pasta, P. and Carrea, G. (1986) "Preparative-scale regio-and stereospecific oxidoreduction of cholic acid and dehydrocholic acid catalyzed by hydroxysteroid dehydrogenases" J. Org. Chem. 51, 2902-2906.

2) Riva, S., Carrea, G., Veronese, F.M. and Bückmann, A.F. (1986) "Effect of coupling site and nature of the polymer on the coenzymatic properties of water-soluble macromolecular NAD derivatives with selected dehydrogena se enzymes" Enz. Microb. Technol. 9, 556-560.

3) Ottolina, G., Riva, S., Carrea, G., Danieli, B. and Palmisano, G. (1987) "Alkylation of NAD, adenine and adenosine with 1,3-propane sultone and study of the pro ducts with FAB-MS and ^{1}H NMR". Commission of the European Communities, BAP-Meeting of Contractants, Capri, Italy.

4) Riva, S., Carrea, G., Pasta, P., Veronese, F.M. and Bückmann, A.F. (1987) "Effect of coupling site and nature of the polymer on the coenzymatic properties of water-soluble macromolecular NAD derivatives with selec ted dehydrogenase enzymes". Commission of the European Communities, BAP-Meeting of Contractants, Capri, Italy.

5) Bückmann, A.F., Carrea, G. and Kylbe, K.D. (1987) "NAD(P)(H) dependent oxidoreductases for the synthesis of fine chemicals" Commission of the European Communities, BAP-Meeting of Contractants, Capri, Italy.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)	Yes	
Joint meeting(s)	Yes	

Descriptive information for the above data.

NAD and NADP functionalized at the N-1 and N^6 posizion with ethyleneimine were provided by A.F. Bűckmann (G.B.F., Braunschweig) to G. Carrea (C.N.R., Milano). The coenzymatic properties of these derivatives were tested with many dehydrogenases. Some of the experiments were carried out jointly in Milano in the period 6:10:86-10:10:86.

The partners of the joint project (A.F. Bűckmann, G. Carrea, K. Kulbe) met at the Fraunhofer-Institut (Stuttgart) on March 10, 1987 to discuss the results obtained (presented in a joint poster at the BAP meeting held in Capri, 1987) and future perspectives.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Fraunhofer Gesellschaft, Stuttgart

Contract no.: BAP - 0059 - D

Project leader: K.D. KULBE

Scientific staff: C.P. Haller, H. Hasenfratz, A. Heinzler, M. Howaldt, W. Gudernatsch, G. Knopki-Fobo, A. Schädel, H. Weber

Address: Fraunhofer-Institut für Grenzflächen- und Bioverfahrenstechnik
Nobelstrasse 12
D - 7000 STUTTGART 80

Telephone no.: +49.711.6868 EXT.440

Telex no.: 7255168 IZS D

Other contractual partners in the joint project:

A.F. Bückmann, Ges. für Biotechnologische Forschung mbH (Braunschweig)
G. Carrea, C. N. R. (Milano)

Title of the research activity:

Continuous synthesis of fine chemicals by cofactor dependent enzymes with simultaneous cofactor regeneration.

Key words:

Oxidoreductases (NAD/NADP/FAD), Enzymatic cofactor regeneration, Enzyme-membrane reactor, Gluconic acid, Mannitol, Sorbitol, L-sorbose, L-ascorbic acid, Electrodialysis

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The long term objective is to develop economic methods for the continuous synthesis of fine chemicals by cofactor (NAD, NADP, FAD) dependent oxidoreductases, namely of 12-Keto-cheno-deoxycholic and 12-Keto-urso-deoxycholic acid; gluconic acid and mannitol from glucose-fructose mixtures; L-ascorbic acid from D-glucose, D-gluconic or D-glucuronic acid; oxidoreduction of poorly water soluble substrates in organic solvents and biphasic organic aqueous media. Common to the proposed processes is the development of continuous cofactor regeneration systems in membrane reactors by using molwt. enlarged NAD(P); improvement of microbial strains, fermentation and enyzme preparation procedures; hollow-fiber membranes; and reactor integrated downstream processing based on membrane technology.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

1. Improvement of the gluconic acid/mannitol process, by membrane reactor development to overcome the strong product inhibition of mannitol dehydrogenase from S. cerevisiae; screening for MDH enzymes insensitive to product inhibition; synthesis of polymer bound NAD(P)-derivatives; testing of retainment of native NAD and negatively charged low molwt. derivatives in reactors with cation-exchange UF membranes,
2. development of a new process technique for FAD-dependent oxidases to minimize enzyme deactivation by H_2O_2 (uncoupling of prosthetic group; coimmobilization of redox dyes or H_2O_2 converting auxiliary enzymes),
3. screening of microorganisms for enzymes to be used in processes for cofactor dependent synthesis of L-sorbose and L-ascorbic acid.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Methodology

The prinicple of intrasequential or conjugated cofactor-regeneration was used to synthesize a series of added value sugar derivatives by action of cofactor dependent oxidoreductases. Screening programs were carried out to find pairs of enzymes with corresponding coenzyme- and pH-dependence. Thus, two desired products are formed within one reaction sequence. For use in membrane reactors cofactors enlarged in molecular weight by coupling to polyethylene-glycol or polyethylene-imine have been prepared (3,15,20). Replacement of cofactors by redox dyes was tested (23).

In theoretical studies it was shown that the efficiency of an enzyme process may depend strongly on the kinetics (substrate and product inhibition, K_M, K_i) and on the type of reactor employed (2,5,8).
A simulation model for the GDH/MDH-system in two different membrane recycle reactors, the PFR and the CSTR, was developed. The residence time distribution of these reactors ($\longrightarrow$ Peclet-No. = 100 for the PFR) was determined. For tubular membrane reactors with a separate filtration

unit, the performance depends strongly on the volumes of the reactor, the membrane unit and the recycle. This influence on the attainable conversion was experimentally verified in a plug flow reactor with a hollow fiber membrane unit (10,13,18).

Electrodialysis was intensively studied for the separation of charged products (gluconic acid, glucuronic acid) from uncharged compounds (6,9).

2. Results

I. Simultaneous production of gluconic acid and mannitol

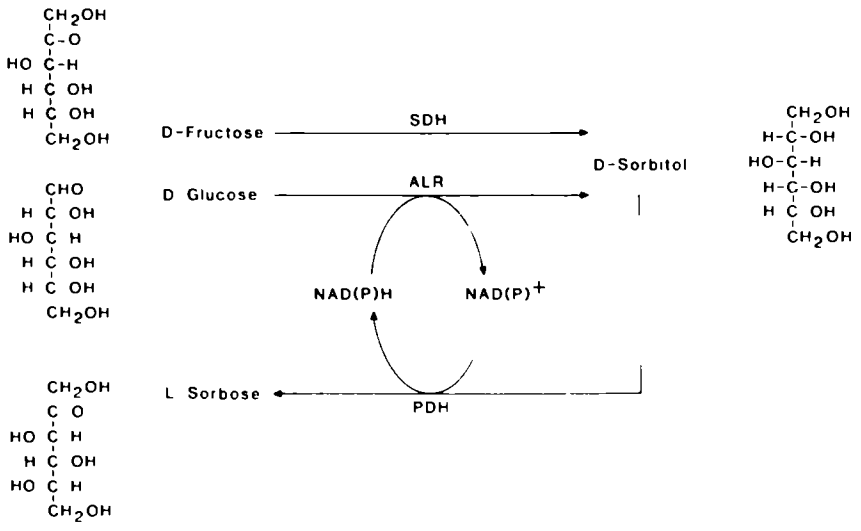
Production of gluconic acid and mannitol were poor when NAD^+-derivatives replaced the true coenzymes (high K_M, low v_{max}). As the up to now used mannitol dehydrogenase shows strong product-inhibition, screening for other NAD^+-dependent MDHs with a pH-optimum at 6.5-8 and without product-inhibition was started (1,3,4,15-17,19,20).

II. Gluconic acid from glucose by glucose oxidase

Heterocyclic and quinoid redox dyes can serve as artificial electron acceptors with respect to glucose oxidase and increase production of gluconic acid under aerobic conditions. In long-term measurements an improvement of enzyme stability is observed on addition of these dyes. Co-immobilized H_2O-producing phenoloxidases reduce the demand for quinones to catalytic amounts (11,23).

III. a) L-sorbose from glucose (figure 1)

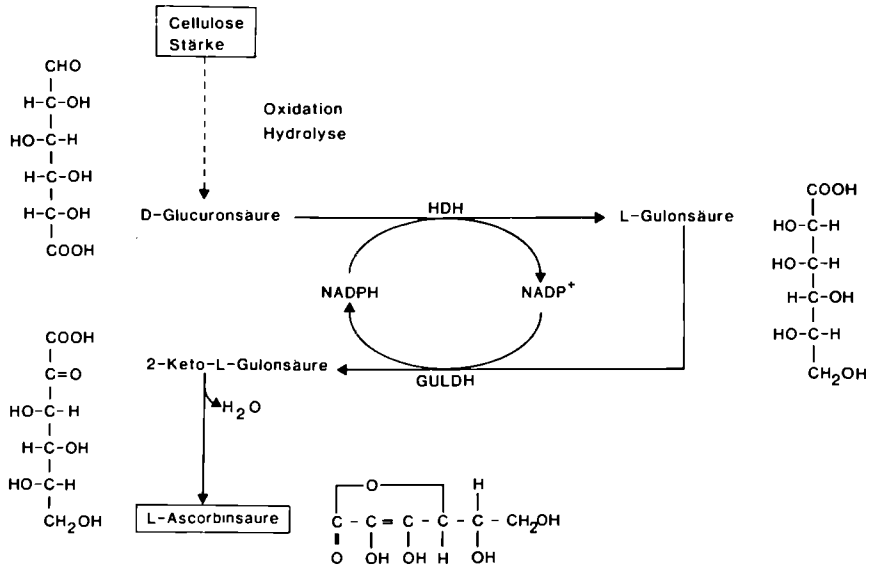
A screening program was performed for sorbitol dehydrogenase (SDH; D-fructose ⟶ D-sorbitol), aldose reductase (ALR; D-glucose ⟶ D-sorbitol), and polyol dehydrogenase (PDH; D-sorbitol ⟶ L-sorbose). Both bacteria and yeasts gave some positive results. Especially the crude extract at the yeast Pichia ambrosiae contained good enzyme activities. The cultivation medium for this microorganism was optimized. Unfortunately, the enzymes proved to be unstable. Therefore, further investigations are required on stabilization and purification of these enzymes (12,16,17,19,21).



b) L-ascorbic acid from uronic acids (figure 2)

Screening programs for L-hexonate dehydrogenase (HDH) and for L-gulono-γ-lactone dehydrogenase/L-gulono-γ-lactone oxidase, respectively, have been carried out. NADPH-dependent activity was found in crude extracts of Lipomyces starkeyi. Cultivation conditions for this yeast have been optimized. Purification as against crude extract was up to 220fold (14,16,19).

Up to now, only FAD or cytochrome c-dependent L-gulono-γ-lactone oxioxidases could be detected in several yeasts. L-gulono-γ-lactone dehydrogenase was found in germinating peas, beans and cress (22). There is some evidence that especially lipid producing yeasts (as Candida sp., Lipomyces sp., Pichia sp.) possess enzymes for ascorbic acid synthesis. A process technique similar to that for glucose oxidase (see chapter II) is going to be applied.



3. Discussion

Activity of GDH from B. megaterium with high molecular weight cofactor derivatives turned out to be low. Therefore, other procedures will be taken into account. For example, cofactors might be coupled to the enzyme protein. Another possibility is to perform separations with charged membranes to achieve retention of likewise charged cofactors.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1 Publications in Scientific Journals, Monographs

(1) K.D. Kulbe, U. Schwab, M. Howaldt and H. Kimmerle
Anwendung von Membranverfahren bei der simultanen Herstellung von Mannitol und Gluconsäure aus Saccharose durch konjugierte NAD^+-abhängige Dehydrogenasen.
In: "Technische Membranen in der Biotechnologie"
(M.-R. Kula, K. Schügerl, Ch. Wandrey, eds.), GBF-Schriftenreihe, VCH Verlagsgesellschaft Weinheim, 186-200 (1986).

(2) M. Howaldt, K.D. Kulbe and H. Chmiel
Choice of reactor to minimize enzyme requirement
I. Mathematical model for one-substrate Michaelis-Menten-type kinetics in continuous reactors.
Enzyme Microb. Technol. 8, 637-631 (1986).

(3) K.D. Kulbe, U. Schwab, M. Howaldt, H. Hasenfratz, K. Kimmerle, W. Gudernatsch and M.K. Otto
Continuous enzymatic conyersion of glucose-fructose mixtures into gluconic acid and sorbitol or mannitol within a hollow fiber membrane reactor.
In: Biomolecular Engineering in the European Community"
(E. Magnien, ed.), Martinus Nijhoff Publishers, Dordrecht, pp. 197-215 (1986).

(4) K.D. Kulbe, U. Schwab and M. Howaldt
Conjugated NAD^+-dependent dehydrogenases for the continuous production of mannitol and gluconic acid from glucose-fructose mixtures in a membrane reactor.
Ann. N.Y. Acad. Sci., 501, 216-223 (1987).

(5) M. Howaldt, K.D. Kulbe, and H. Chmiel
Minimizing enzyme requirement by choice of appropriate reactor type. Computer simulation and experimental results.
Ann. N.Y. Acad. Sci., in press (1987).

(6) K.D. Kulbe, U. Schwab and W. Gudernatsch
Enzyme catalyzed production of mannitol and gluconic acid. Product recovery by several procedures.
Ann. N.Y. Acad. Sci., in press (1987).

(7) U. Schwab, W. Hammes and K.D. Kulbe
A NAD^+-dependent Mannitol Dehydrogenase from Saccharomyces cerevisiae sp.: Isolation, molecular and kinetic properties.
Appl. Microbiol. Biotechnol., in preparation (1987).

IV.2 Short Communications, Internal Reports

(8) M. Howaldt, K.D. Kulbe, and H. Chmiel
Minimizing enzyme requirement by choice of appropriate reactor type. Computer simulation and experimental results.
Proc. V. Conference on Biochemical Engineering (Henniker/New Hampshire, 1986), p. 123.

(9) K.D. Kulbe, U. Schwab and W. Gudernatsch
Enzyme catalyzed production of mannitol and gluconic acid. Product recovery by several procedures.
Proc. V. Conference on Biochemical Engineering (Henniker/New Hampshire, 1986), p. 75.

(10) M.W. Howaldt, H. Chmiel and K.D. Kulbe
Optimization of reactor performance for coenzyme dependent enzyme reactions: computer simulation and experiments.
Proc. 4th Europ. Congr. Biotechnol. (Amsterdam), 1, 257 (1987).

(11) A. Schädel and K.D. Kulbe
Immobilized artificial electron acceptors to improve the long-term performance of oxidase-catalyzed reactions.
Proc. 4th Europ. Congr. Biotechnol. (Amsterdam), 2, 291 (1987).

(12) K.D. Kulbe, I. Haug and A. Heinzler
Enzymatic synthesis of L-sorbose from D-glucose with intra-sequential coenzyme regeneration.
CEC Meeting "Enzyme Engineering: Protein design and application in biocatalysis", 73 (Capri/I., 1987).

(13) M. Howaldt and K.D. Kulbe
Optimization of enzyme reactors with an external filtration unit.
CEC Meeting "Enzyme Engineering: Protein design and application in biocatalysis", 74-75 (Capri/I., 1987).

(14) G. Knopki-Fobo and K.D. Kulbe
Enzymatic synthesis of L-ascorbic acid via uronic acids. 2. L-hexonate dehydrogenase.
CEC Meeting "Enzyme Engineering: Protein design and application in biocatalysis", 73-74 (Capri/I., 1987).

(15) A.F. Bückmann, G. Carrea and K.D. Kulbe
NAD(P)H-dependent oxido-reductase for the synthesis of fine chemicals.
CEC Meeting "Enzyme Engineering: Protein design and application in biocatalysis", 68 (Capri/I., 1987).

(16) K.D. Kulbe
Continuous hydrogen and phosphate transfer reactions in enzyme catalyzed carbohydrate conversion processes.
Carbohydrates 1987: Abstr. 4th European Carbohydrate Symposium (Darmstadt, 1987), F.W. Lichtenthaler, K.H. Neff, Editors, D-16.

(17) K.D. Kulbe and H. Chmiel
Coenzyme-dependent carbohydrate conversions with industrial potential.
Proc. IXth Intern. Enzyme Engineering Conference (Santa Barbara/USA, 1987), accepted.

(18) M.W. Howaldt, K.D. Kulbe and H. Chmiel
Coupled enzyme systems with conjugated coenzyme regeneration in membrane reactors with an external filtration unit.
Proc. IXth Intern. Enzyme Engineering Conference (Santa Barbara/USA, 1987), accepted.

(19) K.D. Kulbe
Enzymatic Transformation of Carbohydrates by continuous Redox and Phosphate Transfer Processes.
Abstr. ACHEMA 88: Internationales Treffen für Chemische Technik und Biotechnologie (Frankfurt/M. 1988), eingereicht.

IV.3 Patents deposited

-

IV.4 Doctorate Thesis (Ph.D) and Degree Thesis

(20) U. Schwab
Zur enzymatischen Umwandlung von Glucose-Fructose-Gemischen in Gluconsäure und Mannit bzw. Sorbit.
Ph.D thesis, Universität Hohenheim, 141 S. (1986)

(21) I. Haug
Wege zur enzymatischen Synthese von L-Sorbose über D-Sorbitol.
Diploma thesis, Universität Hohenheim, 56 S. (1987)

(22) I. Walz
Untersuchungen an pflanzlicher L-Galactono-γ-lacton-Dehydrogenase.
Diploma thesis, Universität Hohenheim, 105 S. (1987)

(23) A. Schädel
Stabilisierung und Cofaktorregeneration H_2O_2-bildender Oxidasen am Beispiel Glucoseoxidase.
Ph.D thesis, Universität Hohenheim, 143 S. (1987)

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

Joint Meeting with G. Carrea (Milano) and F. Bückmann (Braunschweig) at the FhIGB in Stuttgart (9./10.3.87): Discussion of results, also such obtained with the materials (e.g. NAD-derivatives) exchanged; planning of a common publication for the Capri-Meeting in May 1987 (15). Discussion of a new strategy for continuous coenzyme regeneration: free coenzyme or negatively charged coenzyme hold within an enzyme-reactor by identically charged membranes.

Visit of K. D. Kulbe at the laboratory of Mosé Rossi (11./12.6.87, Napoli), Lecture on "Monosaccharide conversion reactions catalyzed by C1- and C2-oxidoreductases". Exchange of information about oxidoreductases. Working discussion on thermophilic enzymes for carbohydrate conversions and planning of exchange of staff.

In collaboration with GBF Braunschweig (H. Hustedt) a two-phase-system was studied for the purification of MDH from S. cerevisiae. A much higher yield of enzyme was obtained. This work will be continued.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: R.W.T.H., Aachen

Contract no.: BAP - 0054 - D

Project leader: W. HARTMEIER

Scientific staff: T. Döppner, U. Drucks, M. Reiss, U. Siemensmeyer

Address:
RWTH Aachen
Institut für Mikrobiologie
Worringer Weg 1
D - 5100 AACHEN

Telephone no.: +49.241.806601

Telex no.: 0832704

Other contractual partners in the joint project:

P.G. Rouxhet, U. C. L. (Louvain-la-Neuve)

Title of the research activity:

Control of the microenvironment of biocatalysts by coimmobilization.

Key words:

Alkaloids, Bacillus, Bacitracin, Claviceps, Co-immobilization, Co-entrapment

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Microenvironmental factors are of major importance for the reaction kinetics and productivity of immobilized biocatalysts. However, only little is known about the structure of microenvironment and its controlled change to improve bioctalytical processes. We thought advisable to investigate microenvironmental effects of oxygen as one factor of basic and industrial relevance as well. The aim is to create, around microorganisms, a defined microenvironment with regard to oxygen. Long-term objectives are to create complex multienzyme biocatalysts, which can be applied in bioreactors for industrial applications. Examples chosen for this project are a bacitracin forming *Bacillus species* and a *Claviceps species* producing ergot alkaloids.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The investigations during the reporting period were directed to evaluate basic data of the systems selected. The microbial and analytical methods as well as immobilization techniques had to be elaborated. It was intended to find out to which extent bacitracin formation of *Bacillus licheniformis* and alkaloid formation of *Claviceps purpurea* depend on oxygen concentration. Both systems had to be tested in native and matrix-entrapped form. First trials to coimmobilize the living organisms with additional catalase and to supply oxygen via hydrogen peroxide were planned. There, entrapment of the biocatalysts with less dense alginate and subsequent membrane enclosement of the alginate beads were envisaged.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

BACITRACIN FROM *BACILLUS LICHENIFORMIS* CO-IMMOBILIZED WITH CATALASE

Bacitracin, a peptide antibiotic, is usually produced in batchwise fermentation with *Bacillus subtilis* or *Bacillus licheniformis*. It is known that bacitracin can also be made using cells immobilized in polyacrylamide gel. Our purpose was to entrap bacitracin forming bacteria into alginate beads and to investigate the influence of oxygen supply on the bacitracin production. In a forthcoming phase of evaluation, it is intended to coimmobilize the cells with catalase and to influence the oxygen content of the cell microenvironment by controlled addition of hydrogen peroxide.

Methodology

Bacillus licheniformis DSM 603 was grown on starch bouillon. Bacitracin was determined by agar diffusion test using *Micrococcus luteus DSM 1790* as test organism. Formation of the antibiotic began in the transition state between logarithmic and stationary phase.

For immobilization, cells from the logarithmic phase were collected, 3 % sodium alginate was admixed, and the suspension was sprayed into a dispersion of acrylic/methacrylic copolymer in $CaCl_2$ solution. The cationic copolymerisate deposited on the anionic alginate beads as a semi-permeable membrane (see Fig. 1), which lead to stabilization of the beads.

Results

Bead Properties and Medium Improvement

Normal alginate beads were not strong enough for the very mobile cells of *B. licheniformis*. The membrane-enclosed beads (see Fig. 1) led to very much better retainment of cells. However, even with additonal mebrane enclosement, leakage could not be completely prevented.

For bacitracin production by immobilized cells we searched for a fermentation medium which inhibited cell growth but preserved metabolic activity. Good results were obtained with 1 % peptone medium.

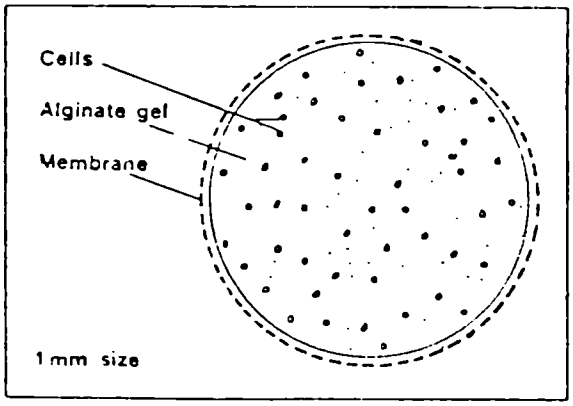


Fig. 1. Membrane-covered alginate bead

Batch Fermentations

In stirred reactors of 1 liter volume bacitracin formation was found to depend considerably on the aeration rate (see Fig. 2). Under the conditions prelimary optimized the specific productivity amounted to 79 u/g·h.

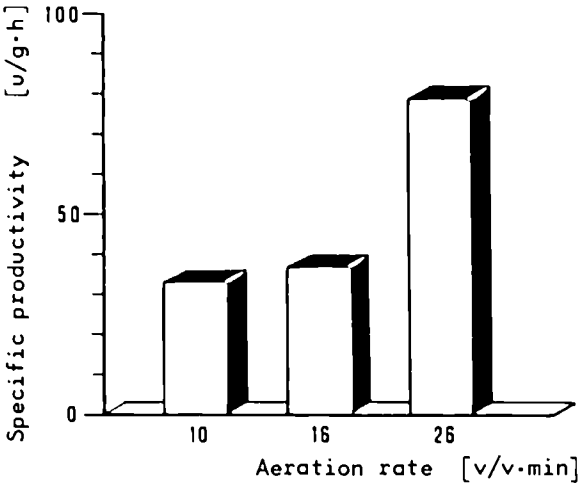


Fig. 2. Bacitracin productivity as a function of aeration rate

Semi-continuous Production

Since bacitracin formation using the embedded cells only occured in a very short phase of the fermentation, a semi-continuous procedure was tried. The beads,

even when membrane-enclosed, swelled during the fermentation process to the fourfold of their original diameter. The mechanical stability decreased and cells were washed out in all cases. Bead damage could be prevented by washing with $CaCl_2$ solution every 8 h. In this way a specific productivity of 85 u/g·h was obtained.

Discussion

From the batch- and semi-continuous trials it can be derived that the alginate-entrapped cell system needs further improvement in both mechanical stability and oxygen supply. Both purposes will be followed in the forthcoming investigations.

ALKALOID FORMATION BY IMMOBILIZED AND CO-IMMOBILIZED SYSTEMS

It is already known that the productivity of the formation of clavin alkaloids can be enhanced by entrapment of *Claviceps* mycelia in alginate matrices. We wanted to find out now, if the productivity of a *Claviceps* strain, forming the pharmaceutically more important peptide alkaloids, could also be improved by means of entrapment. Furthermore, it should be investigated to what extend oxygen supply would influence the productivity and the alkaloid spectrum of the entrapped mould mycelia. As an alternative to conventional aeration we intended to supply oxygen by means of H_2O_2 as oxygen donor. In the latter case we planned to use mycelia coimmobilized with additional catalase.

Methodology

Claviceps purpurea 61 E 213, a generous gift of Schering AG Berlin, was used for our investigations. This strain produces preferably peptide alkaloids. Mycelia of this strain were applied in different form (see Fig 3).

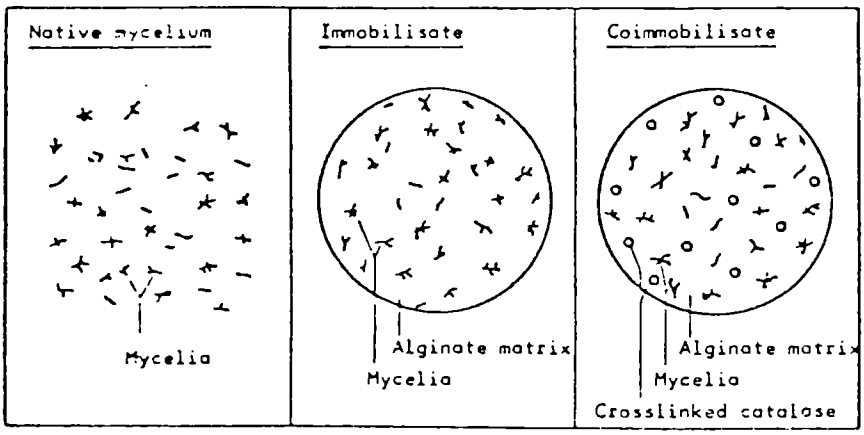


Fig. 3. Biocatalyst forms applied

The mycelia provided for immobilization or coimmobilization were first homogenized by means of an Ultra-Turrax and then entrapped into alginate beads of 1 mm diameter. In case of coimmobilization crosslinked catalase from *Aspergillus niger* origin was applied additionally to the fragments of the *Claviceps* mycelia.

The alkaloids were quantitatively determined by HPLC. The fermentations were carried out in stirred tank reactors of 200 ml working volume. Oxygen was supplied in these trials, controlled by an electrode, either by controlled addition of air, pure oxygen, or hydrogen peroxide.

Results

Entrapment of the mycelia into alginate beads led to a considerable increase of the specific alkaloid formation as calculated per gram dry matter of the mycelium. Furthermore, better alkaloid productivity was obtained by intensifying the aeration, that means with increased dissolved oxygen content of the fermentation medium (see Fig. 4)

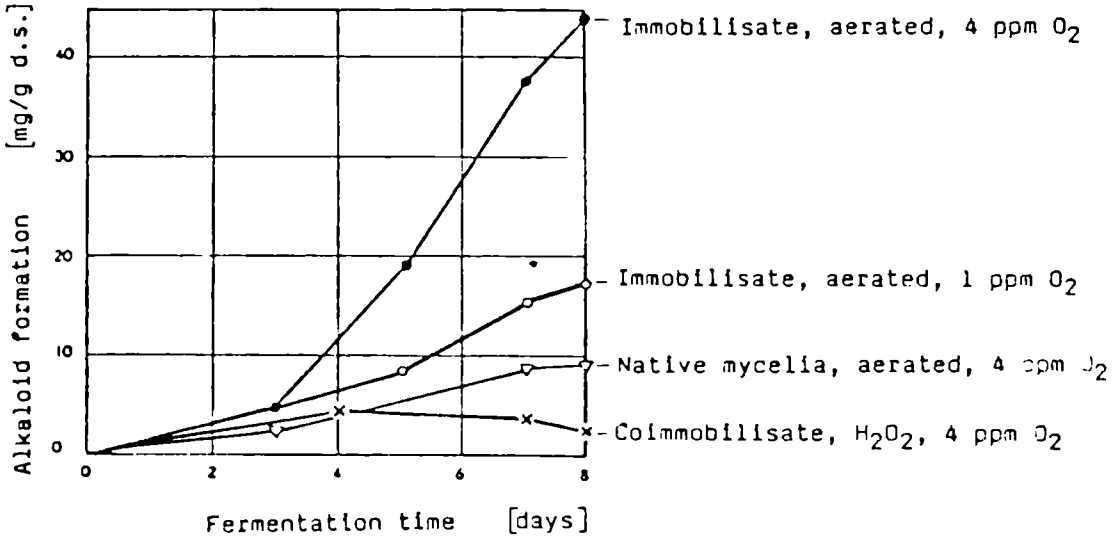


Fig. 4. Peptide alkaloids produced by the different forms of *C. purpurea*

While the absolute yield of alkaloids varied very much with the different forms in which the mycelia were applied, there were only slight differences in the alkaloid spectra. In all cases examined, ergocryptine was the main component of the alkaloids formed. Even when coimmobilized with additional catalase the mycelia were partly inactivated in case of oxygen supply by hydrogen peroxide. Thus, the alkaloid production rate under these conditions was lower than under conventional aeration. The addition of hydrogen peroxide considerably reduced the respiration rate of the mycelia.

Discussion

Increased alkaloid formation of *C. purpurea* is to a considerable extent a function of oxygen supply. Therefore the system will be further examined under the aspect of influencing the oxygen content in the microenvironment of the mycelia. Due to the sensitivity against hydrogen peroxide new concepts must be evaluated to eliminate the poisoning effects in cases where H_2O_2 is used as oxygen donor.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

Publications in scientific journals or books

Hartmeier W: Coimmobilisierung von Enzymen und ganzen Zellen. - Biotech-Forum 1987 (in press).

Hartmeier W, Heinrichs A, Döppner T: Coimmobilization of gluconate-forming bacteria and fungal catalase. - Proc. Congress Biochemical Engineering, Stuttgart, 1987 (in press).

Hartmeier W, Schmitz M: Peptidalkaloide mit immobilisierten Mycelien von *Claviceps purpurea*. - Proc. Congress Biochemical Egineering, Stuttgart, 1987 (in press).

Oral papers

Hartmeier W: Coimmobilization of enzymes and whole cells. - Seminar of the Academy of Sciences of CSSR, Prague, 10.12.1986.

Hartmeier W: Gezielte Umsetzungen mit immobilisierten Biokatalysatoren. - Chemisch-technisches Kolloquium der RWTH, Aachen, 05.02.1987.

Posters

Siemensmeyer U, Drucks U, Hartmeier W: Bacitracin formation using alginate-entrapped *Bacillus licheniformis*. - BAP-Meeting, Capri, 1987.

Hartmeier W, Schmitz M: Basic data on alkaloid formation by immobilized *Claviceps purpurea*. - BAP-Meeting, Capri 1987.

Thesis

Altemüller A: Immobilisierung und Coimmobilisiserung von alkaloidbildenden Mycelien von *Claviceps purpurea*. - Diploma thesis, RWTH Aachen, 1986.

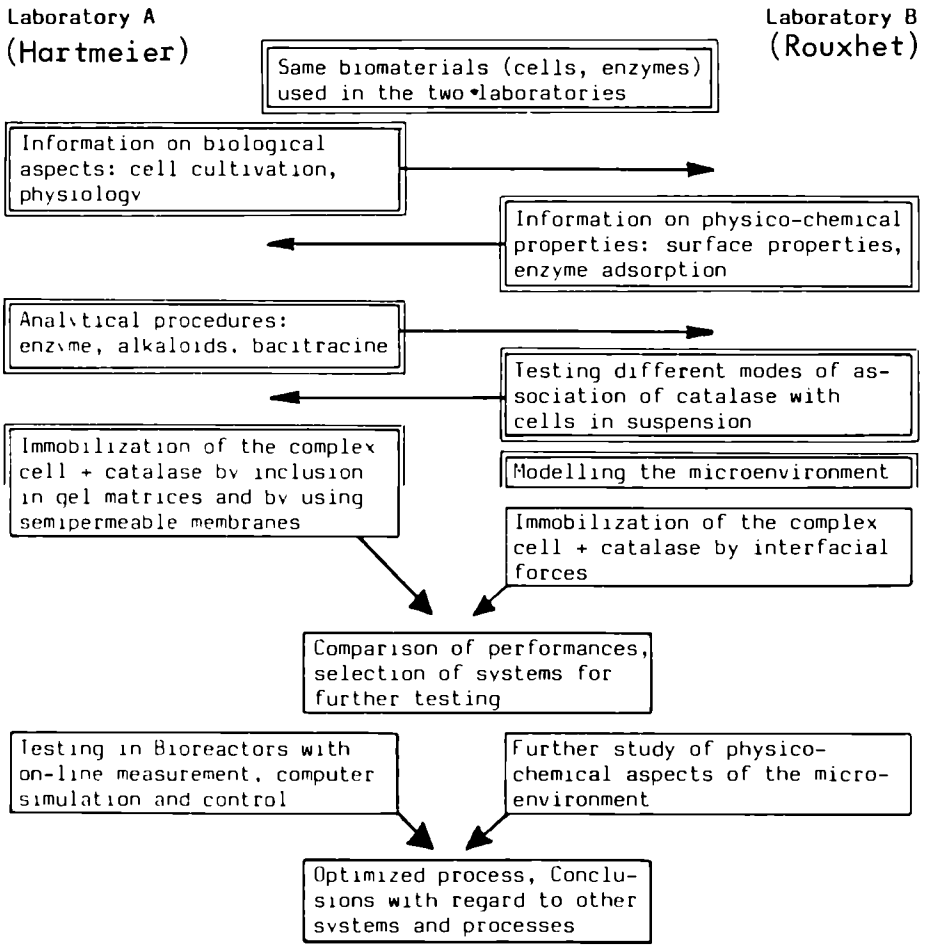
Drucks U: Versuche zur Immobilisierung eines bacitracinbildenden Stammes von *Bacillus licheniformis*. - Diploma thesis, RWTH Aachen, 1986.

TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

Exchange of material and regular meetings were done with the group of Prof. Rouxhet (Louvain la Neuve). In the plan shown below the subjects marked with double lines around ▭ have been carried out already.



Besides the close cooperation with Rouxhet's group, regular contact to European companies has been kept. Schering AG (W-Berlin, Germany) gave us a strain of *Claviceps purpurea*. John & E. Sturge (England) supplied us with catalase from *Aspergillus niger*.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: U.C.L., Louvain-la-Neuve
Contract no.: BAP - 0069 - B

Project leader: P.G. ROUXHET
Scientific staff: N. Mozes, O. Lardinois, D. Masy (part-time)

Address: Université Catholique de Louvain
Unité de Chimie des Interfaces
1, Place Croix du Sud
B - 1348 LOUVAIN-LA-NEUVE

Telephone no.: +32.10.433587

Telex no.: 59037 UCL B

Other contractual partners in the joint project:

W. Hartmeier, R. W. T. H. (Aachen)

Title of the research activity:
Control of the microenvironment of biocatalysts by coimmobilization.

Key words:
Bacillus, Bacitracin, Co-immobilization, Catalase, Hydrogen peroxide

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Microenvironmental factors are of major importance for the reaction kinetics and productivity of immobilized biocatalysts. However, only little is known about the structure of microenvironment and its controlled change to improve biocatalytical processes. It was thought advisable to investigate microenvironmental effects of oxygen as a factor of basic interest and industrial relevance as well. The aim is to create, around microorganisms, a defined microenvironment with regard to oxygen. Long-term objectives are to create complex multienzyme biocatalysts, which can be applied in bioreactors for industrial applications. Examples chosen for this project are Bacillus species forming bacitracin and a Claviceps species producing ergot alkaloids. Controlled change of oxygen around the living cells will be carried out by coimmobilization of the cells with additional catalase and addition of hydrogen peroxide.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The investigations are dedicated to the Bacillus-catalase system. The contribution of this laboratory is to investigate physico-chemical aspects which must play a determining role in the efficiency of the complex biocatalysts and to use interfacial phenomena (adsorption, adhesion, flocculation) for achieving coimmobilization. During the reporting period, one objective has been to clarify the constraints imposed by the reactivity of hydrogen peroxide on catalase and on bacitracine. The other objective has been to immobilize catalase by adsorption.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. CATALASE ACTIVITY AND STABILITY IN SOLUTION

Catalase from Aspergillus niger has been selected because of its highest stability as established by literature data and complementary tests. Half life times measured in H_2O_2 2mM and phosphate buffer 50 mM, pH 7, at 30°C, were 607, 39, 27 and 18 min for catalases from Aspergillus niger, bovine liver, Rhodopseudomonas capsulata and Escherichia coli, respectively.

Below a substrate concentration of 300 mM H_2O_2, the kinetics is characterized by apparent K_m=280 mM and V_{max}=1.82 mmol O_2 $s^{-1}.mg^{-1}$; however examination of a broader range of concentrations has shown a negative cooperativity.

Measurements of deactivation rate in presence of H_2O_2 have shown that the enzyme is irreversibly deactivated by its substrate (fig 1). The process shows a Michaelis-Menten kinetics

$$-\frac{dE}{dt} = \frac{k_d.S.E}{1+ S/K_m}$$

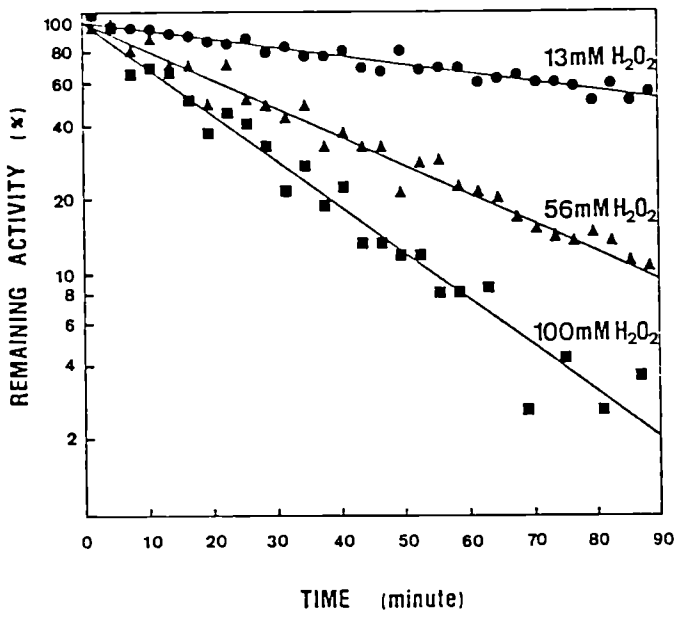


Fig 1 : Deactivation kinetics of Aspergillus niger catalase at various H_2O_2 concentrations.

where S and E are the concentrations in H_2O_2 and active enzyme respectively; the K_m constant is equal to that of the catalytic reaction in the range of hydrogen peroxide concentration 1 to 300 mM. The rate constant k is equal to 9.5 10^{-3} s^{-1} M^{-1} which corresponds to a half life time of 24 min in H_2O_2 50 mM. Two kinetic schemes can be considered to describe the phenomenon :

a) Substrate binding to enzyme stabilizes the enzyme

$$E_{free} + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E_{free} + P$$

$$+S \downarrow K_{d_1}$$

$$E_{inactive}$$

b) Free enzyme is more stable than enzyme substrate complexes. This scheme is consistent with a deactivation provoked by the formation of a toxic intermediate in the course of the catalytic transformation.

$$E_{free} + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E_{free} + P$$

$$\downarrow K_{d_2}$$

$$E_{inactive}$$

Cyanide was used to discriminate between the two mechanisms. This non-competitive inhibitor decreases markedly the rate of deactivation, which indicates that the deactivation is directly related to the existence of enzyme substrate complexes (scheme b).

The molecular weight of the native and deactivated enzyme has been determined by gel permeation chromatography and found equal to 396000 in both cases. This indicated that deactivation is not related to splitting of the molecule into subunits.

The modification of the UV spectra as a result of oxidation differs from that described for mammalian catalase : the absorption coefficient of the Soret band at 405 nm is decreased but no shift is observed. FTIR spectra of the native and deactivated enzyme are similar, indicating that no marked change of the secondary structure of the protein occurs during the deactivation.

2. CATALASE ADSORPTION

Activated carbons, with an isoelectric point near or below pH 3, have been used as a support for the catalase. Adsorption takes place mainly on the external surface of the particles. The adsorbed amount increases as the pH is lowered from 7 to 3 and as the ionic strength decreases from 10^{-1} to 10^{-3}, indicating the influence of electrostatic interactions on the adsorption process.

The immobilized catalase activity is in the range of 0.5μmol O_2 $s^{-1}.g^{-1}$ support for a H_2O_2 concentration 1 mM; this indicates that 1 g of support would allow for a renewal time of 1 min for the oxygen dissolved in 10 ml of solution. The amount of enzyme which can be adsorbed is not a critical factor and attention must be focused on the operational stability.

3. STABILITY OF BACITRACINE WITH RESPECT TO HYDROGEN PEROXIDE

In presence of H_2O_2, bacitracine undergoes irreversible oxidation. The native (bacitracine A) and oxidized (bacitracine F) forms have been characterized using differential pulse polarography and ultraviolet absorption. The halfwave potential and the maximum absorptivity of the two forms differ markedly. The antimicrobial activities of both forms have been determined by the agar diffusion method. A good correlation was observed between the concentration of bacitracine A and the antimicrobial activity; the oxidized form (F) has no antimicrobial activity.

The half life time of the bacitracine activity at 30°C in presence of H_2O_2 50 mM is of the order of 100 min.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

PUBLICATIONS IN SCIENTIFIC JOURNALS

N. Mozes and P.G. Rouxhet (1987) - Methods for measuring hydrophobicity of microorganisms. J. Microbiol. Methods 6, 99-112

C. Changui, A. Doren, W.E.E. Stone, N. Mozes and P.G. Rouxhet (1987) - Surface properties of polycarbonate and promotion of yeast cells adhesion. J. Chim. Phys. 84, 275-281

P.G. Rouxhet and N. Mozes (1987) - Physico-chemical bases of microbial adhesion. Anaerobic digestion : results of research and demonstration projects (Eds. M.P. Ferranti, G.L. Ferrero and P. L'Hermite), Elsevier, p. 218-229

N. Mozes, F. Marchal, M.P. Hermesse, J.L. Van Haecht, L. Reuliaux, A.J. Léonard and P.G. Rouxhet (1987) - Immobilization of microorganisms by adhesion : interplay of electrostatic and non-electrostatic interactions. Biotechnol. Bioeng., in press.

ORAL COMMUNICATIONS, POSTERS, CONFERENCES

O. Lardinois and P.G. Rouxhet - Etude de l'activité et de la stabilité d'une catalase fongique. 13ème Forum des Jeunes Chercheurs, Toulouse (France), 8-11 July 1986

D. Amory - Propriétés de surface de microorganismes : relation entre la composition élémentaire, l'hydrophobicité et la charge électrique.
C. Changui - Propriétés de surface du polycarbonate et promotion de l'adhésion de cellules de levures.
M.P. Hermesse - Influence du milieu de culture sur l'adhésion d'Acetobacter aceti : altération de la charge du support par adsorption de constituants du milieu.
Colloque Etude des phénomènes physico-chimiques aux interfaces et adhésion des microorganismes aux surfaces, Massy (France), 11-12 September 1986.

P. Rouxhet - Use of silica as support for biocatalysts. EUCHEM Conference on Silica, Strasbourg (France), 22-24 September 1986.

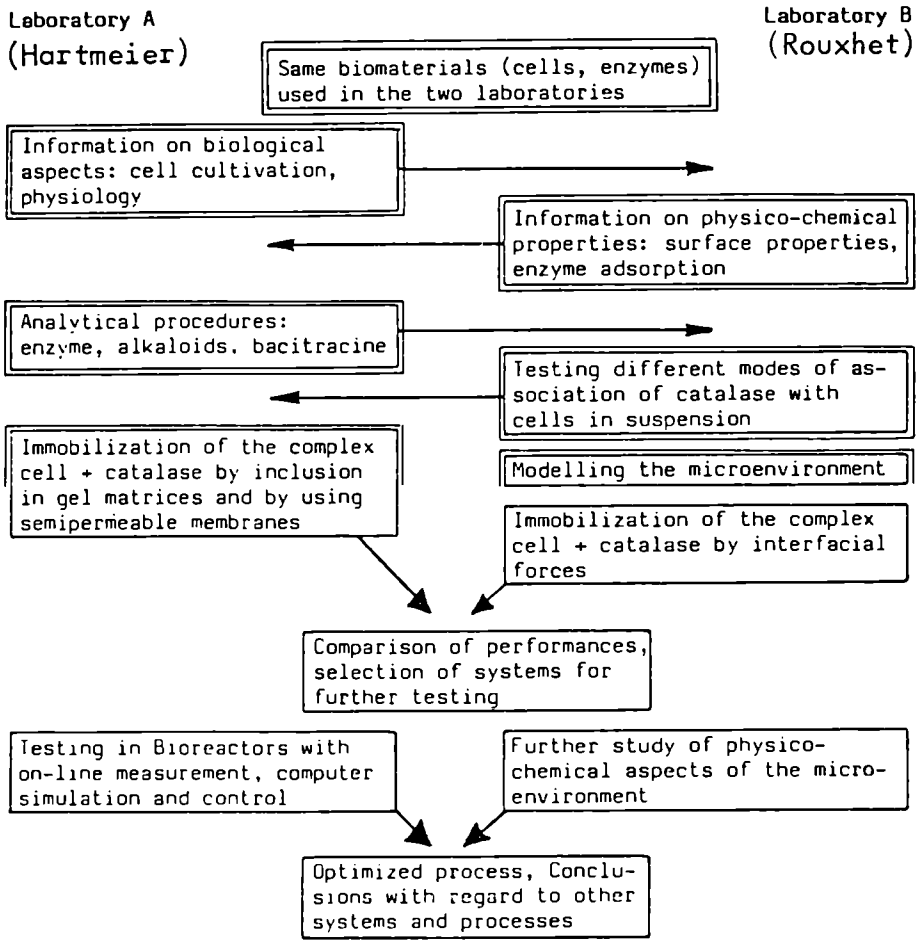
O. Lardinois, P. Herben, D. Masy and P.G. Rouxhet - Coimmobilization of Bacillus licheniformis with catalase : control of the oxygen supply to the bacteria. BAP Meeting, Capri (Italy), 2-6 May 1987.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	No
Joint experiment(s)	No
Joint meeting(s)	Yes

Descriptive information for the above data.

Exchange of material and regular meetings were done with the group of Prof. Hartmeier (Aachen). In the plan shown below, the subjects marked with double lines have been developed.



BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: U. T. C., Compiègne
Contract no.: BAP - 0053 - F

Project leader: M-D. LEGOY
Scientific staff: D. Thomas, S. Pulvin, C. Bourdillon and numerous graduate students

Address: Université Technologie
de Compiègne
Laboratoire de Technologie Enzymatique
B.P. 233
F - 60206 COMPIEGNE CEDEX

Telephone no.: +33.44200037

Telex no.: 150110 UNITECH F

Other contractual partners in the joint project:

M. Rossi, Università di Napoli
F. Kolisis, The National Hellinic Research Foundation (Athens)
K. Schügerl, Universität Hannover

Title of the research activity:
Bioconversion of hydrophilic and hydrophobic compounds by enzyme systems.

Key words:
Cofactor regeneration, Multiphasic reactors, Water activity, Lipases, Alcohol dehydrogenase

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The aims of the project are :

- Production of high added value long chain aldehydes from alcohols by using alcohol dehydrogenase
- Malic acid conversion
- Production of high added value lipids
- L-amino-acid synthesis by using liquid membranes.

These four points are linked to the problems of cofactor regeneration, enzyme stability and use of enzymes in multiphasic reactors.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Until now, in our laboratory, the research has been focused :

- at first, on the production of high added value long chain aldehydes by using two types of alcohol dehydrogenase and different multiphasic reactors,
- and then, on the production of high added value lipids by lipases working for unusual reactions of interesterification and synthesis.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

- Production of high added value long chain aldehydes from alcohols by using alcohol dehydrogenase

Two types of alcohol dehydrogenase have been used : horse liver alcohol dehydrogenase (HLADH) and the thermostable *Sulfolobus solfataricus* (SSADH) enzyme gives by Pr ROSSI from the University of Naples.

These enzymes need NAD as cofactor and set together the problem of cofactor regeneration and of the use of organic solvents since most of the substrates and products are insoluble in aqueous phase. Owing to the fact that ADH is very unstable in free form, it has been studied in an immobilized form for the model reaction of oxidation of geraniol.

Among three cofactor regenerating methods (chemical, bienzymatic and coupled substrate regeneration), we have chosen to work with the coupled substrate regeneration where ADH works alternatively for the oxidation of the alcohol and for the reduction of the acetaldehyde.

HLADH coimmobilized with NAD is used in fixed bed reactors. The optimization of the reactor shows unusual behavior explained by an increase in the mass-transfer resistance at low flow rates. This transition can be characterized by calculating the Damkölher number which compares the mass-transfer rate to the reaction rate. It may be possible to improve the mass transfer by changing the organic phase and/or the solid hydrated phase.
However, in spite of the possibility of realizing this type of reactors, synthesis capacities of ADH present important limitations due to the operational instability of the enzyme and to the insolubility of most of the substrates and products in water which necessitates work in emulsions or organic solvents. So, we have elaborated another system for using immobilized enzymes with substrates and products in gaseous form. Batches and fixed bed reactors have been tested with HLADH and NAD or NADH coimmobilized. In the reactors, gaseous substrates flow through the solid support, react with the enzyme-coenzyme system and gaseous products leave the reactor.
The feasibility of the system has been tested in batches for the reduction of ethanal, butanal, pentanal and hexanal with NADH coimmobilized with HLADH. Then column reactors have been studied at 60 °C with the system : the oxidation of butanol coupled with the reduction of acetaldehyde for NAD regeneration.
In such a system, the limiting step is always the HLADH instability. This problem can be solved by using the thermostable enzyme of *Sulfolobus solfataricus* . This microbial enzyme is stable at high temperature and gives activity yield of 80 % after immobilization when HLADH gives only 10 %. Moreover, this enzyme reacts with a wide range of substrates and will allow to optimize the system and to generalize its use.
We have tested this SSADH for the oxidation of butanol coupled with the reduction of acetaldehyde. A butanal production of 6 mmoles par unit of enzyme has been obtained with the *Sulfolobus* enzyme whereas only 0.2 mmole were obtained per unit of HLADH.

- Production of high added value lipids

Unusual reactions of interesterification and synthesis catalyzed by microbial lipases have been tested in reverse microemulsions . The microemulsions used are made of fatty acids or triglycerides, the enzyme dissolved in a very low water quantity, Brij 35 as surfactant and an alcoholic cosurfactant. In such systems, fats and alcohols are both the substrates of the enzyme and the microemulsion components. Microemulsions are monophasic, isotropic and stable at the thermodynamical and kinetical point of view. In a microemulsion there is no "visible" physical interface but the contact area between the organic and the aqueous phase is very large and exchanges are very rapid.
In our case, reverse microemulsions permit not only the solubilization of substrates and

products but also by using very low water quantity the obtention of non conventional reactions of interesterification and synthesis.

- interesterification :

The interesterifications of triolein and triglycerides having chain lengths from C_4 to C_9 have been studied for two microbial lipases : the non specific *Candida cylindracea* lipase and the 1-3 specific *Rhizopus arrhizus* lipase. The results show that in any case interesterification occurs with a 40 % yield for the 1-3 specific lipase, when the non specific one reacts less with triglycerides having short chain length.

In such systems, one of the fundamental parameter of the reaction is the water activity A_w.

In microemulsions it is possible by using always the same water amount to obtain different A_w by varying the ratio surfactant/cosurfactant/lipidic phase. The best results of interesterification (triolein-triheptanoin) are obtained for an $A_w = 0.63$. For higher A_w, hydrolysis is favored and for smaller A_w, interesterification does not occur.

The effect of the ratio surfactant/cosurfactant has been studied for the same reaction for microemulsions of given A_w (= 0.63). The lower the surfactant amount, the faster the interesterification reachs its maximum but, after the maximum the interesterified triglycerides decrease. When the surfactant amount increases, the maximum interesterification is obtained latter but is higher. It seems that the surfactant insures for the lipase a good catalytic efficiency in the time by a regulation of the available water.

- synthesis :

The synthetic capacities of the lipase in microemulsions have been studied. 98 % heptyl-oleate synthesis have been obtained after 18 days of reaction. This reaction time can be considerably reduced by using lipase directly in presence of the substrates (oleic acid and heptanol) without addition of organic solvent or water .

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

Publications in Scientific Journals

- PULVIN S., LEGOY M.D., LORTIE R., PENSA M. and D. THOMAS
  Biotechnol. Lett., 1986, 8, 11, 783

- LEGOY M.D., BELLO M., PULVIN S. and D. THOMAS
  Studies in Organic Chemistry, 1987, 29, 97

- BELLO M., THOMAS D. and M.D. LEGOY
  Biochem. Biophys. Res. Commun., in press

Communications

- Deuxième Congrès National de la Société Française de Chimie.
  8-12 septembre 1986. Paris, France

- International Symposium on Biocatalysis in Organic Media.
  7-10 december 1986. Wageningen, The Netherlands

- Meeting of Contractants
  2-6 may 1987. Capri, Italy

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff	Yes	
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

exchange of materials : the thermostable alcohol dehydrogenase and the Sulfolobus solfataricus strain have been given by the laboratory of Naples.

exchange of staff : a student from the laboratory of Pr Rossi has spent 10 months in our laboratory.

Joint meeting : a joint meeting has been held in Capri (Italy) at the beginning of may.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Univ. degli Studi di Napoli

Contract no.: BAP - 0052 - I

Project leader: M. ROSSI

Scientific staff: S. Bartolucci, M. de Rosa, A. Gambacorta, G. Iorio, R. Rella

Address: Università di Napoli
Dipartimento di Chimica
Organica e Biologica
Via Mezzocannone 16
I - 80134 NAPOLI

Telephone no.: +39.81.206411

Telex no.:

Other contractual partners in the joint project:

M.D. Legoy, Université de Technologie (Compiègne)
F. Kolisis, The National Hellenic Research Foundation (Athens)
K. Schügerl, Universität Hannover

Title of the research activity:

Bioconversion of hydrophilic and hydrophobic compounds by enzyme systems.

Key words:

Archaebacteria, Thermostable enzymes, Alcohol dehydrogenase, Malic enzyme, β-galactosidase

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The development of many industrial enzymatic processes is connected with enzymes stability, coenzymes regeneration and the use of enzymes in organic solvents. Stability can be achieved by immobilization or by using special enzymes such as those extracted from extreme thermophilic bacteria. Coenzymes can be macromolecularized and regenerated enzymatically also in the case when it is necessary to use two phase systems consisting of water and water immiscible organic solvents selecting enzymes able to operate in such environmental conditions.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Aim of the project was to study the potentiality of some extreme thermophilic organism's cells and enzymes which are thermostable and resistent to commmon protein denaturing agents and to organic solvents. Enzymes models were: a new alcohol dehydrogenase, malic enzyme, ß-galactosidase and DNA polymerase. A gene bank of Sulfolobus solfataricus DNA had to be constructed and methods developed for cloning the above mentioned enzymes in E.coli.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Methodology

The organism S.solfataricus was grown at 87°C as described in the proceeding BEP progress reports. A system was developed to separate chromatographically different enzymatic activities from a single homogenate. The new alcohol dehydrogenase was purified to homogeneity according to the procedure published in European Journal of Biochemistry and immobilization on Eupergit C was performed as described. Antibodies against ß-galactosidase and alcohol dehydrogenase were obtained in rabbits. The NH_2 terminal sequence of ß-galactosidase was performed with a gas-phase sequencer and oligo-nucleotides were obtained from Applied Biosystem.

Results

Alcohol dehydrogenase

A novel NAD^+-dependent alcohol dehydrogenase was obtained in homogeneous form from S.solfataricus. The dimeric enzyme, was thermophilic, thermostable, resistent to organic solvents and had a quite broad substrate specificity. The enzyme thermophilicity was unusual because the activity increased with temperature up to 95°C and its considerable thermostability was not affected by the presence of certain organic solvents. Ethyl acetate at 10% in the standard assay mixture did not affect the enzyme activity.

The stereospecificity was tested on 3-Methyl-butal-2-one with Eupergit C immobilized enzyme. NADH regeneration was achieved in situ with the coupled substrate approach using propan-2-ol as oxidable co-substrate in 13-fold molar excess with respect to ketone. It was demonstrated that the reduction involves a hydride attack to the re face of the carbonyl to produce the corresponding S-alcohol.

Malic enzyme

A thermophilic and thermostable malic enzyme was obtained in homogeneous form and its molecular structure and properties studied as published. The enzyme can use NAD^+ or $NADP^+$ as coenzymes although with different efficiency. NAD^+ and $NADP^+$ were macromolecularized by covalent coupling to PEG (Mr 17,000 or 20,000) in collaboration with A. Bückman and G. Carrea. The kinetic parameters of these derivatives in different operational conditions are under investigation in a reactor having coenzyme immobilized in the form of a gel layer onto the inner surface of a capillary membrane.

From studies on the activity and stability of the malic enzyme in denaturing agents and organic solvents it can be assessed that this enzyme is not only stable but even more active in the presence of different organic solvents.

Construction and screening of a S.solfataricus genomic library.

A S.solfataricus genomic library was constructed in the expression vector λgt11 which contains a single ECO R I cleavage site within the lac Z gene, 53 base pairs upstream from the ß-galactosidase translation codon. Foreign DNA sequences inserted in this site have the potential to be expressed as

fusion proteins with ß-galactosidase. Genomic DNA libraries constructed in λgtll can therefore be screened with antibody probes for antigens produced by specific recombinant clones. λgtll can accommodate up to 7 kb insert DNA but clones carrying shorter inserts express fusion proteins more efficiently. To construct the genomic library, DNA was isolated from S. solfataricus and digested with deoxyribonuclease. Fragments ranging between 1.0 and 2.0 Kb were purified by preparative electrophoresis: this length seemed appropriate to avoid gene splitting into too many pieces and yet to obtain an efficient expression of fusion proteins. Blunt end fragments were generated by T4 DNA polymerase and ligated to ECO R I linkers: these fragments were subsenquently digested with ECO R I and ligated to λgtll arms. Recombinant phages were packaged in vitro, plated and directly screened. Polyclonal antibodies raised against homogeneous ß-galactosidase and alcohol dehydrogenase from S.solfataricus were used for the immunoscreening of the library. Using ß-galactosidase and alcohol dehydrogenase antisera, respectively, 7 and 3 clones were isolated out of 10000 recombinant clones. A mixture of 53-mer oligonucleotides, whose sequence was deduced from the aminoterminal sequence of ß-galactosidase, was synthesized and used to screen the same library: 14 positive clones were found out of 15000 screened. The purified ß-galactosidase clones have been characterized and partially sequenced.

Work is in progress to determinate a partial aminoacid sequence of alcohol dehydrogenase against which oligonucleotides will be constructed.

Discussion

A novel alcohol dehydrogenase has been discovered and purified from S. solfataricus. This enzyme which is different from those of other thermophilic organisms, has been characterized in the free and immobilized state and in the cells. Studies are in progress concerning the effect of organic solvents on stability and activity of alcohol dehydrogenase, malic enzyme and ß-galactosidase.

A genome library of S.solfataricus has been constructed and positive clones found for ß-galactosidase and alcohol dehydrogenase.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

R. Rella, F.M. Pisani, C. Raia, C. Vaccaro, M. De Rosa, A. Gambacorta, M. Rossi: Thermophilic and thermostable alcohol dehydrogenase from archaebaterium Sulfolobus solfataricus.
17th FEBS Meeting Berlin (West), August 24-29, 1986, Abstr., **FRI 06.01.23**:334.

C.A. Raia, R. Tarantino, R. Rella, M. Rossi: Purification and characterization of a metal-chelator-sensitive peptidase from an extreme thermophile.
17th FEBS Meeting Berlin (West), August 24-29, 1986, Abstr., **FRI 06.03.52**:366.

C.A. Raia, R. Tarantino, R. Rella, R. Nucci, M. Rossi: Characterization of a partially purified cobalt peptidase from an extreme thermophile.
Conferenza Nazionale su "Meccanismi del riconoscimento in macromolecole biologiche: aspetti molecolari ed applicazioni biotecnologiche", Siena 4-6 Settembre 1986, Abstr. pag.44.

S. Bartolucci, A.M. Guagliardi, M. Moracci, A. Gambacorta, M. De Rosa, M. Rossi: Sulfolobus solfataricus malic enzyme.
XIV International Congress of Microbiology Manchester, England, Sept. 7-13, 1986, Abstr. **P.B18-10**:106.

R. Rella, F.M. Pisani, C.A. Raia, C. Rozzo, A. Gambacorta, M. De Rosa, M. Rossi: Purificazione all'omogeneità e caratterizzazione di una ß-galattosidasi dall'archeobatterio S.solfataricus.
32° Congresso SIB, Messina - Giardini Naxos, 28 Sett. - 1 Ottobre 1986, Abstr. **E-10**:168.

S. Bartolucci, A. Guagliardi, M. Moracci, M. Rossi: L'ossalacetato come substrato dell'enzima malico da Sulfolobus solfataricus.
32° Congresso SIB, Messina - Giardini Naxos, 28 Sett. - 1 Ottobre 1986, Abstr. **E-16**:174

M. Rossi: Aspetti della sintesi del DNA ad alta temperatura.
Atti XXIII Convegno SIBBM, Pavia 13-15 Ottobre 1986, **12**.

R. Rella, M.F. Pisani, M. Rossi: Preparation and properties of an alcohol dehydrogenase activity from the extreme thermophilic bacteria Sulfolobus solfataricus.
Biocatalysis in Organic Media, Wageningen (The Netherland) December 7-10, 1986, Abstr. **P9**:43.

M. Rossi: Applications Potentielles des Procaryotes Thermophiles.
Biofutur **53**:39-42, 1987.

R. Rella, C.A. Raia, A. Trincone, A. Gambacorta, M. De Rosa, M. Rossi: Properties and specificity of an alcohol dehydrogenase from thermophilic archaebacterium Sulfolobus solfataricus.
Biocatalysis in Organic Media - Studies in Organic Chemistry, **29**:273-278, 1987 Edited by C. Laane, J. Tramper and M.D. Lilly.

A. Guagliardi, S. Bartolucci, M. Moracci, M. Rossi: Activity and stability of an archaebacterial malic enzyme.
Riunione SIB Campania: Calabria-Puglia-Sicilia, Aprile 1987.

M. Rossi, R. Rella, C.A. Raia, F.M. Pisani, C. Vaccaro, R. Nucci, A. Gambacorta, M. De Rosa, S. Bartolucci, A. Trincone, L. Lama, V. Lanzotti, C. Rozzo: Purification, structure and properties of a novel archaebacterial NAD^+ dependent alcohol dehydrogenase.
BAP, Capri 2-6 May, 1987, Abstr. pagg. 86-87.

R. Rella, C.A. Raia, F.M. Pisani, C. Vaccaro, R. Nucci, A. Gambacorta, M. De Rosa, M. Rossi: Specificity and stability in organic solvents of a novel archaebacterial NAD^+ dependent alcohol dehydrogenase.
4th European Congress on Biotechnology, (ECB4), Amsterdam, June 14-19, 1987, Abstr. TUP-143:336-339.

S. Bartolucci, A.M. Guagliardi, M. Moracci, M. Rossi: Activity and stability of an archaebacterial malic enzyme in denaturing agents and organic solvents.
18th FEBS Meeting Ljubljana, June 28 - July 3, 1987.

R. Rella, C.A. Raia, S. D'Auria, R. Nucci, C. Vaccaro, A. Trincone, M. Rossi: Struttura e specificità di una nuova alcool deidrogenasi: biotrasformazioni ad alta temperatura e in presenza di solventi organici.
33° Congresso Nazionale SIB, Brescia-Gardone, 26-28 Settembre 1987.

S. Bartolucci, R. Rella, A.M. Guagliardi, C.A. Raia, A. Gambacorta, M. De Rosa, M. Rossi: Malic enzyme from archaebacterium Sulfolobus solfataricus: purification, structure and kinetic properties.
J. Biol. Chem. **262**:7725-7731, 1987.

R. Rella, C.A. Raia, M. Pensa, F.M. Pisani, A. Gambacorta, M. De Rosa, M. Rossi: A novel archaebacterial NAD^+-dependent alcohol dehydrogenase. Purification and properties.
Eur. J. Biochem. (in press).

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

A collaboration on the new alcohol dehydrogenase has been started with Dr. Legoy's group, in Compiègne. This collaboration is of particular interest since S.solfataricus strain and the purified enzyme have been given to the Compiègne's group to be used in a gas-phase reactor already set up. In addition, Dr. M. Pensa from our laboratory spent about one year working in Compiègne getting acquainted with gas-phase reactor technique.

During the Capri meeting in May, the contractual partners met and planned a meeting in 1988. The Naples group discussed with Prof. Shügerl the methodology to experiment in the liquid membranes system their stable enzymatic activities.

In addition to contractual partners, the Naples group is in contact with Prof. Külbe, Dr. Bückmann and Dr. Carrea for a collaboration on macromolecularized coenzymes. In June Prof. Külbe come to visit our laboratory and to give a seminar: in two days the development of the collaboration was discussed.

In addition Dr. R. Rella visited and made experiments in the laboratory of Prof. A. Fontana, in Padova, to follow by optical method the denaturation of the thermostable alcohol dehydrogenase by heat and denaturing agents such as urea and guanadine hydrochloride.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: The Nat. Hellenic Research Foundation, Athens

Contract no.: BAP - 0051 - GR

Project leader: F. KOLISIS

Scientific staff: A. Xenakis, Th. Valis, F. Kolisis

Address: The National Hellenic Research Foundation
Center of Biological Research
Department of Biochemistry
48, Vas. Constantinou Ave.
GR - 11635 ATHENS

Telephone no.: +30.1.7239965

Telex no.:

Other contractual partners in the joint project:

M.D. Legoy, Université de Technologie (Compiègne)
M. Rossi, Università di Napoli
K. Schügerl, Universität Hannover

Title of the research activity:
Bioconversion of hydrophilic and hydrophobic compounds by enzyme systems.

Key words:
Lipases, Microemulsions

Reporting period: June 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Lipases will be used for the production of high added value lipids from olive oil.Processes will be optimized in systems containing only few per cent of water for hydrolysis,transesterification and synthesis of lipids. Lipases from different sources immobilized or not will be tested.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

In this work we attempt spectroscopic studies on lipase molecule in microemulsions formulated with water/sodium-di-ethylhexyl sulfoccinate(AOT)/ isooctane,by using various probes.Application of this technique to protein containing microemulsions yields informations about the interactions between protein molecule and the surfactant membrane separating the oil/water phases.Because the dimensions of the water droplets and the protein molecules are similar,these interactions can be very important and can have a dramatic influence on the enzymatic activity.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

In a number of studies it has been shown that many enzymes retain enzymatic activity when they are incorporated in microemulsions.Microemulsions can be defined as systems of water/oil/amphiphile,which are optically isotropic and thermodynamically stable liquid solutions.One interesting class of these systems is water in oil microemulsions where oil consists the continuous phase,while water is located in the centre of spherical droplets formated by amonolayer of surfactant molecules.The fact that these systems combined two distinct regions with complet properties permits their application in various domains.Thus,enzymatic studies have been recently performed in microemulsions showing that enzymes are active in such media.Furthermore,the optical transparency of microemulsions permits processing spectroscopic studies on the enzyme hosted in the microdroplet.

1.METHODOLOGY

Triolein hydrolysis in AOT/isooctane microemulsions

Following partial purification of the enzyme,a sample of concentrated solution of lipase was added into a solution of 50 mM AOT in isooctane.The mixture was vortexed for 15 sec to obtain clear miccelar solution.The R value (R = $(H_2O)/(AOT)$) was equal to 20.The AOT/isooctane solution contained the substrate (10%,w/v).

Free fatty acids determination:A desired amount of the enzyme was poured in to the solution of AOT/isooctane and the reaction was initiated by vortex mixing.In selected periods of time 0.4 ml of the reaction mixture was added to the test tube containing 4.6 ml isooctane and 1 ml cupricacetate-pyridine solution.The mixture was immediately vortexed to stop the reaction and to determined the content of free fatty acids liberated,according to the method of Lowry et al.

Enzymatic hydrolysis in emulsions

The substrate (triolein) was placed in 35 ml glass-stopered flask.Phosphate buffer (0.1 M,pH 7.5) was added (1.85 ml).The desired amount of lipase and sufficient distilled water were also added to bring the total aqueous phase to 3.25 ml.The flask was placed on a stirring plate at constant temperature and at prediscribed periods the reaction was stoped by inactivation of the enzyme with the addition of 1:1 mixture of acetone and ethanol to the flask.

Free fatty acids determination:The solution was titrated with standard 0.1N NaOH solution.Blank determinations were made in the mixture of the reactans, which was inactivated immediately with the acetone-methanol mixture and titrated as above.

Tributirin hydrolysis in emulsions

The method is similar to the previous described.100 µl of tributirin were added into 15 ml sodium phosphate buffer (0.1 M,pH 7.0,$CaCl_2$ 0.1 mM).The reaction mixture was sonicated 4 x 30 sec and the desired amount of enzyme was added.The FFA content was immediately measured by the usual titrimetric method.For the hydrolysis of tributirin in the presence of eosine,the desired amount of eosine was added in the concentrated enzyme solution and the mixture was added in the buffer-substrate solution.

Spectroscopic studies

Absorption spectra of fluoresceine-isothiocyanate (FITC) were obtained by using

FITC in the presence or absence of the enzyme in solution and microemulsions

Absorption spectra of eosine in phosphate buffer (0.1 M,pH 7.0) were obtained by using 77 μM eosine in a 1 mm cuvette in absence or presence of 58 μM lipase.Also,absorption spectra of eosine in AOT/isooctane/water reversed micellar solution (AOT 50 mM,R = $(H_2O)/(AOT)$=10) were obtained by adding 90 μM (total)eosine in a 1 mm cuvette or by adding 180 μM eosine (total) in the presence of 0.104 μM lipase.The spectra were recorded on a Perkin-Elmer 356 double beam spectrophotometer.

Fluoresence studies were performed by using Rhodamine-B as a probe and the spectra were recorded on a Perkin -Elmer 650-40 Fluoresence spectrophotometer.

2.RESULTS AND DISCUSSION

During standarization of the various assay procedures pancreatic lipase was used partial purified by three centrifugation steps in 4,000,16,000and 19,000 rpm respectively,following by two ultrafiltration steps using XM-300 and UM-200 ultrfiltration filters.The final preparation was 10 times pure giving two distinct bands of the enzyme in SDS polyacrylamide gel electrophoresis.

In the spectroscopic studies it was used lipase from Rhizopus delemar. Spectroscopic studies on the enzyme molecule in solution and in microemulsions were performed in the presence or absence of fluoresceine-isothiocyanate and eosine.

A slight difference of 2 nm in the absorption spectra of eosine was observed in the presence of lipase in microemulsions in comparison to the eosine spectra in the buffer solution.This is probably due to the lower polarity of the water cores of the microdroplets.It seems that the enzyme molecule is located in close viccinity of the surfactant membrane.

Fluoresence studies were performed using rhodamine-B as a fluorophore in the presence of enzyme,substrates such as triolein,oleic acid etc.The differences in the spectra of the probe obtained in aqueous solution and in microemulsions are used in the studies of the conformation of the enzyme molecule during its catalytic action in such media.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

Lipases in microemulsions:Spectroscopic studies.T.P. Valis,A.Xenakis and F.N. Kolisis.Meeting of contractans,Capri,2-6 May,1987.

Enzymatic catalysis in organic solvents.T.P. Valis and F.N. Kolisis Chimica Chronica.in press (Greek)

Applications of heterogeneous enzymatic catalysis in Biotechnology. T.P. Valis,A. Xenakis and F.N. Kolisis.International Congressin Biology and Production.Organized by Panhellenic Union of Biologists,October 1987,Athens Greece.

. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	No
Exchange of staff	Yes	No
Joint experiment(s)	Yes	No
Joint meeting(s)	Yes	No

Descriptive information for the above data.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Universität Hannover

Contract no.: BAP - 0070 - D

Project leader: K. SCHÜGERL

Scientific staff:

Address: Universität Hannover
Institut für Technische Chemie
Callinstrasse 3
D - 3000 HANNOVER

Telephone no.: +49.511.762 EXT.2253

Telex no.: 923868 UNIHN D

Other contractual partners in the joint project:

M.D. Legoy, Université de Technologie (Compiègne)
M. Rossi, Università di Napoli
F. Kolisis, The National Hellenic Research Foundation (Athens)

Title of the research activity:

Bioconversion of hydrophilic and hydrophobic compounds by enzyme systems.

Key words:

Enzyme immobilisation, Liquid membrane reactor, Liquid membrane emulsions, Cofactor regeneration, Enantioselective synthesis

Reporting period: July 1986 - July 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The long term objective of this study is the development of a liquid membrane reactor for the enantioselective synthesis of chemicals and pharmaceuticals. A new immobilisation technique for enzymes and enzyme systems will be studied intensively. Up to four enzymes will be immobilised and continuous cofactor recycling systems will be used. The three phase immobilisation system is easy to perform and reaction details of the enzyme, the transport reaction and the purification reaction can be studied as well as the use of this type of second generation bioreactor for industrial purposes.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

During the reporting period a new method for the montioring of the leaking behaviour of the liquid membrane emulsions was established. A system with three enzymes and contiuous cafactor recycling was investigated for the l-alanine production. In addition the continuous ATP-regeneration in liquid membranes was studied. Different analysis techniques were applied (AAS, amino acid analysis with pre column derivatisation, HPLC).

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1.Methodology

A liquid membrane reactor system was set up for studies with enzyme emulsions for L-alanine production and continuous ATP-regeneration. These systems can be run batch-wise or continuously. Different on-line analytical detection systems (polarimeter and UV-spectrophotometer) were applied to the reactor to monitor the process. The amino acid analysis was performed in an amino acid analyser system (OPA-method), other components like ATP, G6P, glucose and acetyl phosphate were analysed enzymatically. An AAS was used for the determination of the potassium concentration during the leak rate experiments

2. Results

Liquid membrane emulsion systems are suitable for the encapsulation of enzyme and

enzyme/coenzyme systems. Earlier investigations have shown, that L-leucine can be produced in a continuously working liquid membrane reactor (based on the results of Kula and Wandrey). During the reporting period two liquid membrane reactor systems for the continuous regeneration of ATP and the production of L-alanine as well as mass transport studies in liquid membrane emulsions were investigated.

Enzyme emulsions are prepared by emulsifying enzyme solutions into suitable organic phases. This organic phase separates the enzyme phase from the outer substrate phase, thus preventing the mixing of both miscible liquids. Tensides are added to attain suitable and stable emulsions. The substrates or the products are transported through the membrane due to their physical solubility or by carrier transport. Liquid secondary or tertiary ammonium salts are used as anion exchangers.

Different enzymes are involved in the ATP-regeneration system. Glucose is converted to glucose-6-phosphate by hexokinase. During this reaction ATP is consumed and continuously regenerated by the dephosphorylation of acetyl-phosphate to acetate. This reaction is catalyzed by acetatekinase.

Different organic systems were tested to get a suitable membrane phase. The best results were obtained with xylol and cyclohexane phases. The enzymes and the native ATP were encapsulated into this membrane phase and the emulsion was dispersed in a substrate phase. Glucose-6-phosphate is enriched in the inner phase and can be recovered at the end of the reaction by breaking down the emulsion in a high frequency electrical field.

L-alanine can be produced from D,L-lactate. This substrate is converted to pyruvate by D- and L-lactatedehydrogenase. The produced NADH is oxidized during the reductive amination of pyruvate to L-alanine by alaninedehydrogenase.

The enzyme system can be encapsulated in a kerosene membrane (5% Span 80 and 1% Adogen 464). The whole reaction rate depends mainly on the ratio of ALDH to D,L-LDH .

The modelling of liquid membrane reactors is difficult because different transport phenomena can be observed. Substances can be transported from one phase to the other by permeation (solubility or carrier transport) or by leaking of the emulsion. The leaking behaviour of emulsions is a function of the membrane composition as well as the composition of the aequous phases. The influence of the enzyme stabiliser glycerine on the membrane stability was tested. Potassium ions were encapsulated together with the enzymes in the inner phase. The metal cations can only get into the outer phase by leaking of the emulsion. Anions like the produced L-alanine were also transported by the carrier. By measuring the potassium and amino acid concentration in the outer phase, the leak rate as well as the permemation could be detected at the same time and the negative influence of the glycerine on the membrane stability could be studied.

3.Discussion

The liquid membrane reactor can be used for the production of other amino acids like L-alanine and for reactions with continuous ATP-regeneration. A basis for an efficient modelling, leak and peremation rate measurements can be performed at the same time. The use of the reactor system for other enzyme systems will be extended and several reaction parameters (membrane stability) will be investigated during the next research period

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

Th. Scheper, Z. Likidis, K. Makryaleas, Chr. Nowottny, K.Schügerl,
Three different examples of enzymatic bioconversion in liquid membrane reactors, Enzyme and Microbial Technology, in press.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	No
Exchange of staff	No
Joint experiment(s)	No
Joint meeting(s)	Yes

Descriptive information for the above data.

We met in Capri during the sectorial meeting and the future colaboration was discussed. We will get enzymes from Prof. Kolisis, Dr. Legoy and Prof. Rossi to test them in the liquid membrane reactor.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: C. N. R. S., Tours

Contract no.: BAP - 0067 - F

Project leader: C. ROPARS

Scientific staff:

Address: Laboratoire de Biopharmacologie
Transfusionnelle - CNRS
Centre Régional de Transfusion Sanguine
2, Brd. Tonnellé
F - 37044 TOURS

Telephone no.: +33.47376683

Telex no.: 750 605 F (Telefax n° : 47.61.08.32)

Other contractual partners in the joint project:

A. de Flora, Univesità di Genova
L. Silengo, Università di Torino
G. Fornaini, Università degli Studi di Urbino

Title of the research activity:

Construction of enzyme-loaded erythrocytes as bio-reactors.

Key words:

Bioreactors, Erythrocyte encapsulation, Enzymes, Targeting, Pro-drugs

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Development of circulating bioreactors by encapsulation of enzymes in lysed and resealed RBC.
The main project is the encapsulation of hexokinase and its production by genetic engineering.
However, the feasibility of encapsulation of other enzymes is investigated in our laboratory :
- asparaginase (treatment of LAL and some lymphosarcoma)
- di amino oxidase (histamine metabolism)
- acetaldehyde deshydrogenase (genetic defect in severe alcoolic patients)

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

- Development of the methodology of large scale encapsulation of substances in red blood cells (using Desferal and Inositol Hexaphosphate as molecules to be entrapped).
- Encapsulation of yeast hexokinase
- Encapsulation of asparaginase
- Analytical methods for Diamino oxidase
- Evaluation of feasibility of encapsulation of acetaldehyde deshydrogenase.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1 - Methodology

a) Technology of encapsulation.

- Large scale encapsulation of Desferal and Inositol hexaphosphate have been extensively investigated, by us in association with the Centre National de Transfusion Sanguine, leading to the elaboration of satisfactory protocols, in order to obtain blood products suitable for human transfusion (Desferal) or physiological investigations in animals (IHP).

- The parameters to be used in an automatic or semi automatic process have been studied.

- Preliminary, in vivo, experiments have been performed in humans using red blood cells loaded with Desferal in Phase I and Phase II clinical studies in Paris and in association with Dr Zanella, Blood Transfusion Center - Milan (Italy).

b) Analytical Methods

Several analytical methods have been extensively studied and adapted in our Laboratory :

- dosage of Hexokinase activity using a spectrophotometric method
- measurement of :
  - . glycolysis shunt activity in RBC
  - . total glycolysis in RBC
  - . glucose consumption
- HPLC of RBC metabolites using acid or basic pH precipitation of total blood.
- Glucose oxidase activity using oxygen consumption.
- Diamino oxidase activity :
  - . liquid scintillation of putresceine ^{14}C
  - . spectrophotometric dosage of NADH
  - . correlation between the two techniques
- Asparaginase activity. - Spectrophotometric method.
- Asparagine dosage in blood and plasma using HPLC.

c) Small scale encapsulation of enzymes in human or animal RBC.

- Encapsulation of yeast hexokinase, glucose oxidase and asparaginase in human, mouse and rat RBC.
- Measurement and possible correction of non specific absorption at the surface of RBC.

d) In vivo measurement of mouse and rat RBC life span.

Using ^{51}Cr labelling for

- . controls
- . RBC loaded with asparaginase

e) Determination of asparaginase activity encapsulated in RBC.

Pharmaco-Kinetic of free or encapsulated asparaginase. Effect on circulating asparagine in mouse.

2 - Results.

a) Development of the methodology is successfully in progress and in vivo experiments in human have been performed using the present protocols. Further developments of devices are linked to pre-industrial investigations on Desferal and IHP modified RBC.

b) Encapsulation of Hexokinase from yeast.

Such encapsulation has been performed, with final molecular yields of about 20 %. However, a non-specific absorption (3 to 14 %) is observed, depending on the RBC used, with yeast HK. Which seriously hampers the interest of encapsulation for this particular molecule.
We have recently received human HK from our partners and the investigation using this new material has now begun.

Yeast HK encapsulation lead as expected to :
. increased glucose consumption
. increased total glycolysis
In vivo experiments have to be performed in mouse.

c) Encapsulation of Asparaginase.

Remarquable results have been obtained using Asparaginase encapsulated in RBC.

- Normal in vivo life span of modified cells (mouse)
- The activity of Asparaginase in vivo parallels the RBC concentration
- Total suppression of Asparagine in the plasma, with a single injection, during the period of observation.

Other data obtained in USA indicate a protective effect of encapsulation against anaphylactic reactions in guinea pig.

All these results, to be published, will allow us to investigate clinical trials in human in the coming months expecting to prevent the adverse reactions in the patients with a very important increase in the efficacity of the treatment, possibly associated with reduction of the dose injected.

d) Diamino oxidase - acetaldehyde deshydrogenase.

Preliminary experiments have been performed on these molecules in vitro.

Discussion :

Very encouraging results have been obtained :

- Our partners have very rapidly investigated human HK preparation and cloning which will allow us to continue our own program on this specific molecule.
- Asparaginase results will allow the development of human clinical trials in the near future.
- Other enzymes under investigation are potentially of high interest for human therapy.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV 2/

- C. ROPARS, M. CHASSAIGNE, Ch. BAILLEUL and M.C. VILLEREAL.
Methodology of loading xenobiotics in Red Blood Cells.
BAP First sectorial Meeting - European Communities.
CAPRI. May 2-6 1987.

- M. JRADE, C. ROPARS, C. VANVOOREN and M. CHASSAIGNE.
Technical aspects of human red blood cells carriers.
IInd International Meeting - Red Blood Cells as carriers for drugs.
TOURS April 7-9 1987.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Exchange of materials.

We have received human HK from placental origin from our partners in the recent period of time.

Exchange of staff.

- M. LAGUERRE from our laboratory have spent 1 week in Pr DEFLORA laboratory (GENOA) to learn analytical methods for HK measurements and standardization of yeast HK samples.

- U. BENATTI from Pr DEFLORA laboratory came in our laboratory to learn the techniques of in vivo experiments in mouse.

- C. ROPARS has made 2 visits in GENOA
1 visit in URBINO

- A. DEFLORA, M. MAGNANI, U. BENATTI, H. ZOCCHI and L. GUIDA have attended the IInd International Meeting "Red Blood Cells as carriers for drugs" organised in TOURS, France, by C. ROPARS, with large discussions on the joint program.

Joint Experiments.

Encapsulation of yeast hexokinase, with Pr DEFLORA laboratory, comparison of results and standardization.

Joint Meetings.

1°) IInd International Meeting on "Red Blood Cells as carriers for Drugs" organized in TOURS France by C. ROPARS.

Attended by partners (see above).

- Other participations indirectly linked to the project was from P. ZANELLA and Co workers. Blood Transfusion Center, Milano, Italy, and Staff from Centre National de Transfusion Sanguine, PARIS France. These teams are associated with us for the development of the methodology of encapsulation and its applications to Desferal and IHP transport for therapeutic purposes.

- Several other participants to the Meeting have presented their own results on encapsulation of enzymes in RBC :

- C.A. KRUSE (USA)	*- Arginase*
- J.L. WAY (USA)	*- Rhodanese*
- A.M. Del C. BATTLE (Arg.)	*- Amino-levulino dehydratase.*

2°) Participation of the partners to the BAP - 1st sectorial Meeting CAPRI May 2-6 1987.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Univ. degli Studi di Genova

Contract no.: BAP - 0056 - I

Project leader: A. DE FLORA

Scientific staff: U. Benatti, E. Zocchi, L. Guida, M. Tonetti, C. Polvani

Address: Università di Genova
Istituto Policattedra di Chimica Biologica
Viale Benedetto-XV 1
I - 16132 GENOVA

Telephone no.: +39.10.515365

Telex no.:

Other contractual partners in the joint project:

G. Fornaini, Università degli Studi di Urbino
L. Silengo, Università di Torino
C. Ropars, C. N. R. S. (Tours)

Title of the research activity:
Construction of enzyme-loaded erythrocytes as bio-reactors.

Key words:
Bioreactors, Erythrocyte encapsulation, Enzymes, Targeting, Pro-drugs

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Red blood cells (RBC) can be engineered to behave, a) as vehicles for chemicals to be disseminated in circulation, b) as circulating bioreactors for display of unusual functions. The latter objective can be achieved by entrapment of bioregulators, or of suitable pro-drugs to be converted to active drugs inside RBC, or of enzyme proteins degrading membrane-diffusible blood metabolites. This project aims at encapsulating human hexokinase (HK) in human RBC, in order to construct "super-RBC" useful in transfusion technology. In addition, other enzyme-loaded RBC systems will be constructed and characterized as bioreactors.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

A) Development and refinements of the technique of encapsulation of proteins and chemicals within RBC (hypotonic hemolysis-isotonic resealing).
B) Encapsulation of Glucose oxidase (GOD) as a model enzyme and characterization of the biochemical properties of the GOD-containing RBC both "in vitro" and "in vivo" (mouse).
C) Targeting of suitably manipulated carrier RBC (i.e., hemolyzed and resealed) to either liver or spleen in mice.
D) Preliminary assessment of the ability of engineered RBC to produce and release antineoplastic drugs from encapsulated pro-drugs.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY

The technique of encapsulation was based on transient hypotonic hemolysis of both human and mouse RBC, followed by isotonic resealing and reannealing at 39°C. The biochemical properties of the RBC subjected to the encapsulation technique were investigated according to standardized procedures. Following entrapment of both HK and GOD, non-specific adsorption to the RBC surface was ruled out by immunofluorescence experiments. HK activity was assayed by a coupled spectrophotometric procedure in the presence of $NADP^+$ and G6PD, while GOD activity was measured by oxygen consumption. The "in vivo" survival of the variously manipulated RBC (e.g., loaded with GOD or fragilized by energy depletion, treatment with neuraminidase and exposure to a $NaN_3+H_2O_2$ mixture) was estimated after ^{51}Cr labelling and intravenous re-infusion in mice. Analysis of metabolites of antineoplastic drugs (Adriamycin) and pro-drugs (F-deoxyuridine monophosphate, FdUMP) was carried out on methanol/chloroform and perchloric acid extracts, respectively, by automated HPLC techniques.

2. RESULTS

Encapsulation of GOD (from Aspergillus niger) in human and mouse RBC allowed us to construct a suitably fragilized RBC model. Fragilization was explored in human GOD - containing RBC by conventional analytical procedures and revealed an intraerythrocytic burst of H_2O_2 that was clearly related to the levels of encapsulated GOD activity. In the mouse, the GOD-loaded RBC showed rapid removal from circulation and concomitant uptake by the spleen.

Other procedures were developed in order to achieve selective targeting of fragilized mouse RBC to either the spleen or the liver. Splenic uptake of "carrier" RBC was also induced by exposure of RBC "in vitro" to an oxidative challenge represented by a mixture of NaN_3 and H_2O_2. Conversely, rapid hepatic sequestration of "carrier" RBC was obtained following either ATP depletion at 42° C or removal of sialic acid by incubation of RBC with neuraminidase. EM analyses of livers from mice receiving energy-depleted RBC i.v. revealed massive congestion within sinusoids and remarkable hyperactivity of Kuppfer cells.

Studies on the potential use of RBC as carriers or bioreactors in antineoplastic therapy were performed with Adriamycin and with FdUMP. Mouse RBC loaded with Adriamycin proved to be an effective system for targeting this drug to selected organs, notably liver, as compared with intravenous administration of free Adriamycin. Preliminary studies on the fluoropyrimidine compounds showed the competence of FdUMP-loaded human RBC in producing the potent antineoplastic drug, F-deoxyuridine (FUdR), by means of an intracellular phosphatase. The newly formed FUdR is then released to the outside through a nucleoside transport system present in the membrane. Accordingly, the FdUMP-loaded RBC seem to behave as endogenous bioreactors for effective production and delivery of FUdR in the circulatory system.

3. DISCUSSION

The results obtained so far bear relevance to the general objective of constructing and characterizing engineered RBC as bioreactors, although the specific issue of HK-enriched RBC is still at a preliminary step.

Entrapment of GOD as a foreign enzyme protein in human and mouse RBC is a first example of "ad hoc" manipulated RBC. These RBC acquire new biochemical properties that make them a promising model for investigating the mechanisms both of RBC aging and of oxidative hemolysis resulting in their splenic sequestration. Potential applications of GOD encapsulation in mouse RBC (and of exposure of RBC to NaN_3+H_2O_2 as well) concern the development of new immunization technologies based on still unexplored procedures for presentation of co-encapsulated antigens.

The variety of methodologies developed in Genoa to achieve selective targeting of manipulated RBC within the reticuloendothelial system opens, in addition, new perspectives for addressing drugs and bioregulators to either the spleen or the liver.

The complement of resident enzymes makes RBC potential bioreactors for the intraerythrocytic production and delivery of drugs starting from RBC-encapsulated pro-drugs. This turns to be the case for the formation of the highly toxic antitumour drug, FUdR, whose release from engineered RBC might be regulated in order to meet pharmacokinetic requirements and to achieve selective organ targeting (e.g., liver).
Research on HK is now in progress, in close collaboration with the three partners of this joint project (see section V). Preliminary results are very encouraging.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV. 1.

1. Comparative tissue distribution and metabolism of free versus erythrocyte-encapsulated adriamycin in the mouse.
   Benatti, U., Zocchi, E., Tonetti, M., Guida, L., and De Flora, A.
   Advances in Biosciences (C. Ropars, M. Chassaigne, and C. Nicolau, eds.) Pergamon Press, Oxford, in press.
2. Encapsulation of Glucose oxidase in mouse erythrocytes: an experimental model of oxidant-induced cytotoxicity and a means for splenic targeting of carrier erythrocytes.
   Zocchi, E., Benatti, U., Guida, L., Tonetti, M., Damonte, G.L. and De Flora, A.
   Advances in Biosciences (C. Ropars, M. Chassaigne, and C. Nicolau, eds.) Pergamon Press, Oxford, in press
3. Engineered red blood cells as carriers of drugs. An "in vitro" study.
   De Flora, A., Benatti, U., Zocchi, E., and Guida, L.
   Biotechnology in Clinical Medicine, (Albertini, A., et al., eds.), Raven Press, New York, in press.
4. Hepatic or splenic targeting of carrier erythrocytes: a murine model.
   Zocchi, E., Guida, L., Benatti, U., Canepa, M., Borgiani, L., Zanin, T., and De Flora, A.
   Biotechnol. Appl. Biochem., in press.

IV. 2.

1. Glucose oxidase-loaded human and mouse red blood cells (RBC): A selective way for RBC fragilization and targeting to spleen.
   De Flora, A., Zocchi, E., Guida, L., Benatti, U.
   BAP First Sectorial Meeting - E.E.C., pp 93-94 (1987).
2. Entrapment of antineoplastic drugs and pro-drugs in human red blood cells (RBC): An "in vitro" study.
   Zocchi, E., Guida, L., Benatti, U., De Flora, A.
   BAP First Sectorial Meeting - E.E.C., pp. 94-95 (1987).

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Exchange of materials
Purified HK form human placenta was supplied from M. Magnani since October 1986.
Exchange of staff and joint experiments
E. Zocchi was in L. Silengo laboratory for 1 week to learn screening techniques of cDNA libraries from human placenta. U. Benatti spent three different periods in C. Ropars laboratory to learn the technique for estimating the half-life of ^{51}Cr-labelled erythrocytes in circulation. M. Laguerre, from C. Ropars laboratory, spent 1 week in our laboratory to learn analytical methods for measuring HK and GOD activities and for assay of RBC metabolites and to standardize samples of yeast HK to be encapsulated within human and mouse RBC. L. Rossi, from G. Fornaini lab, spent 1 week in our laboratory performing the preliminary joint experiments on the biochemical properties of human RBC loaded with human HK. C. Ropars made 2 visits to our laboratory to discuss the joint project and to develop research strategies involving exchange of staff. L. Silengo made 5 visits to our laboratory to discuss progress of the joint project.

Joint meetings
2nd International Meeting on "Red Blood Cells as carriers for Drugs", organized by Dr. C. Ropars, Tours, France, 7-9 April, 1987. This was attended, among others, by A. De Flora, U. Benatti, E. Zocchi, and L. Guida.
International Meeting "RIA '87: Biotechnology in Clinical Medicine", Rome, Italy, 13-15 April, 1987. This was attended by C. Ropars, A. De Flora and U. Benatti.
B.A.P. First Sectorial Meeting on "Enzyme engineering: Protein design and Applications in biocatalysis", Capri, Italy, 2-6 May, 1987. This was attended by C. Ropars, L. Silengo, M. Magnani, A. De Flora and U. Benatti.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Univ. degli Studi di Torino

Contract no.: BAP - 0068 - I

Project leader: L. SILENGO

Scientific staff: A. Daniele, F. Altruda, P. Cervella, M. Ferrone, P. Defilippi

Address: Università di Torino
Istituto di Igiene
Via Santena 5bis
I - 10126 TORINO

Telephone no.: +39.11.6963106

Telex no.:

Other contractual partners in the joint project:

C. Ropars, C. N. R. S. (Tours)
A. de Flora, Università di Genova
G. Fornaini, Università degli Studi di Urbino

Title of the research activity:
Construction of enzyme-loaded erythrocytes as bio-reactors.

Key words:
Hexokinase, Expression vector, cDNA, Immunological screening, Fusion protein

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Red blood cells (RBC) can be engineered to behave, a) as vehicles for chemicals to be disseminated in circulation, b) as circulating bioreactors for display of unusual functions. The latter objective can be achieved by entrapment of bioregulators, or of suitable pro-drugs to be converted to active drugs inside RBC, or of enzyme proteins degrading membrane-diffusible blood metabolites. This project aims at encapsulating human hexokinase (HK) in human RBC, in order to construct "super-RBC" useful in transfusion technology. In addition, other enzyme-loaded RBC systems will be constructed and characterized as bioreactors.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

To clone the gene coding for human HK we proceed through the following steps:

a) construction of a cDNA library in the expression vector gt11 from human placenta polyA+ RNA.

b) screening with specific antibodies against HK supplied by the laboratory in Genoa and Urbino.

c) nucleotide sequencing of the clone. This analysis will allow the identification of the primary structure of the protein.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

In mammalian tissues the phosphorilation of glucose is catalyzed by four different hexokinase isozymes. At present,hexokinase activity was found in a soluble form in human placenta:70% is hexokinase type I while the remaining 30% is hexokinase type II.

Hexokinase I was purified and shows only one band in non denaturing polyacrilamide gel electrophoresis; however two components (with m.r. 112,000 and 103,000) were constantly seen in SDS-gel electrophoresis.

Monospecific antibodies have been raised in rabbit against purified hexokinases subtypes. Nothing is known about the aminoacid composition; the only information about aminoacid sequence comes from yeast hexokinases (1) where the cloning and sequence determination of the genes specifying hexokinase A and B has been determined. Our goal is to isolate the cDNA coding for the human hexokinase from a placenta cDNA library in order to overexpress this enzyme in a prokaryotic system using an appropriate expression vector. In addition we should learn about the hexokinase primary structure by sequencing the cDNA isolated.

In order to isolate the cDNA coding for the hexokinase we decided to screen a human placenta cDNA library by immunological method (2) using polyclonal antibodies against the enzyme.
The present study proceeds through the following steps:
1) Construction of the cDNA library.
2) Screening of the library with specific antibodies.
3) Isolation of the lysogens from immunoreacting clones.
4) Western blot of the fusion protein and test with two different preparations of polyclonal antibodies.
5) Experiments of immunocompetition using the purified hexokinase and the fusion protein from positive clones (this part of the work has been done by the group in Urbino and we will not refer it in detail).

METHODOLOGY

1) Construction of the cDNA library. We have constructed a cDNA library in the expression vector lambda gt11 (2). RNA was prepared from human placenta by the guanidine thiocyanate method (3). Poly(A)$^+$RNA was purified by chromatography on oligo(dT)-cellulose. Single-strand cDNA was prepared using reverse transcriptase primed by oligo(dT), and double-stranded cDNA was synthesized by using RNase H and DNA polymerase Klenow fragment (4); the cDNA prepared in this way was to at least 3 kb long. The cDNA was methylated with EcoRI methylase and then ligated with EcoRI octamer linkers. The linkers were then cleaved with EcoRI, and cDNA was ligated with EcoRI-digested phosphatase-treated lambda gt11 DNA into the beta-galactosidase structural gene lac-Z.
In this way we have obtained a human placenta cDNA library
containing 10^5-10^7 indipendent recombinant phages.
2) Screening of the library with specific antibodies.
The placental cDNA library was screened with polyclonal antibodies obtained from rabbits immunized against purified human placental hexokinase and purified on a column of *E. coli* extract coupled to sepharose. The screening was carried out using biotinylated second antibody and peroxidase-conjugated strepto- avidin to detect bound antibody.
3)-4) The positive clones were lysogenized in *E. coli* strain Y1089 (2). Lysogens were cultured at 30°C, induced for 20 min at 45°C and then the synthesis of the fusion protein was induced with IPTG for 1 hr at 37°C. The cells were harvested by centrifugation, washed, and lysed by sonication. Induced lysogens were dissolved in electrophoresis sample buffer, and the proteins were separated on 7% SDS-polyacrylamide gels (5). The proteins were transferred to nitrocellulose filters that were incubated for 1 hr at room temperature in TBS containing 3% bovine serum albumin and then with affinity-purified antibodies for 1 hr. After washing the filters and incubation with horseradish peroxidase-conjugated goat anti-rabbit serum for 1 hr, bound antibodies were detected with naphthol.

RESULTS

We screened approximately 1×10^6 independent recombinants clones and 12 positives were obtained. The fused protein of

each clone reacts
with polyclonal antibodies specific for human hexokinase. In particular one clone (n. 16) reacts with two different preparation of polyclonal antibodies, with polyclonal antibodies against yeast hexokinase and a monoclonal antibodies against human hexokinase. The inserts were sized, after digestion with the enzyme EcoRI, on agarose gel. They are extimated to be in the range between 1600 bp and 600 bp. The cDNAs were then subcloned into the plasmid Bluescribe VCS for further analysis.
We are currently characterizing the clones by DNA sequencing using the chain termination method of Sanger et al. (6).

Since no aminoacid sequence of the human hexokinase is known we perfomed some experiments of immunocompetition using purified hexokinase and the fusion protein synthetized by our selected clones. The data obtained by the group of Urbino on the immunocompetition indicate that clone n. 16 gives a strong competition with the purified hexokinase in an Elisa assay. Taken together these results show that at least one clone (n. 16) is the putative cDNA coding for the human hexokinase.
In order to identify this cDNA we will prepare antibodies against the partially purified fused protein from clone n. 16. They will be used to recognize the placenta hexokinase in a Western blot.

REFERENCES

(1) Stachelek,C., Stachelek,J., Swan, J., Botstein, D. & Konigsberg, W. - Nucl. Ac. Res. 14, 2 pp. 945-963.(1986)
(2) Young,R.A., and Davies,R.W.,- Proc. Natl. Acad. Sci. USA 80,pp. 1194-1198.(1983)
(3) Hynes,R.O., Schwarzbauer,J.E. & Tamkun,J.W. - Methods in Enzymol., in press.
(4) Chirgwin,J.M., Przybyla,A.E., MacDonald,R.J. & Rutter,W.J. - Biochemistry 18, pp.5194-5296.(1979)
(5) Gubler,U., and Hoffman,B.J. - Gene 25, pp.263-269.(1983)
(6) Laemmli,U.K. - Nature 227, pp.680-685.(1970)
(7) Sanger,F., Nicklen,S., & Coulson,A.R. - Proc. Natl. Acad. Sci. USA 74, pp. 5463-5466.(1977)

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	No

Descriptive information for the above data.

Exchange of materials
Purified HK policlonal antibodies were supplied from M. Magnani.
Exchange of staff and joint experiments
E.Zocchi, from A.De Flora lab,spent 1 week in our laboratory to learn screening techniques of cDNA libraries from human placenta.
Extracts from lysogenic positive clones isolated in our lab were antigenically characterized in M.Magnani lab.
The fusion proteins purified from the extracts will be used to prepare policlonal antibodies by the group in Urbino.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Univ. degli Studi di Urbino

Contract no.: BAP - 0055 - I

Project leader: G. FORNAINI

Scientific staff: M. Magnani, V. Stocchi, A. Accorsi, A. Fazi, P. Ninfali, L. Chiarantini, L. Rossi, G. Serafini

Address: Università degli Studi di Urbino
Istituto di Chimica Biologica
Via A. Saffi 2
I - 61029 URBINO PS

Telephone no.: +39.722.320188

Telex no.:

Other contractual partners in the joint project:

L. Silengo, Università di Torino
C. Ropars, C. N. R. S. (Tours)
A. de Flora, Università di Genova

Title of the research activity:

Construction of enzyme-loaded erythrocytes as bio-reactors.

Key words:

Bio-reactors, Erythrocyte - loading, Enzymes

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Red blood cells (RBC) are ideal vehicles for a number of chemicals to be disseminated via circulation and, in addition, may become valuable bio-reactors performing unusual metabolic functions "in vivo". The rationale for use of RBC as bio-reactors is the entrapment of specific enzyme proteins within RBC, with the aim of achieving overconsumption of elevated blood metabolites. This project, whose objective is the entrapment of human hexokinase (HK), is a specific example of this emerging trend. The aims of this project are: a) attempts at achieving some control of moderate hyperglycemias b) development of a useful cell system for investigating erythrocyte aging c) construction of new blood products useful in transfusion technology.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

A) Selection of an easily available human tissue containing enough hexokinase (HK) type I to be purified, the isoenzyme on which the project is based (human tissues contain four different HK isoenzymes encoded by different genes, each tissue containing one or more isoenzymes).
B) Purification, possibly in homogeneous form, of human HK I. This will be done by conventional methods (ion-exchange chromatography, gel filtration, etc.) and modern techniques (affinity and dye-ligand chromatography, HPLC).
C) Production of anti-HK antisera.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Human placenta was chosen as a starting material for the purification of hexokinase type I because of its availability and relative high-proportion of type I isoenzyme. In fact, in human placenta 85% of total hexokinase activity was found in a soluble form. Of this, 70% is hexokinase type I while the remaining 30% is hexokinase type II. All the bound hexokinase is of type I. Soluble hexokinase I purified 11,000-fold by a combination of ion-exchange chromatography, affinity chromatography, and dye-ligand chromatography. The specific activity was 190 units/mg protein with a 75% yield. The enzyme shows only one band in non denaturating polyacrylamide gel electrophoresis that stains for protein and enzymatic activity, however two components (with Mr 112,000 and 103,000) were constantly seen in sodium dodecyl sulfate-gel electrophoresis. Many attempts were made to separate these two proteins under native conditions, however only one peak of activity was obtained when the enzyme was submitted to gel filtration (Mr 118,000), preparative isoelectric focusing (pI 5.9), anion exchange chromatography, hydroxylapatite chromatography and affinity chromatography on immobilized dyes and immobilized glucosamine. The high and low Mr

hexokinases show the same isoelectric point under denaturating conditions as determined by two dimensional gel electrophoresis. Each hexokinase sub-type was obtained by preparative sodium dodecyl sulfate electrophoresis followed by electroelution. Monospecific antibodies raised in rabbits against electroeluted high Mr and low Mr hexokinases were not able to recognize the native enzymes but each of them detected both hexokinases on immunoblots. The amino acid compositions and peptide mapping by limited proteolysis of the high and low Mr hexokinases were also performed suggesting a strong homology between these two sub-types of human hexokinase I.

During the course of this work it became evident that starting from the same human tissue, several other enzymes involved in glucose metabolism could be simultaneously purified. In this respect we have developed procedures for the simultaneous purification of hexokinase, phosphoglucomutase 1 and 2 aldolase, glucose phosphate isomerase and glucose 6-phosphate dehydrogenase. All the enzymes mentioned were obtained in homogeneous forms, in one week, with average recoveries over 40%. So the procedures utilized seem to be useful in obtaining large amounts of enzymes from an easily available human tissue.

The second important step in this work was the determination of a partial amino acid sequence useful to synthesize a synthetic oligo deoxynucleotide probe for identifying the human hexokinase I gene. This work is complicated by the availability of small amounts of protein, and moreover by the presence of hexokinase in two molecular forms inseparable by conventional procedures. Therefore, we have obtained them separately after preparative electrophoresis followed by electroelution. As a first step in their characterization, techniques have been developed to hydrolyze and analyze protein samples in microgram quantities.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

1. M. Magnani, V. Stocchi, G. Serafini, L. Chiarantini, G. Fornaini
   Human placenta hexokinase type I. Purification, properties and evidence for two sub-types. (1987) submitted for publication
2. A. Fazi, M. Magnani, A. Accorsi, P. Ninfali, G. Fornaini
   Simultaneous, preparation from human placenta of several enzymes of glucose metabolism. (1987) submitted for publication.
3. M. Magnani, V. Stocchi, A. Fazi, A. Accorsi, P. Ninfali, G. Fornaini
   Construction of enzyme-loaded erythrocytes as bio-reactors BAP-meeting Capri 2-6 May, 1987; pp 97-98

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Exchange of material(s)

Purified hexokinase I was supplied for metabolic studies to A. De Flora (Genova) and for standardizing the entrapment procedures to C. Ropars (Tours).

Purified rabbit anti-human hexokinase antibodies (IgG) were provided to L. Silengo (Torino) for screening of the cDNA libraries.

Exchange of staff and joint experiments

Dr. L. Rossi was in the A. De Flora laboratory from May 11 to May 21, 1987 performing the initial joint experiments on the "in vitro" metabolic properties of human erythrocytes loaded with human hexokinase I.

Joint meeting

Prof. M. Magnani partecipate at the IInd International Meeting on Red Blood Cells as carriers for Drugs Potential Therapeutic Applications (Tours 7-9 April, 1987) organized by C. Ropars (Tours).

PROTEIN ENGINEERING

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Univ. of Newcastle-upon-Tyne

Contract no.: BAP - 0042 - UK

Project leader: R.H. PAIN

Scientific staff:

Address: The University of Newcastle-upon-Tyne
Department of Biochemistry
Ridley Building
UK - NEWCASTLE-UPON-TYNE NE1 7RU

Telephone no.: +44.632.328511 EXT.3437

Telex no.: 53654

Other contractual partners in the joint project:

G. Schumacher, Boehringer Mannheim GmbH (Tutzing)
A. Böck, Ludwig-Maximilians-Universität (München)

Title of the research activity:

Folding, assembly, stability and genetic modification of penicillin acylase and its precursor.

Key words:

Penicillin acylase, Precursor, Folding, Stability, Assembly

Reporting period: October 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The objectives are to lay the basis for more effective production and use for penicillin acylase, for improving stability and for extending future capabilities of the antibiotic industry by engineering penicillin acylase for new applications in the modification of penicillins and cephalosporins. The short term objectives are to study the cloning, expression and secretion of the enzyme and to study its folding and assembly in both wild type and modified forms.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The objectives are to begin to characterise the physical properties and conformation of penicillin acylase.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Despite repeated advertisement it has not been possible to find a suitable candidate for the Newcastle work until June 1987. This is because of the shortage of postdoctoral scientists in the physical biochemistry area. Dr. C. Lindsay has just been appointed and has started work. There has at this point been no opportunity for significant results to be obtained.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

Nil

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)	Yes	
Joint meeting(s)	Yes	

Descriptive information for the above data.

Samples of purified enzyme have been received from Boehringer Mannheim. Samples of precursor have been received from Professor Böck, ready for future experiments which will be complementary to those in his laboratory on the secretion and processing of penicillin acylase.

A joint meeting is being arranged in July 1987 to review progress and reassess objectives for the coming year.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Ludwig-Maximilians-Universität München
Contract no.: BAP - 0040 - D

Project leader: A. BÖCK
Scientific staff: D. Sizmann

Address: Ludwig-Maximilians-Universität München
Institut für Genetik/Mikrobiologie
Maria-Ward-Strasse 1a
D - 8000 MÜNCHEN 19

Telephone no.: +49.89.177084

Telex no.: 529860

Other contractual partners in the joint project:

R.H. Pain, The University of Newcastle-upon-Tyne
G. Schumacher, Boehringer Mannheim GmbH (Tutzing)

Title of the research activity:
Folding, assembly, stability and genetic modification of penicillin acylase and its precursor.

Key words:
Penicillin acyclase, Secretion, Processing

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The technically important enzyme penicillin acylase displays a unique gene-protein relationship: The two subunits of the enzyme are processed from a single precursor polypeptide concomitant with its export into the periplasm. It is the aim of the joint project (i) to study the path of export and processing of this precursor, (ii) to analyse folding and assembly of the subunits, and (iii) to produce genetically modified enzymes with desired stability and specificity properties.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The biochemistry of proteolytic processing was analyzed with special emphasis on the connection between proteolysis and membrane translocation. A procedure was established to prepare precursor protein in large amount. The purified precursor was used as substrate for localization of the proteases involved in cleavage. Genetically modified precursors were used for elucidating primary structure requirements for export and processing.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

RESULTS AND DISCUSSION

Penicillin acylase (PA) catalyzes the hydrolysis of penicillin G to phenylacetic acid and 6-aminopenicillanic acid, a crucial intermediate in the production of semisynthetic penicillins. The enzyme, located in the periplasmic space, consists of two non-identical subunits, α and ß. Both subunits are derived from a single precursor polypeptide, comprising a signal sequence and the two subunits, separated by 54 amino acids (the endopeptide), which are absent in the mature enzyme. Processing of the precursor seems to occur together with export.

Construction of a plasmid (G. Schumacher, Boehringer, Tutzing) coding for a PA precursor without a signal peptide resulted in the intracellular accumulation of the translation product. This precursor is enzymatically inactive and it is not processed within the cytoplasm. Processing starts immediately, when a crude extract is prepared from these cells. This in vitro processing pathway proceeds as follows: a first rapid cleavage step yields ß subunit and a 29 kD protein corresponding to α plus endopeptide; in a second step the endopeptide is removed to give mature α via an intermediate (27 kD) composed of α and part of the endopeptide. Thus mature subunit results either from a distinct two-step removal of the endopeptide or from unspecific and stepwise C-terminal proteolytic degradation which gives rise to the 27 kD protein.

Plasmids bearing the genetic information for either α or ß subunit under control of the tac promoter have been constructed (G. Schumacher, Boehringer, Tutzing). While the ß subunit is produced in amounts that lead to precipitation and formation of inclusion bodies in the cells, α is detectible only in comparatively small quantities, due to rapid proteolytic degradation. This can be cicrumvented by extending the N-terminus of α by met-tyr-tyr-phe (G. Schumacher, Boehringer, Tutzing). This prolonged α is synthesized in comparable amounts to ß and forms inclusion bodies. Reconstitutions of penicillin acylase activity could neither be achieved by mixing α (or stabilised α) and ß containing crude extracts nor in cells carrying two compatible plasmids with the information for α and ß, respectively. Thus the endopeptide might be crucial for the formation of a correct tertiary structure of the subunits. In order to test this hypothesis, the stabilised α and the ß subunit have been purified as inclusion bodies to 90 % homogeneity and are studied by Prof. R. Pain in Newcastle Upon Tyne by biophysical techniques.

For die identification and characterization of the PA processing activity purified precursor protein (lacking the signal peptide) is the essential substrate. However, only small amounts of precursor protein are accumulated in the cells due to rapid turnover and immediate processing after disruption. Two approaches were successful:

a) Transfer of the plasmid into a heat shock response defective strain. An estimated 5 - 10 % of these cells showed precursor-inclusion bodies.

b) Stabilization of the precursor was obtained in an analogous way to the α subunit, namely by extension of the N-terminus. This resulted in a highly stable precursor polypeptide that precipitates to form multiple inclusion bodies within the cells. These inclusion bodies were purified to yield a precursor preparation of at least 90 % homogeneity.

In both preparations the precursor protein is present in a denatured form. Solubilization in 8 M urea and removal of the urea by dialysis in presence of 0.2 % Triton X100 provided sufficient though small amounts of soluble precursor for processing experiments. The results obtained so far are consistent with prior findings that processing of the precursor withouth signal peptide occurs only when cells are disrupted. The data point to (a) processing enzyme(s) attached to the cytoplasmic membrane facing the periplasm.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1 G. Schumacher, D. Sizmann, H. Haug, P. Buckel and A. Böck: Penicillin acylase from E. coli: unique gene - protein relation. Nucleic Acids Research 14, 5713-5727 (1986).

IV.2 D. Sizmann, G. Schumacher, P. Buckel and A. Böck: Penicillin Acylase aus E. coli: Analyse des Prozessierungs- und Exportmechanismus. Gemeinsame Frühjahrstagung der VAAM und der Sektion I der DGHM (Münster, 1986).

D. Schumacher, D. Sizmann, H. Haug, P. Buckel and A. Böck: Folding, assembly, stability and genetic modification of penicillin acylase and its precursor. In: Enzyme Engineering: Protein design and applications in biocatalysis (Capri, 1987).

/. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

Precursor of penicillin acylase and inclusion bodies of the individual subunits were prepared in our laboratory and provided to Dr. R. Pain for folding studies. Boehringer Mannheim provided plasmids with desired penicillin acylase constructs as well as oligonucleotides. Several meetings were arranged between the three BAP contractors of this joint project.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: State University Utrecht

Contract no.: BAP - 0071 - NL

Project leader: G.H. DE HAAS

Scientific staff: H.M. Verheij, O. Kuipers

Address: State University of Utrecht
Laboratory of Biochemistry
Padualaan 8
Transitorium III
NL - 3584 CH UTRECHT

Telephone no.: +31.30.533186

Telex no.: 70353 BITRA NL

Other contractual partners in the joint project:

R. Verger, C. N. R. S. (Marseille)

Title of the research activity:

Conversion of pancreatic phospholipase A2 into triglyceride-degrading lipase.

Key words:

Phospholipase, Lipase, Phospholipids, Lipolysis

Reporting period: August 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

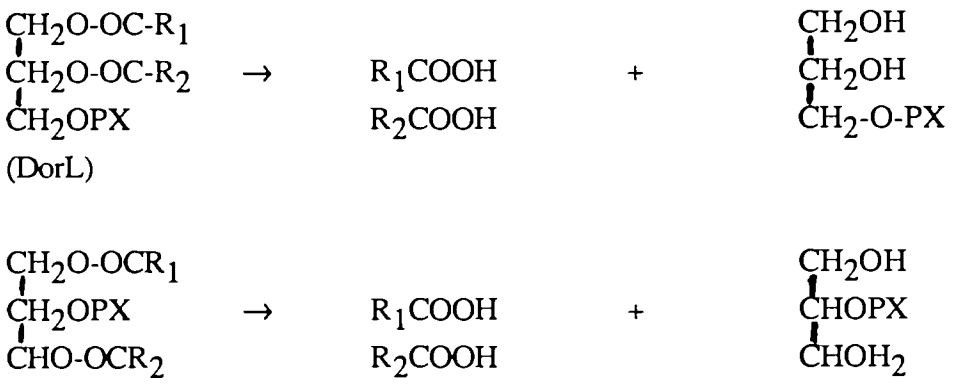
Lipolytic enzymes catalyze the hydrolysis of water-insoluble fats and phospholipids. Phospholipases A_1 and A_2 are Ca^{2+}-dependent enzymes and attack phospholipids, whereas lipases do not need Ca^{2+} and hydrolyze preferentially di- and triglycerides. The stability of these enzymes varies from rather low (lipases) to very high (phospholipases A_2). Stable, aspecific fat-degrading enzymes may be of interest as additives in detergents and may form a excellent tool for the synthesis of (phospho)lipids in enzyme reactors.
To bridge the gap between phospholipase and lipase, pancreatic phospholipase will be modified by site-directed mutagenesis to create an enzyme with the stability of the parent molecule but with a broader substrate specificity.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

In the past year we purified a membrane bound phospholipase A from *E. coli* and determined its substrate specificity. Crystallization experiments were undertaken (Prof. Drenth, Groningen) which yielded crystals of this enzyme.
Similarly in collaboration with Dr. Götz (München) a lipase from *S. hyicus* was purified and its substrate specificity was determined.
Regarding the pancreatic phospholipase A_2 it was desirable to extend the possibility to express the enzyme to other systems than *E.coli*. As a first approach to modify the substrate specificity modifications had to be considered which modified the substrate binding but where the active site residues involved in bond-breaking and bond-making remained unchanged.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Using an overproducing clone the phospholipase A (PLDA) from the outer membrane of *E. coli* was purified to homogeneity with a yield of about 1.5 mg pure enzyme per liter of culture medium. The enzyme is devoid of endogeneous phospholipids and can be kept in solution in the presence of detergents only. (Publication 1).
Crystallization attempts carried out in the lab of Prof. Drenth yielded crystalls with dimensions 0.5 x 0.5 x 0.04 mm which diffracted to a resolution of 2.5 Å . The unit cell has dimensions 72 x 107 x 76 Å (space group C_{2221}). The crystalls showed some disorder. Attempts are going on to obtain more homogeneous and larger crystalls.
Using a large number of synthetic substrates we were able to determine in detail the substrate specificity of this enzyme. In contrast to reports in literature where the enzyme was reported to be specific for hydrolysis of fatty acyl ester bonds at the *Sn* -1 position of natural phospholipids we found the enzyme to be quite aspecific.
Hydrolysis occurs of both fatty acid ester bonds in phospholipids. Not only the natural L -α phospholipids are hydrolyzed but also D-α and β phospholipids are hydrolyzed.



Neutral lipids like triglycerides and diglycerides are not degraded or at least 10^3 times slower than phospholipids.
The enzyme needs Ca^{2+} ions for activity and is completely inactive in the presence of chelators like EDTA. Of all kations ions tested only Sr^{2+} could replace Ca^{2+} with appreciable (25%) activity.

S hyicus contains a lipase which has been cloned and overexpressed in *S. carnosus* by Dr. F. Götz (München). This strain excretes the enzyme as a protein with an apparent MW of 86 KD. Attempts to purify this enzyme failed since the protein appeared to be very sensitive to proteolytic cleavage which resulted in 5 bands (MW 50 - 65kD) all showing enzymic activity.
In vitro cleavage with chymotrypsin yielded one band (MW 50KD) which is about 2 times as active as the 86 KD protein. This band was purified to homogeneity and its substrate specificity was determined.
Using synthetic substrates we could show that this acid labile lipase not only hydrolyses natural triglycerides (olive oil) and short-chain triglycerides but also hydrolyses very efficiently (short-chain) phospholipids. Both in neutral lipids and in phospholipids the enzyme attacks all ester bonds yielding fatty acids and fully deacylated products. Both Ca^{2+} and Sn^{2+} ions enhance the enzymatic activity even though a considerable rest activity is observed in the presence of EDTA.
Since this enzyme is about two times more active on phospholipids than on triglycerides it can be designated as a phospholipase rather than a lipase. Determination of its X-ray structure would be very informative and we hope to obtain crystals in the next report period.

Pancreatic phospholipase A_2 can be expressed in *E. coli* as a fusion protein from which it can be cleaved off by chemical (hydroxylamine) or protolytic (trypsin) means. Since in *E. coli* the phospholipase does not contain disulfide bridges *in vitro* reduction / reoxidation and renaturation has to be carried out to obtain full enzymatic activity. This procedure is time-consuming and although it gives good results with native enzyme and simple mutants it might be less succesfull in mutants lacking one or two cysteines.
We thus developed an expression system for expression in bakers yeast which is capable of *in vivo* disulfide bridge formation. We thus fused the gene coding for prophospholipase A_2 to the secretory peptides of the yeast α-mating factor. In this way prophospholipase A_2 is secreted into the culture medium at a level of 0.5 - 2 mg/liter. The isolated and purified prophospholipase A_2 was converted into active PA_2 which was indistinguishable from native PLA_2. This proves that yeast indeed is capable of producing correctly folded proteins with a disulfide-bridge content as high as seven. (Publication submitted to EJB).

In a first attempt to change the substrate specificity of phospholipase A_2 we started to modify residues 31 (Leu in WT enzyme) and 69(Tyr in WT enzyme). Based on the X-ray model of bovine pancreatic PLA_2 these residues are in close contact with each other thus preventing the entry of substrate to the active site. X-ray data suggest that Tyr^{69} might be involved in binding of the substrate via a formation of a hydrogen bridge between the phenolic OH and the oxygens of the phosphate.
We have replaced Leu^{31} by Trp, Ala and Arg (frequently occurring in snake venom phospholipases) and Ser, Thr and Gly. This was done in one experiment using a sixfold degenerated oligonucleotide where CTA-(Leu) was replaced by - A C / T G G / G -.

Tyr^{69} has been replaced by Phe and Lys respectively.
At present we have purified PLA_2 -Ala^{31} and PLA_2-Trp^{31} and the purification of the other mutants is in progress.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

1) P. de Geus, N.H. Riegman, A.J.G. Horrevoets, H.M. Verheij and G.H. de Haas (1986) Eur. J. Biochem. 161, 163-169.

2) P. de Geus, C.J. van den Bergh, O. Kuipers, H.M. Verheij, W.P.M. Hoekstra and G.H. de Haas (1987) Nucl. Acids Res. 15, 3743-3759.

3) C.J. van den Bergh, A.C.A.P.A. Bekkers, P. de Geus, H.M. Verheij and G.H. de Haas, submitted for publication.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

1) Exchange of materials:
Purified *E. coli* phospholipase A_2 has been sent to Dr. Verger (Marseilles) for a kinetic characterization using the monolayer technique.

1&2) One of the members of the Utrecht groep (Verheij) has spent a week in the lab of Dr. Götz (München) to isolate and precipitate lipase from *S. carnosus* supernatant and to introduce a chromogenic lipase activity test in München. The precipitated material was taken to Utrecht for purification.

3) In Utrecht a plasmid construction was made which was designed to export pancreatic $proPLA_2$ as a fusion product with *S. hyicus* lipase to the culture medium. Transformation and screening was done in München; enzymatic activity of the resulting clones was quantitatively determined in Utrecht.

4) Sectorial meeting on Enzyme Engineering and related fields in the subprogramme Contextual Measures in the Biotechnology Action Programme (BAP held at Capri, Naples, Italy from May 2nd 1987 to May 6th 1987.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: C. N. R. S., Marseille
Contract no.: BAP - 0062 - F

Project leader: R. VERGER
Scientific staff: G. Piéroni, C. Rivière, Y. Gargouri, H. Moreau, N. Rolland, A. Moulin

Address: Centre de Biochimie et de Biologie Moléculaire du CNRS
31, Chemin Joseph-Aiguier
B.P. 71
F - 13402 MARSEILLE CEDEX 9

Telephone no.: +33.91715857

Telex no.: 430225 CNRSMAR F

Other contractual partners in the joint project:

G.H. de Haas, State University of Utrecht

Title of the research activity:
Conversion of pancreatic phospholipase A2 into triglyceride-degrading lipase.

Key words:
Lipase, Phospholipase, Lipid monolayers, Enzyme kinetics, Surface tension

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

On an industrial scale, so far, mainly proteases and carbohydrate-hydrolyzing enzymes are being used. Active research, however, is going on in several laboratories to use lipases in detergent industry, pharmacology, food and oil technology. Lipases are highly active enzymes and can be produced at relatively low cost. Their major drawback is a low stability at high temperature and sensitivity to detergents. Therefore, the final aim of the present joint project is the construction, by genetic engineering or chemical modification, of hybrid enzymes sharing the stability properties of phospholipases A_2 with the broad substrate specificity of lipases. To bridge the gap between the knowledge of phospholipases and lipases, phospholipase A_1 from E. coli will be studied in detail. Very often, the detection of lipolytic activities represents the critical step in understanding the structure function relationships of these enzymes. We will thus develop new methodologies for studying lipase kinetics at the triacyl-glycerol-water interface.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The conversion of pancreatic phospholipase A_2 into triglyceride-degrading lipase requires advanced and specific kinetic assays. As can be seen from literature (1-5), numerous techniques are available for measuring lipase activity. They can be classified into three groups on the basis of either substrate consumption, product formation, or modification with time of one physical property, such as conductivity, turbidity or interfacial tension.

Two new methods, the so-called "oil-drop" and "teflon plunger" methods, were designed to monitor lipase activity through the enzymatic hydrolysis of natural long-chain triacyl glycerols during which the oil/water interfacial tension decreases with time.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Methodology.

The "Teflon plunger" method.

As shown in figure 1A, the "Teflon plunger" is made of a Teflon cylinder, the lower part of which is conical in shape to permit smooth passage through the oil phase. Interfacial tension is measured by pushing the cylinder (attached to the beam of a KSV 2200 electromicrobalance) down through the oil-water interface. The variation with time of interfacial tension is continuously recorded with an accuracy of $\pm$ 0.05 dyn x cm^{-1}.

The "oil-drop" method.

The oil-drop is formed within an aqueous enzyme solution by pushing oil up with a syringe. Absolute values of interfacial tension were derived from the measurements of equatorial diameter (D) and secant (d) of the drop, as illustrated on figure 1B. Accurate determinations ($\pm$ 10 µm) of D and d were performed each 30 sec using a Nikkon profile projector model 6CT2.

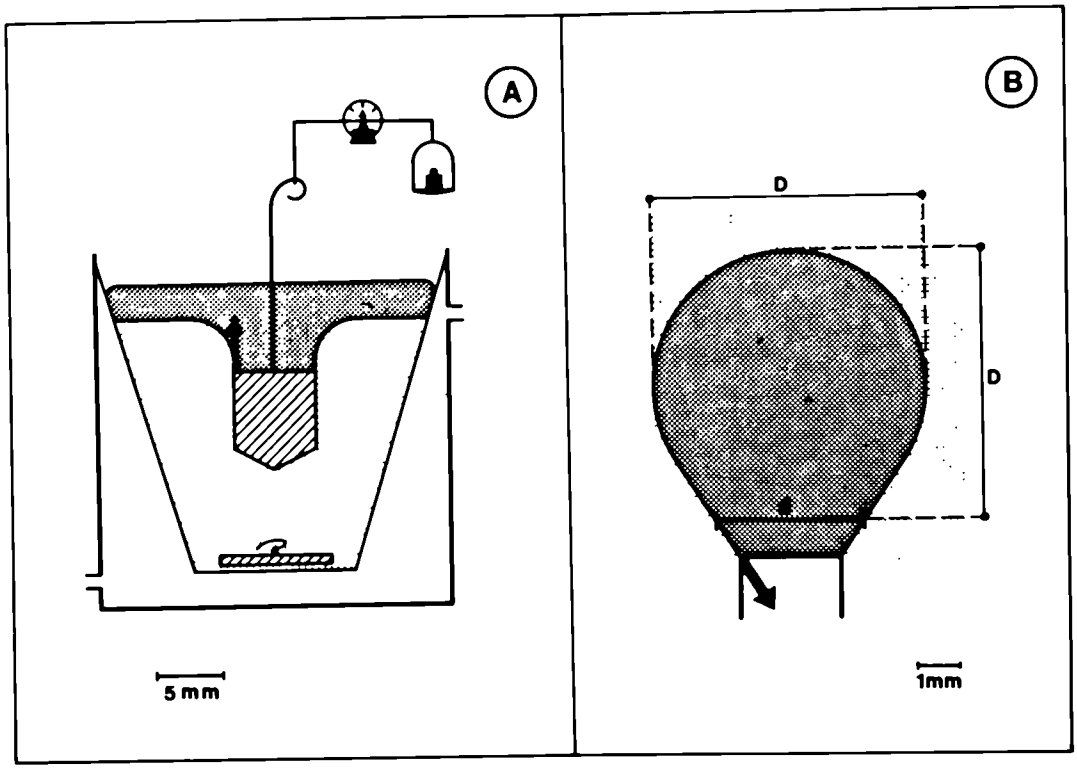


Figure 1 : Schematic view of the "teflon plunger" (A) and "oil drop" (B) methods.

2. Results.

Optimal pH conditions for human gastric lipase.

In line with previous investigations by Gargouri (6, 7), we studied human gastric lipase (HGL) in a reaction mixture containing 1 mM NaTDC. Initial rates of variation of γ o/w were linearly dependent upon HGL concentrations ranging from 1 to 20 Units x ml^{-1} (data not shown). The influence of the pH on these initial rates show an optimal value around pH 5.5.

Effect on tensioactive agents on Human gastric lipase.

In view of the fact that HGL activity is modified by tensioactive agents in emulsified triacylglycerol systems (7), variable NaTDC concentrations were used to change the initial oil-water interfacial tension. These studies were performed at the optimal pH value of 6.0. The influence of increasing NaTDC concentrations on the initial rate of decrease of γ o/w is shown in figure 2.

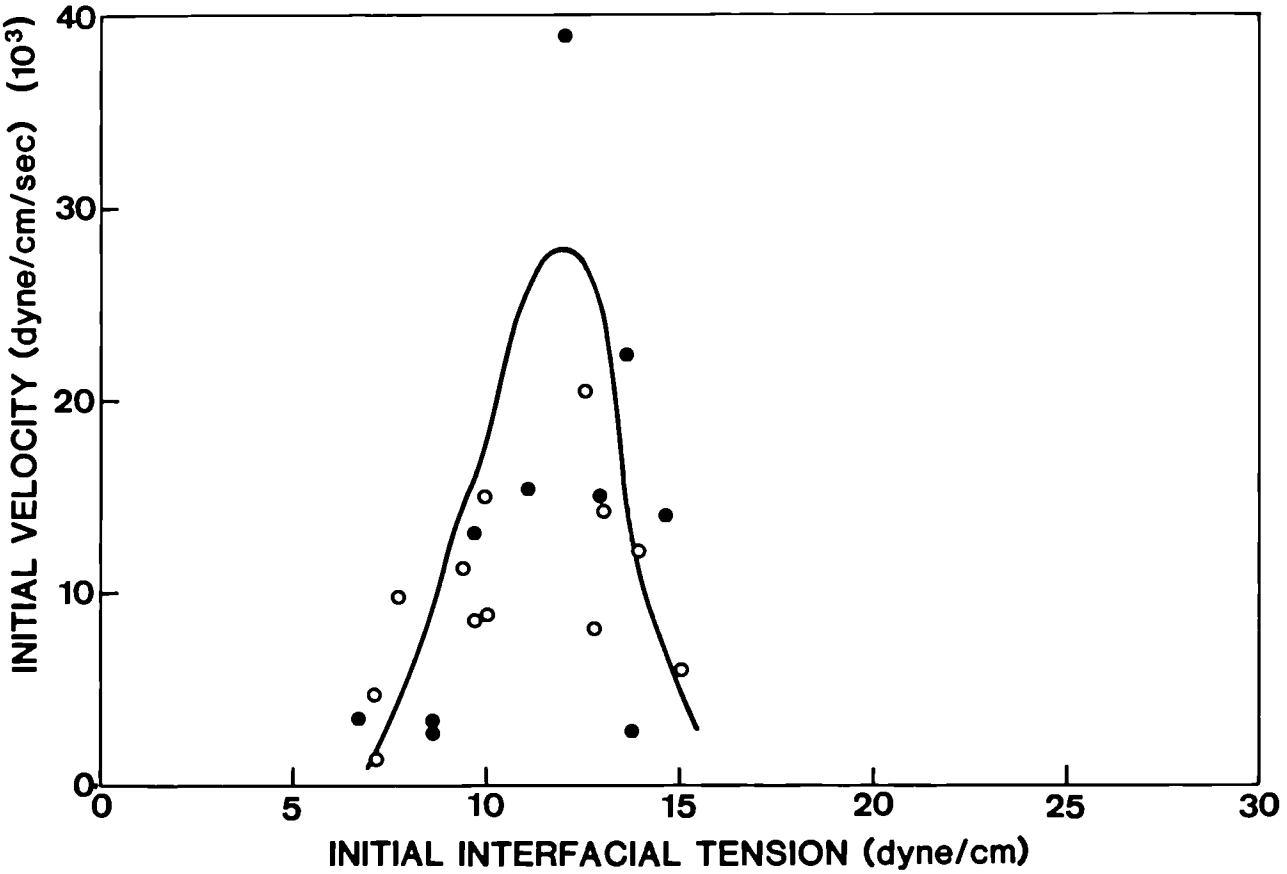


Figure 2 : Dependence on initial interfacial tension of the initial rate of interfacial tension decrease. The initial interfacial tension was adjusted by varying the NaTDC concentration. HGL concentration : 2.5 Units x ml^{-1}. "Oil drop" method : (o). "Teflon plunger" method : (o).

3. Discussion.

These new methods give linear responses with porcine pancreatic lipase concentrations ranging from 1 x 10^{-3} to 30 Units x ml^{-1}. Optimal pH conditions for human gastric lipase were found to range around 5. In the presence of variable concentrations of sodium taurodeoxycholate, both methods show that human gastric lipase is active in the 8-13 dyn x cm^{-1} range of interfacial tension. It is concluded that these two methods, based upon variations with time of the oil-water interfacial tension, constitute reliable, sensitive and convenient means of investigating lipase kinetics.

REFERENCES

1. H. Brockerhoff and R.G. Jensen, Lipolytic enzymes, Academic Press, New York, N.Y. (1974).

2. R. Verger and G.H. de Haas, Ann. Rev. Biophys. Bioengin. 5 (1976)77.

3. H.L. Brockman, Methods Enzymol., 71 (1981) 619.

4. R.G. Jensen, Lipids, 18 (1983) 650.

5. R. Verger, in : B. Borgström and H.L. Brockman (eds.), Lipases, Elsevier, Amsterdam (1984).

6. Y. Gargouri, G. Piéroni, C. Rivière, J.F. Saunière, P.A. Lowe, L. Sarda and R. Verger, Gastroenterology 91 (1986) 919.

7. Y. Gargouri, G. Piéroni, P.A. Lowe, L. Sarda and R. Verger, Eur. J. Biochem. 156 (1986) 305.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

- Lipase kinetics at the triacylglycerol/water interface using surface tension measurements. S. Nury, G. Piéroni, C. Rivière, Y. Gargouri, A. Bois and R. Verger (1987) Chem. Phys. Lipids accepted for publication.

- Gastric lipase : a kinetic study with dicaprin monolayers. Y. Gargouri, G. Piéroni, F. Ferrato and R. Verger (1987) Eur. J. Biochem. (in press.

- Brevet Européen d'Invention entitled : " Dosage des activités lipasiques par la méthode de la goutte d'huile".

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

An intensive kinetic analysis of the pure E. coli phopspholipase A_1 started in Marseille by the presence of Dutch specialists (Dr. H.M. Verheij and A. Horrevoets) in France. A close collaboration was initiated by an exchange of staff and materials (enzymes and substrates) in order to perform joint experiments.

Synthetic substrates and substrate analogs, which are usually not commercially available, were provided by the Utrecht's group. A. Horrevoets achieved in Utrecht the difficult task of purifying the membrane bound phospholipase from E. coli outer membrane. During his 3 months stay in Marseille, A. Horrevoets was trained in the monolayer methodologies by N. Rolland. This team succeeded in co-spreading at a constant surface pressure an enzyme/substrate complex at the air/water interface. After film aspiration, they could show that the membrane associated phospholipase was still active.

Frequent meetings in Utrecht and Marseille have taken place between staff members of both groups : Prof. G.H. de Haas, Dr. H.M. Verheij, Dr. R. Verger and Dr. G. Piéroni.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Aarhus Univ. Contract no.: BAP - 0058 - DK

Project leader: B.F.C. CLARK
Scientific staff: T. la Cour
J. Nyborg

Address: Aarhus University
Department of Chemistry
Division of Biostructural Chemistry
Langelandsgade 140
DK - 8000 AARHUS C

Telephone no.: +45.6.124633

Telex no.: 64767 AAUSCI DK

Other contractual partners in the joint project:

L. Bosch, University of Leiden
A. Parmeggiani, Ecole Polytechnique (Palaiseau)

Title of the research activity:
Construction and biological function of altered proteins defined by their spatial structure.

Key words:
Protein engineering, Molecular graphics, Elongation factor Tu, GTP-binding proteins, Site-directed mutagenesis

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The general goal of this programme is to study how alterations in a protein's structure can modify its biological function. The protein selected for study is the elongation factor EF-Tu. This was chosen because of its own fundamental biological importance, because of its recently-recognized significance as a member of a group of GTP-binding proteins of medical relevance, and finally because of existing experience and expertise with this protein in the laboratories participating in the collaboration. The specific aim of the joint research programme was to study this factor with special reference to modification of its substrate specificity, its sensitivity towards antibiotics and its thermal stability.

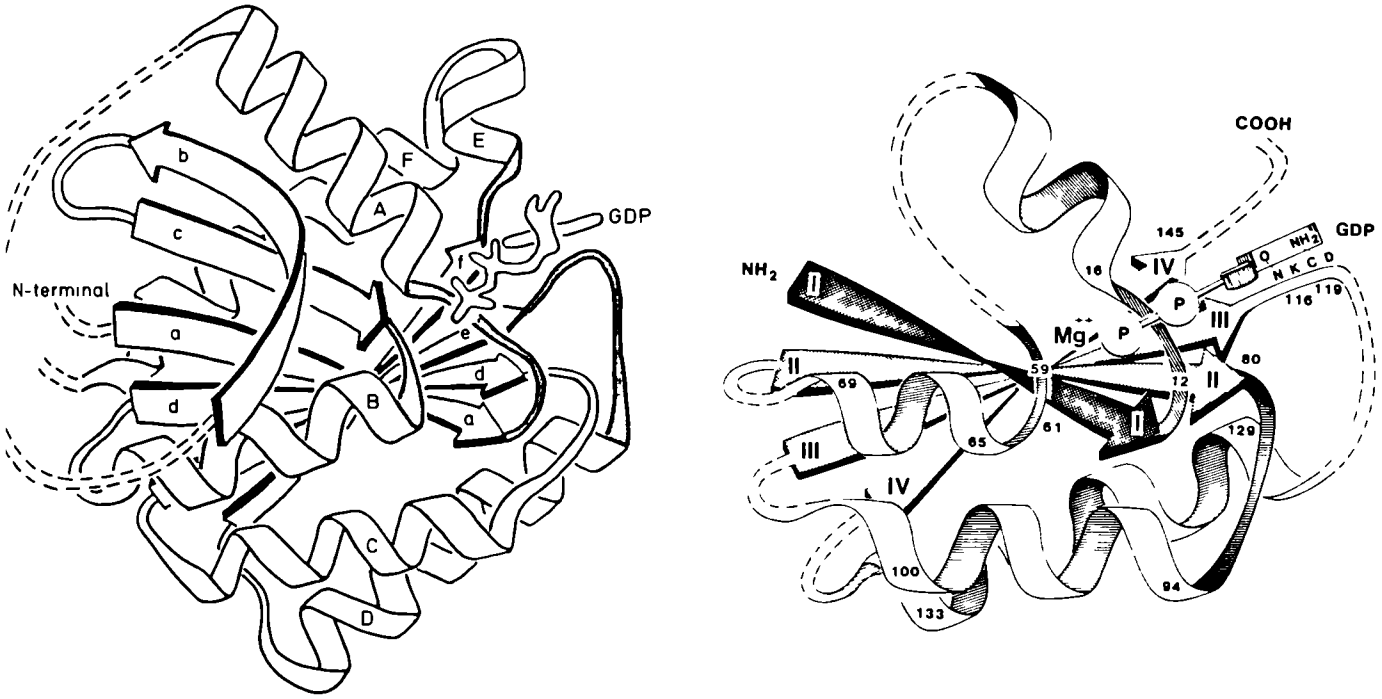
II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

During the first year it has been our aim to introduce alterations into the amino-acid sequence of EF-Tu by the technique of site-directed mutagenesis (mainly in Paris) and by other molecular-genetic methods (Leiden). The altered proteins that arise are being studied by structural and functional tests and by using their homology relationships with other GTP-binding proteins (mainly in Aarhus). A derivative of particular interest is the GTP-binding domain (G-domain) of EF-Tu, which comprises only one-third of the native molecule, but which can be isolated as a separate entity. The isolated G-domain is currently being prepared in large quantities for exhaustive and detailed structural investigation including X-ray crystallography.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

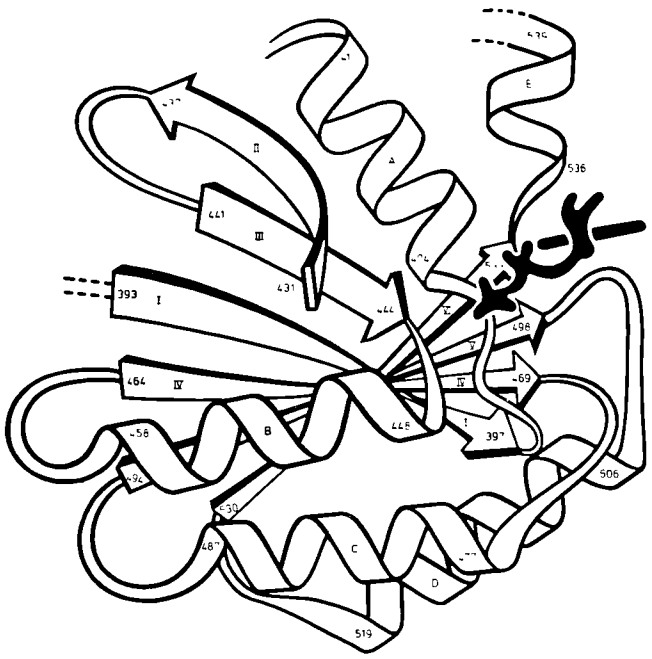
The EF-Tu molecule is a monomer consisting of 393 amino acids. In earlier work, we showed that these fall into three domains of tertiary structure. Domain I contains the N-terminal 200 amino acids. This domain binds GTP and GDP and has been re-named the G-domain.

A structure model for this domain based upon our X-ray studies was proposed by la Cour *et al.*. (EMBO J. **4**, 2385 (1985)) and is shown below (left).



Beside it is shown a similar model which was proposed in collaboration with F. McCormick (Science **230**, 78 (1985)) for the structure of the cancer-related *ras* protein p21. The latter model is based upon a combination of (i) the known X-ray structure of EF-Tu, (ii) the observed sequence homology between EF-Tu and p21, and (iii) the use of a computer programme to fit the p21 sequence to the EF-Tu structure and then to optimize the result. The fact that the computer arrives at a similar result is a clear indication that there is at least some truth in the assumption of a similar tertiary structure related to the similarity in function (here, the binding of guanine nucleotides).

We have used the same approach to predict the structure of the region in the initiation factor IF2 that is analogous to the G-domain of EF-Tu, as shown below.



Our predictions based upon three-dimensional structure have recently been confirmed by independent work. First of all, W.C. Merrick and co-workers (Proc. Natl. Acad. Sci. U.S.A. **84**, 1814 (1987)) have completed an analysis of all currently known protein sequences and have shown that certain amino-acid sequence elements are common to all proteins that bind GTP/GDP and probably only to these. Merrick's consensus elements occur in loops of EF-Tu that are indeed in contact with GDP in the X-ray crystal structure. This is shown in the following Table. In the Table, the loops connecting α-helix and β-sheet are defined by appropriate upper- and lower-case letters; for example, aA is the loop running from the C-terminal end of strand a in the β-sheet to the N-terminal end of α-helix A. It is seen that the residues found to be conserved (left column) reflect well the residues found to be in contact with the GTP (right column, large capitals).

Consensus Sequence (Merrick *et al.*)	Loop sequence (Aarhus)
GXXXXGK	aA = G_{18}HVDHGK_{24}
DXXG	cB = D_{80}CPG$_{83}$
NKXD	eD = N_{135}KCD$_{138}$
	fE = S_{173}ALK$_{176}$

A second source of confirmation is provided by recent work by Wagner *et al.* (EMBO J. **6** (1987), to be published) with the *ras*-related yeast protein YPT. This protein binds and hydrolyses GTP. However, if the lysine or the aspargine corresponding to the K (top line in the Table) or the N (third line) is mutated, the protein cannot bind GTP and the mutation is lethal. However, mutations of two of the variable X residues influenced GTP binding and GTPase activity only to a relatively slight extent.

Thirdly, we have collaborated with the group of Dr. Grunberg-Manago (IMBC, Paris) in a functional investigation of initiation factor IF2. It was shown (Cenatiempo *et al.*, 1987) that removal of a 289-amino-acid-long N-terminal fragment from IF2 leaves most of the functions of IF2, in particular the binding or hydrolysis of GTP, unchanged. However, the retention of GTP binding and hydrolysis is in agreement with our postulate that the G-domain of IF2 is analogous with that of EF-Tu, since our postulated G-domain begins at residue 393, so it is not removed by the cleavage at residue 289.

Potentially interesting positions for site-directed mutation of EF-Tu can therefore be predicted, on the basis of the above model, with some confidence (e.g. Clark *et al.*, 1987).

In initial mutagenesis experiments on EF-Tu, we aimed at various putative functional amino-acid residues in the G-domain, such as those involved in guanine base recognition and specificity, Asn-135 and Lys-136; those involved in binding and hydrolysis of the phosphates, Val-20, Asp-80, His-84 and Glu-117; and, finally, one residue believed to be involved in tRNA binding, Cys-81.

In order to change the nucleotide specificity, Asn-135 was replaced with aspartic acid. Model studies predicted a decreased affinity for guanine as Asn-135 hydrogen-bonds to the keto group of the base. On the other hand, Asp at that position could hydrogen-bond to the amino group of adenine. Preliminary results obtained in collaboration with Dr. A. Parmeggiani's group (In "Structure, Function and Genetics of Ribosomes", Springer-Verlag, 1986, p. 672) show that the mutant factor binds GDP/GTP; however, a quantitative determination of nucleotide specificity has still to be made. Homology studies by Leberman *et al.* (EMBO J. **3**, 339 (1984)) indicated the necessity of a second mutation (Lys-136 → Ile) in order to obtain increased affinity to ADP/ATP. This double mutation has been made but remains still to be characterized.

The only mutation obtained so far around the phosphate binding site of EF-Tu, even at DNA level, is the Val-20 → Gly mutation. This position is particularly interesting because the homologous position in the proto-oncogene product p21 is glycine, whereas the corresponding oncogenic protein contains valine at this position. We interpret the difficulties in producing other mutants in the phosphate binding site by assuming that such mutant proteins are deleterious and that their expression is fatal for the host cell. This is in itself interesting, since most of the attempts involved the replacement of an amino-acid residue with glycine - that is, a side-chain was merely pruned away; this would not normally be regarded as a very harmful mutation.

Finally, the mutation Cys-81 → Gly has been produced. The mutated protein, which is expressed, is now being characterized.

We have also obtained and characterized the N-terminal domain of EF-Tu (Parmeggiani *et al.*, 1987; unpublished results by M. Jensen (currently working in the Paris group) which, as discussed above, is also the G-domain. This involved truncating the molecule from 393 to 203 residues, but the methods did not otherwise differ from those used to prepare the point mutants described above.

Functional tests are in progress in Paris, and a large-scale preparation is being undertaken in Aarhus with a view to crystallographic and spectroscopic studies.

First functional tests have so far demonstrated that this isolated G-domain binds GTP, and GDP as described in Prof. Parmeggiani's report below.

Site-specific mutations of the EF-Tu G-domain will be a useful tool in the investigation of the relationships between structure and function in the class of G-binding proteins. It seems that the use of EF-Tu as a model protein will lead to valuable new insight, in particular into the mechanism of action of the factor and in general into the problems associated with the production of mutated proteins, such as harmful effects on the host, unpredictable levels of expression, or the denaturation and/or precipitation of the desired and overproduced product.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

1. Mortensen, K.K. (1987) Dissertation: Protein Engineering on Elongation Factor EF-Tu: Isolation of the G-domain., University of Aarhus.
2. Parmeggiani, A., Swart, G.W.M., Mortensen, K.K., Jensen, M., Clark;B.F.C., Dente, L. & Cortese, R. (1987) Properties of a Genetically Engineered G-Domain of ElongationFactor EF-Tu Proc. Natl. Acad. Sci. U.S.A. **84** 3141-3145.
3. Cenatiempo, Y., Deville, F, Dondon, J., Grunberg-Manago, M., Sacerdot, C., Hershey, J.W.B., Hansen, H.F., Clark, B.F.C., Kjeldgaard, M., la Cour, T.F.M., Mortensen, K.K., Nyborg, J. & Petersen, H.U. (1987) The IF2 G-domain. A study of a functionally active C-terminal 65 kilodalton fragment of IF2 from *E. coli.*, (1987) Biochemistry, in press (28 July 1987).
4. Clark, B.F.C., Grunberg-Manago, M., Kjeldgaard, M., la Cour, T.F.M., Mortensen, K.K., Nyborg, J. & Petersen, H.U. (1987) Protien Engineering and modification of Translation Factors, In Whelan, W. (ed.) Proc. of the F.A.O.B. Congress, 1986.
5. Clark, B.F.C., Grunberg-Manago, M., Kjeldgaard, M., la Cour, T., McCormick, F., Mortensen, K.K., Nyborg, J., Nørskov-Lauritsen, L., Petersen, H.U. & Thirup, S. (1987) G-binding protein. Structural homologies Protein Engineering **1**, 247.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

a)	Exchange of material(s)	Yes	
b)	Exchange of staff	Yes	
	Joint experiment(s)		No
c)	Joint meeting(s)	Yes	

Descriptive information for the above data.

a) E. coli harbouring plasmids containing genes for mutated EF-Tu

b) M. Jensen (9 months in Paris during the period of the contract)

c) June 3-5, 1986 (Århus)
January 29, 1987 (Leiden)
March 16, 1987 (Leiden)
April 15, 1987 (Leiden)

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Rijksuniversiteit Leiden

Contract no.: BAP - 0057 - NL

Project leader: L. BOSCH
Scientific staff:

Address:
University of Leiden
Department of Biochemistry
Wassenaarseweg 64
NL - 2333 AL LEIDEN

Telephone no.: +31.71.272727 EXT.4763

Telex no.:

Other contractual partners in the joint project:

B.F.C. Clark, Aarhus University
A. Parmeggiani, Ecole Polytechnique (Palaiseau)

Title of the research activity:

Construction and biological function of altered proteins defined by their spatial structure.

Key words:

Elongation factor Tu, Kirromycin, Structure-function relationships, Site-directed mutagenesis, Tuf genes, Streptomyces

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Protein engineering of the polypeptide chain elongation factor EF-Tu.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

a) Understanding of the relationship between structure and function of EF-Tu.
b) Biotechnological application: production of the antibiotic kirromycin by Streptomyces ramocissimus.
c) Regulation of the expression of the two genes encoding EF-Tu in E. coli.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. INTRODUCTION AND METHODOLOGY

A basic problem of biotechnology and molecular biology is to comprehend the functional properties of a protein in terms of its three-dimensional structure. The elongation factor Tu (EF-Tu) of E. coli is an ideal model for studies of this type. The primary structure of this moderately sized protein ($M_R \sim$ 44.000) is known and substantial progress has been made with the elucidation of the crystal structure by the group of Dr. Clark in Aarhus. Two genes, designated tufA and tufB code for EF-Tu. These genes have been cloned in various vectors and their nucleotide sequences have been determined. Various mutants of E. coli, altered either in tufA or tufB or in both have been obtained in our group and that of Dr. Parmeggiani. Classical genetic techniques and site-directed mutagenesis have been applied and the structural and functional

consequences have been examined. In this progress report we describe these results obtained with the mutants (I).

The finding by Parmeggiani et al. of an antibiotic with a target site on EF-Tu has greatly facilitated the isolation of mutants and has deepened our insight in the functioning of this multifunctional and versatile protein. This antibiotic, known under the name of kirromycin or mocimycin, is also of great biotechnological interest, however. When added to the food, it increases the biomass of poultry and swine. Since it does not penetrate the wall of the digestive tract, it does not turn up in meat intended for consumption. Up till now its production at an economically rewarding scale has been hampered by the fact that, above a certain level, it inhibits its own producer. Attempts by the industry (Gist-Brocades, Delft, The Netherlands) to render S. ramocissimus resistant to kirromycin, have failed despite many years of research and costly investments. Recently we succeeded in isolating E. coli mutants resistant to kirromycin. Here we report on the cloning and sequencing of *tuf* genes from S. ramocissimus and on our endeavours to render the streptomycete resistant to the antibiotic (II).

A complicating factor in studying EF-Tu mutants and structure-function relationships of the protein is the presence of the two encoding genes *tufA* and *tufB*. Their nucleotide sequences in E. coli are almost identical and their products: EF-TuA and EF-TuB differ in one amino acid residue only, i.e. the C-terminal residue. Characterization of mutant EF-Tu species therefore requires the separation of the two *tuf* gene products that are structurally almost identical. We have found, to our surprise, that *tufB* can be inactivated without significant consequences for growth of the E. coli cells, whereas inactivation of *tufA* is lethal. These intriguing findings not only shed totally new light on *tuf* gene expression, but also have a bearing on our genetic manipulations of the *tuf* genes. We therefore have studied the regulation of the expression of *tufA* and *tufB* in E. coli and report on this in section III.

2. RESULTS

I. Mutants of E. coli altered in *tufA* and *tufB*.

The antibiotic kirromycin binds to EF-Tu in an 1:1 molar ratio,

presumably to the C-terminal domain since we could cross-link it to Lys-357 (compare Figure 1). The complex EF-Tu.kirromycin sticks to the ribosome. Kirromycin immobilizes the ribosome on the mRNA and thus is an effective inhibitor of protein synthesis. Phenotypic expression of kirromycin resistance requires alteration of both tufA and tufB. Sensitivity to the antibiotic dominates resistance. The mutations Ala-375 -> Thr and Ala-375 -> Val confer resistance. The mutation Gly-222 -> Asp is recessive to resistance. EF-Tu (Gly-222 -> Asp) binds kirromycin but in doing so does not immobilize the ribosome.

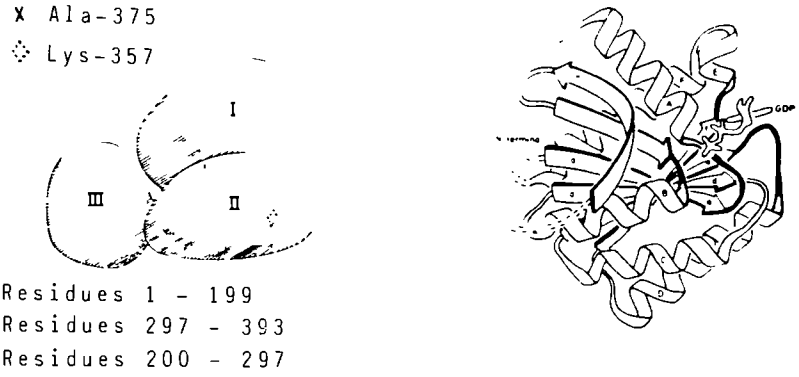


Figure 1. (Left) Three-dimensional representation of the overall shapes of the domains of EF-Tu.GDP. The position of GDP in domain I is indicated by the dotted region. The dotted lines of the symbol for residue 357 in domain II indicate that this residue is located at the back side of the domain. The X-ray diffraction pattern does not yet permit the location of the positions of Lys-208, Gly-222 and Lys-237 in domain III. (Right) Structural cartoon of domain I with arrows representing β-strands and cylinders representing α-helices.

Our studies with EF-Tu mutants have yielded a number of unexpected results: 1) Inactivation of tufA (by insertion of bacteriophage Mu) leads to cell death; inactivation of tufB hardly affects bacterial growth. The tuf gene products EF-TuA and EF-TuB differ only in their C-terminal amino acid residue. 2) Cells harbouring mutants in tufA and tufB suppress nonsense and frameshift mutations. Suppression is most effective when both tuf genes are mutated. 3) A synergistic effect of two mutant EF-Tu species is also observed during in vitro polypeptide synthesis.

II. Genetic manipulation of the tuf genes of Streptomyces ramocissimus.

We have isolated EF-Tu.GDP from S. ramocissimus ($EF\text{-}Tu_{S.R.}$) in a

homogeneous state. EF-$Tu_{S.R.}$ differs slightly from E. coli EF-Tu (EF-$Tu_{E.C.}$) in its molecular weight. It shows cross-reactivity towards antibodies raised against EF-$Tu_{E.C.}$ and is functionally interchangeable with EF-$Tu_{E.C.}$ during protein synthesis on E. coli ribosomes. It binds kirromycin and as a result undergoes a conformation change.

Using E. coli plasmids carrying a tuf gene as a probe, we have cloned two Streptomyces tuf genes in E. coli. Nucleotide sequence analysis is in rapid progress. For raising kirromycin resistance in the streptomycete, our strategy will be to inactivate one of the two tuf genes by mutagenetic insertion and to submit the other gene to mutagenesis. The results have also led to conclusions of more general interest: 1) S. ramocissimus is the first gram-positive bacterium that has been found to harbour two tuf genes. B. subtilis has only one tuf gene. 2) One of the tuf genes in S. ramocissimus is preceeded by an EF-G encoding gene suggesting a similar tuf gene organisation as in E. coli.

III. Regulation of the expression of tufA and tufB.

Knowledge of the regulation of tufA and tufB expression is a prerequisite for genetic manipulation of these genes. We found that tufA and tufB are expressed coordinately at varying growth rates. They are part of two different transcription units, rather distantly located on the E. coli chromosome. By constructing tRNA':lacZ and tRNA-tufB':lacZ operon fusions and a tufB':lacZ' gene fusion we demonstrated that the cellular level of transcripts initiated at the primary promoter of the tRNA-tufB operon, increases with growth rate. In this respect the tRNA-tufB operon resembles the rRNA operons.

The regulation of expression of the tRNA-tufB operon is very complex. A number of transcription signals is presented in Figure 2. Upstream of

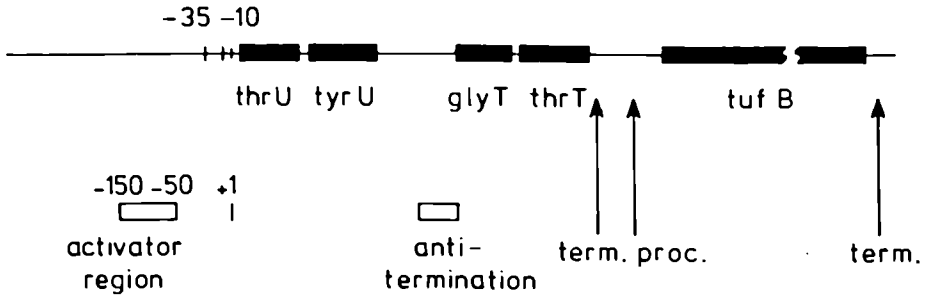


Figure 2. Transcription signals of the tRNA-tufB operon (for experimental details see J.H.M. van Delft, Ph.D. thesis, Leiden University, 1987).

the primary promoter a region is present that enhances the transcription of the operon. Deletion mapping has revealed a terminator of transcription in the intergenic region between thrT and tufB. Evidence for antitermination was also obtained by constructing deletions. A processing site was mapped 72-74 nucleotides upstream of the initiation codon of the tufB cistron by S1 nuclease five mapping. Expression of tufB, not that of tufA, is under autogenous control. Finally expression of the tRNA-tufB operon is regulated by the so-called "ribosome feedback". A post-transcriptional mechanism counteracts the ribosomal feedback effect on transcription.

Conclusions.

Mutants of E. coli affecting EF-Tu have been very useful in studying the relationship between structure and function of this multifunctional protein. They have paved the way also for investigating the regulation of the expression of the two operons carrying an EF-Tu encoding gene. Homologous recombination enables us to transfer mutations, engineered by site-directed mutagenesis on a vector, to both the tufA and the tufB position on the chromosome.

The cloning of the two tuf genes from S. ramocissimus in E. coli has led the foundation for biotechnological improvement of kirromycin production by the streptomycete.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

Van de Klundert, J.A.M., Van der Meide, P.H., Van de Putte, P. and Bosch, L. Mutants of Echerichia coli altered in both genes coding for elongation factor Tu. (1978) Proc. Natl. Acad. Sci. USA 75, 4470-4473.

Swart, G.W.M., Kraal, B., Bosch, L. and Parmeggiani, A. Allosteric changes of the guanine nucleotide binding site of elongation factor EF-Tu. (1982) FEBS Lett. 142, 101-106.

Van der Meide, P.H., Vijgenboom, E., Dicke, M. and Bosch, L. Regulation of the expression of tufA and tufB, the two genes coding for the elongation factor EF-Tu in Escherichia coli. (1982) FEBS Lett. 139, 325-330.

Van Noort, J.M., Duisterwinkel, F.J., Jonak, J., Sedlacek, J., Kraal, B., and Bosch, L. The elongation factor Tu kirromycin complex has two binding sites for tRNA molecules. (1982) EMBO J. 1, 1199-1205.

Bosch, L., Kraal, B., Van der Meide, P.H., Duisterwinkel, F.J. and Van Noort, J.M. The elongation factor Tu and its encoding genes. (1983) Progr. Nucl. Acids Res. Mol. Biol. 30, 91-126.

Van der Meide, P.H., Vijgenboom, E., Talens, A. and Bosch, L. The role of EF-Tu in the expression of tufA and tufB genes. (1983) Eur. J. Biochem. 130, 397-407.

Van der Meide, P.H., Kastelein, R.A., Vijgenboom, E. and Bosch, L. Tuf gene dosage effects on the intracellular concentration of EF-TuB. (1983) Eur. J. Bioch. 130, 409-417.

Duisterwinkel, F.J., Kraal, B., de Graaf, J.M., Talens, A., Bosch, L., Swart, G.W.M., Parmeggiani, A., La Cour, T.F.M., Nyborg, J. and Clark, B.F.C. Specific alterations of the EF-Tu polypeptide chain considered in the light of its three-dimensional structure. (1984) EMBO J. 3, 113-120.

Van Noort, J.M., Kraal, B., Bosch, L., La Cour, T.F.M., Nyborg, J. and Clark, B.F.C. Cross-linking of tRNA at two different sites of the elongation factor Tu. (1984) Proc. Natl. Acad. Sci. USA 81, 3969-3972.

Van Noort, J.M., Kraal, B. and Bosch, L. A second tRNA binding site on EF-Tu is induced while the factor is bound to the ribosome. (1985) Proc. Natl. Acad. Sci. USA 82, 3212-3216.

Vijgenboom, E., Vink, T., Kraal, B. and Bosch, L. Mutants of the elongation factor EF-Tu, a new class of nonsense suppressors. (1985) EMBO J. 4, 1049-1052.

Sam, T., Pingoud, A. and Bosch, L. Mutants species of EF-Tu, altered at position 375, exhibit a reduced affinity for aminoacylated transfer RNAs. (1985) FEBS Lett. 185, 51-56.

Bosch, L., Kraal, B., van Noort, J.M., van Delft, J., Talens, J. and Vijgenboom, E. Novel RNA interactions with the elongation factor EF-Tu: consequences for protein synthesis and tuf gene expression. (1985) Trends Biochem. Sci. 10, 313.

Bosch, L., Kraal, B., van Noort, J.M., Vijgenboom, E., van Delft, J.H.M. and Talens, A. Novel functions of EF-Tu during polypeptide synthesis and tuf gene expression. (1986) In: Structure, Function and Genetics of Ribosomes (eds. Hardesty, B. and Kramer, G.) Springer-Verlag, New York pp 658-671.

Van Noort, J.H.M., Kraal, B., Sinjorgo, K.M.C., Persoon, N.L.M., Johanns, E.S.D. and Bosch, L. Methylation in vivo of elongation factor EF-Tu at lysine-56 decreases the rate of tRNA-dependent GTP hydrolysis. (1986) Eur. J. Biochem. 160, 557.

Van Noort, J.H.M., Kraal, B. and Bosch, L. GTPase center of elongation factor Tu is activated by occupation of the second tRNA binding site. (1986) Proc. Natl. Acad. Sci. USA 83, 4617.

Vijgenboom, E. and Bosch, L. Transfer of plasmid-borne tuf mutations to the chromosome as a genetic tool for studying the functioning of EF-TuA and EF-TuB in the E. coli cell. (1987) Biochimie, in press.

Van Delft, J.H.M., Schmidt, D.S. and Bosch, L. The tRNA-tufB operon. Transcription termination and processing upstream of tufB. (1987) J. Mol. Biol., in press.

Van Delft, J.H.M., de Jong, P.J., Schmidt, D.S., Talens, A. and Bosch, L. Feedback regulation of the Escherichia coli tRNA-tufB operon: in vitro studies. (1987) Mol. Gen. Genet., submitted.

Van Delft, J.H.M., de Jong, P.J., Schmidt, D.S., Talens, A. and Bosch, L. Feedback regulation of the Escherichia coli tRNA-tufB operon: in vivo studies. (1987) Mol. Gen. Genet., submitted.

Van Noort, J.M. Two tRNA binding sites on elongation factor Tu from E. coli, Ph.D. Thesis, Leiden University (1985).

Schilstra, M. Biophysical studies on the elongation factor EF-Tu from Escherichia coli. Ph.D. Thesis, Leiden University (1986).

Swart, G.W.M. The polypeptide chain elongation factor Tu from E. coli. Ph.D. Thesis, Leiden University (1987).

Van Delft, J.H.M. Regulation of expression of the Escherichia coli tRNA-tufB operon. Ph.D. Thesis, Leiden University (1987).

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

a) Exchange of material(s) Yes

b) Exchange of staff Yes

Joint experiment(s) Yes

c) Joint meeting(s) Yes

Descriptive information for the above data.

a) Tuf mutants of E. coli.

b) G. Swart, P. Anborgh and R. Cool (working in Paris)

c) June 3-5, 1986 Aarhus
January 29, 1987 Leiden
March 16, 1987 Leiden
April 15, 16, 1987 Leiden

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Ecole Polytechnique, Palaiseau

Contract no.: BAP - 0066 - F

Project leader: A. PARMEGGIANI

Scientific staff: P.H. Anborgh, J.B. Crechet, R.H. Cool, E. Jacquet, F. Gümüsel, M.Y. Mistou, G. Parlato

Address:
Ecole Polytechnique
Laboratoire de Biochimie
F - 91128 PALAISEAU CEDEX

Telephone no.: +33.1.60194181

Telex no.: 691596 ECOLEX F

Other contractual partners in the joint project:

B.F.C. Clark, Aarhus University
L. Bosch, University of Leiden

Title of the research activity:

Construction and biological function of altered proteins defined by their spatial structure.

Key words:

Elongation factor Tu, G-proteins, Protein designing, Site-directed mutagenesis, Structure-function relationships

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The general goal of this project is to develop basic research methods in a study of how alterations in the protein's structure can change function. Site-directed mutagenesis and protein designing of the EF-Tu molecule, a guanine nucleotide binding protein of great biological significance, are used with the aim of elucidating general mechanisms controlling the properties of an enzyme such as substrate specificity, catalysis, thermosensitivity and proteolysis. Elucidation of structure-function relationships in EF-Tu are expected to contribute to the clarification of the mechanism of action of kirromycin, an antibiotic of industrial interest having EF-Tu as its specific target, as well as the common features of the guanine nucleotide binding proteins, a class of proteins involved in fundamental physiological and pathological processes of the cell.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

In the first year of the project, the group Parmeggiani has given priority to the development of 1) methods for introducing mutations in the *tufA*, one of the two genes encoding EF-Tu in *E. coli*, 2) a suitable system for the expression of the modified *tufA* gene and the overproduction of soluble mutated proteins and 3) a procedure allowing separation of the plasmide-borne, mutated EF-Tu from the chromosomic EF-Tu of the host cell. The first targets chosen for site-directed mutagenesis are : A) deletion of two EF-Tu domains in order to obtain the isolated N-terminal domain (G-domain) containing the substrate binding site and the catalytic center, and B) the introduction of point substitutions in the residues of the substrate binding pocket. The isolation in preparative amounts and the functional characterization of pure, mutant EF-Tu factors have represented major aims, since they pave the way for the next developments of the project.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

The polypeptide chain of EF-Tu (393 amino acid residues, M.W. = 43,000) is folded in three distinct domains (Fig. 1A, ref. 1), of which the N-terminal domain has primary and secondary structure features common to other guanine nucleotide binding proteins. For more information about its structural, functional properties and its genetic organization see the reports of the two partner groups (Profs. L. BOSCH and B.F.C. CLARK).

METHODOLOGY.- For the construction of the EF-Tu mutants, a 2kb DNA fragment containing the structural *tufA* has been cloned into pEMBL9, a vector susceptible to be secreted as single stranded DNA upon superinfection with phage F1 (2-5). Point substitutions or deletion of the *tufA* portion coding for the Middle and C-terminal domain (570 pb) from Glu-203 to the penultimate residue Leu-392 have been introduced by means of synthetic oligonucleotides, using the gapped duplex method (6). Screening of the transformed colonies was performed by cell colony hybridisation and DNA sequencing. For overproduction of the mutated EF-Tu factors, the modified *tufA* gene was cloned into the the expression vector pCP40, in which it is under temperature-inducible λP_L control (7). Shift to 38-42 °C activates λP_L-expression and runaway replication. The precise conditions for obtaining optimal overproduction and solubility of the plasmid-borne, mutant EF-Tu in the host cell, had to be painstakingly determined for each mutant. The host cells were either the kirromycin-sensitive *E. coli* 71/18 (EF-TuAs, EF-TuBs) or the kirromycin resistant *E. coli* PM455 (EF-TuAr) and its recA$^-$ variant

PM1455. The G-domain and its mutants were purified to homogeneity by three chromatoghraphic steps (DEAE-Sepharose, Ultrogel ACA44 and MonoQ on FPLC), whereas mutated EF-Tu factors were purified from the chromosomic EF-TuAr by exploiting their different behaviour on DEAE-Sepharose chromatography in the presence of kirromycin (8). This procedure can be applied to the isolation of any kind of mutant factors regardless of charge differences.

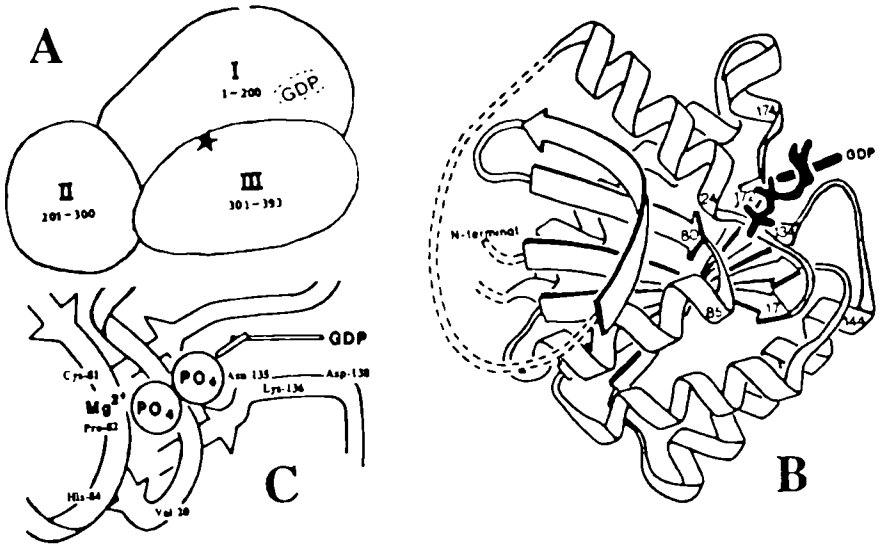


Figure 1.- A : Representation of the overall shapes of the three EF-Tu domains with the GDP binding site in domain I (N-terminal domain or G-domain) and the number of the residues participating in the formation of the respective domains. The star indicates position 375, responsible for kirromycin resistance, situated near the cleft between domain I and III (C-terminal domain). - **B** : Conformation of the N-terminal domain (G-domain) of mildly trypsinized EF-Tu·GDP derived from X-ray diffraction analysis. - **C** : Schematic representation of the four loops constituting the GDP binding pocket, showing the substituted amino acids. Figure 1 is derived from references 1, 9 and 12.

RESULTS.- The N-terminal domain of EF-Tu, in a model refined to 2.9 Å (1) shows an α/β structure, typical for the class of nucleotide binding proteins (Fig. 1B). The GDP binding pocket is constituted by four loops connecting the β-strands with α-helices. The residues Val-20 and His-84 are likely to interact with the phosphoryl groups of GDP/GTP , while the residues Asn-135, Lys-136 and Asp-138 interact with the base ring (9). Figure 1C (from ref. 9, modified) represents schematically these 4 loops, showing the point substitutions introduced sofar. Substitutions Val-20→Gly, Cys–81→Gly and Asn-135→Asp/Lys-136→Ile were introduced in the intact EF-Tu molecule, and substitutions Pro-82→Thr, His-84→Gly and Asp-138→Asn in the G-domain (4,5). The main effects of the different mutations can be resumed as follows :

EF-TuVal-20→Gly. The alignement of the primary structure between EF-Tu and *ras* protein p21 shows that the amino acids Val and Gly in position 20 of EF-Tu correspond to the amino acids Val and Gly in position 12 of the oncogenic and the wild-type p21, respectively (10). The most remarkable effect of the substitution of Val-20 by Gly in EF-Tu is the strong increase of the catalytic activity (5-10 times) .This is in agreement with the results obtained with the *ras* p21, where the wild-type protein (Gly-12) shows much higher GTPase activity than the oncogenic variant (Val-12, ref. 11).

EF-TuCys-81→Gly and **EF-TuAsn-135→Asp/Lys-136→Ile**. The characterization of these two mutants is in progress. They appear to be still able to interact with GTP, aa-tRNA and sustain protein synthesis.

G-domain = EF-Tu (ΔGlu-203-Leu-392), M.W. = 21,000. This homologue of the *ras* p21 is a very stable protein. It binds GDP and GTP, but differently from EF-Tu it does not distiguish between these two nucleotides. The G-domain retains the catalytic properties of EF-Tu (GTPase activity). Of the EF-Tu ligands (ribosomes, aa-tRNA, EF-Ts) and the antibiotic kirromycin only the ribosomes can affect the GTPase activity of the G-domain, inducing a 15-20-fold stimulation. It has been isolated in large quantity. Attempts to obtain crystals suitable for X-ray analysis are presently carried out by the group Clark in Aarhus.

G-domainPro-82→Thr. This substitution induces a pronounced decrease of the GTPase activity (<30%) associated with an increased affinity for the substrate GDP/GTP.

G-domainHis-84→Gly. The affinity for GDP/GTP is essentially unchanged, whereas the GTPase activity is strongly decreased (<10%).

G-domainAsp–138→Asn. The overproduced protein has turned out to be insoluble under all conditions tested.

Conclusions.- As next developments, it is planned to pursue the characterization of the substrate binding pocket. Moreover, mutations will be introduced such to affect A) the stability of EF-Tu to thermal denaturation and B) its sensitivity to proteolytic phenomena. Other methods to facilitate the isolation of mutated EF-Tu, such as gene displacement or overproduction of the mutated EF-Tu in organisms other than *E. coli* will be explored.

References

1) La Cour, T.F.M., Nyborg, J., Thirup, S. & Clark, B.F.C. (1985) *EMBO J. 4*, 2385-2388
2) Dente, L., Cesareni, G. & Cortese, R. (1983) *Nucl. Acid Res.11*, 1645-1656
3) Parmeggiani A., Swart, G.W.M., Mortensen, K.K., Jensen, J., Clark, B.F.C. & Cortese, R. (1987) *Proc. Natl. Acad. Sci. USA 84*, 3141-3145
4) Parmeggiani, A., Anborgh, P.H., Cool, R.H., Jacquet, E., Gümüsel, F., Parlato, G., & Swart, G.W.M. in *Genetics of Translation : New Approaches* (Bolotin-Fukuhara, M. & Picard, M., eds) NATO ASI series 1987, in print
5) Parmeggiani, Jacquet, E., Jensen, M., Anborgh, P.H., Cool, R.H., Jonak, J. & Swart, G.W.M. in *Metabolism and Enzymology of Nucleic Acids Including Gene Manipulations* (Zelinka, J. Balan,J., eds) Vol. 6, Slovak Academy of Sciences, Bratislava 1987, in print
6) Kramer W, Shugart, K., Fritz, H.L.-J. (1982) *Nucl. Acids Res. 10*, 6475-6485
7) Remaut, E., Tsao, H., Fiers, H. (1983) *Gene 22*, 103-113
8) Swart, G.W.M., Parmeggiani, A., Kraal, B. & Bosch, L. (1987) *Biochemistry 26*, 2047-2054
9) McCormick, F., Clark, B.F.C., La Cour, T.F.M., Kjeldgaard, M., Norskov-Lauritsen, L. & Nyborg, J. (1986) Science 230, 78-82
10) Halliday, K.R. (1983) *J. Cycl. Nucl. Protein Phosph. Res. 10*, 6475-6485
11) Scolnick, E.M., Papageorge A.G. A.G., Shih, T.Y. (1979) *Proc. Natl. Acad. Sci. USA 76*, 5335-5339

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1 Parmeggiani, A., Swart, G.W.M., Mortensen, K.K., Jensen, M., Clark, B.F.C. & Cortese, R. (1987) PROPERTIES OF A GENETICALLY ENGINEERED G-DOMAIN OF ELONGATION FACTOR Tu *Proc. Natl. Acad. Sci. USA 84*, 3141-3145

Parmeggiani, A., Anborgh, P.H., Cool, R.H., Jacquet, E., Jensen, M., Gümüsel, F., Parlato, G., & Swart, G.W.M. THE POLYPEPTIDE CHAIN ELONGATION FACTOR Tu : CHARATERIZATION OF MUTANTS AND PROTEIN ENGINEERING in *Genetics of Translation : New Approaches* (Bolotin-Fukuhara, M. & Picard, M., eds) NATO ASI series 1987, in print

Parmeggiani, A., Jacquet, E., Jensen, M., Anborgh, P.H., Cool, R.H., Jonak, J. & Swart G.W.M. SITE-DIRECTED MUTAGENESIS OF ELONGATION FACTOR Tu in *Metabolism and Enzymology of Nucleic Acids Including Gene Manipulations* (Zelinka, J. & Balan, J., eds) Vol. 6, Slovak Academy of Sciences, Bratislava 1987, in print

IV.2 Parmeggiani, A., Anborgh, P.H., Cool, R.H., Jacquet, M., Jensen, M., Merola, M., Mortensen, K.K. & Swart G.W.M. SITE-DIRECTED MUTAGENESIS OF ELONGATION FACTOR Tu in *Enzyme Engineering : Protein Design and Applications in Biocatalysis*, Commission of the European Community Biotechnology Action Programme, Capri, Italy, 2-6 May 1987

IV.4 Swart, G.W.M. THE POLYPEPTIDE CHAIN ELONGATION FACTOR Tu FROM *E. COLI* Doctorate Thesis, University of Leiden, 29th January 1987

Mistou, M.-Y. MUTAGENESE DIRIGEE SUR LE GENE RAS2 DE LA LEVURE *S. CEREVISIAE* ET COMPARAISON DES ACTIVITES BIOCHIMIQUES DES PROTEINES RAS2 ET RAS2Val-19, EF-Tu ET EF-TuGly-20 D.E.A. d'Enzymologie, Université d'Orsay, June 1987

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

a : *tuf* mutants and purified EF-Tu mutants of *E. coli*

b and c : G.W.M. Swart, P.H. Anborgh and R.H. Cool from the Dept. of Biochemistry, University of Leiden, and K.K. Mortensen and M. Jensen from the Dept. of Chemistry, Aarhus University, have worked or are working in the group Parmeggiani in Palaiseau

d : June 3-5, 1986 Aarhus
January 29, 1987 Leiden
March 16, 1987 Leiden
April 15-16, 1987 Leiden

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: U. L. B., Brussels

Contract no.: BAP - 0139 - B

Project leader: S. WODAK

Scientific staff:

Address: Université libre de Bruxelles, CP160
Unité Conformation des
Macromolécules Biologiques
Avenue Paul Héger P2
B - 1050 BRUXELLES

Telephone no.: +32.2.6485200

Telex no.: 23069 UNILIB B

Other contractual partners in the joint project:

A.R. Fersht, Imperial College of Science & Technology (London)
J. Janin, Université Paris-Sud (Orsay)
P. Stanssens, Plant Genetic Systems NV (Gent)

Title of the research activity:

Engineering of an extracellular ribonuclease by gene modification.

Key words:

Proteins, Protein engineering, Ribonuclease, Model building

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The aim of this project is to develop rational approaches for engineering modified proteins using genetic engineering technology.

The system chosen for that purpose is Barnase, a small excreted endonuclease from B. Amiloliquefaciens which cleaves single stranded RNA.

The questions initially addressed concern elucidation of the catalytic mechanism and substrate specificity. Later on, the effect of amino-acid substitutions on thermodynamic and folding properties of the enzymes will also be examined.

The research programme consists on a systematic approach which combines experiments with model building and computer simulations.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Interactive computer graphics and computer calculations have been applied to analyse the known crystal structure of the native Barnase protein.

Special attention has been given to the analysis of enzyme-substrate and enzyme-inhibitor interactions.

Direct comparisons have been performed between the structures of Barnase, which has little cleavage specificity and the homologous enzyme RNase.T1(crystallised with 2'GMP inhibitor), which displays more pronounced specificity towards guanosine.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Methodology

I. Analysis of contacts between Barnase molecules in the crystal has been carried out by generating neighbouring molecules in the unit cell starting from the asymmetric unit which contains 3 independent molecules of Barnase. Computer graphics, distance calculations and computations of accessible surface area were used to analyse intermolecular interactions.

II. Detailed analysis of each monomer in the asymmetric unit has also been performed. It includes structural comparisons of the 3 molecules using coordinate superposition, comparison of crystallographic B-factors for the main chain and side chain atoms, calculation of atomic volumes, characterization of internal cavities and surface area calculations to analyse solvent accessibility of residues in the protein. To further assess possible differences between the three molecules, the conformational energy of each monomer has been computed using the BRUGEL package[1] and energy parameters from the CHARMM library[2].

III. One molecule, molecule C was then chosen to be studied in detail. In particular, we analysed the dihedral angles of the main chain and side chains, the geometry and energetics of H-bonds and interactions with water molecules.

IV. Next we proceeded to perform a detailed comparison between Barnase[3] and RNase.T1[4], in order to study enzyme-inhibitor interactions for which a model exists in the RNase.T1-2'GMP complex. We started by defining the structural core common to Barnase and RNase.T1 using an approach similar to the one described by Chothia & Lesk[5]. Equivalent stretches of the polypeptide chain belonging respectively to the 2 Å resolution structure of Barnase and to the 1.8 Å resolution structure of RNase.T1 in the RNase.T1-2'GMP complex were superimposed individually. Those that matched to within 2 Å root mean square deviation were retained as being part of the core. Interactions between Barnase and 2'GMP(or 3'GMP) positioned after coordinate superposition of the common core of RNase.T1 were analysed and sampling of the conformational space available to 2'GMP and 3'GMP in Barnase and RNase.T1 was then performed.

2.Results

I. Due to crystal packing, two of the three molecules (A and B) which constitute the asymmetric unit were found to have their active site unaccessible to substrates. This is caused by close contacts from an α helix (res 6-18) belonging to a neighbouring molecule.

II. Structural comparison of the three molecules indicates that they are very similar with a root mean square deviation of about 0.3 Å for the C_α atoms and of about 0.6 Å on all heavy atoms. Non-bonded energies of molecules A, B, C computed in absence of solvent are however different with values ranging from -770 to -950 Kcal/mol. The average accessible surface area of Barnase (molecules A, B, C) is 6017 ± 45 Å^2. A number of internal cavities have also been detected. Some of these cavities are common to all three molecules, other are not. Molecules A and B display similar patterns in their B-factors while the pattern of B-factors in molecule C is somewhat different. This molecule displays lower B-factors in the substrate binding region (res 57-60) and in the α helix (res 6-18).

III. The distribution of main chain dihedral angles in Barnase (molecule C) is well confined to allow conformations confirming the relative high quality of the X-ray coordinates. Some "unusual" positive Ψ values have nonetheless been detected but could in all but one case (Trp-94) be rationalized on the basis of rare, yet previously observed[6] main chain conformations. As for side chains, noteworthy is the finding of two buried carboxylates forming no intramolecular H-bonds, belonging to Glu 73 and Asp 75.

IV. The common structural core of Barnase and RNase.T1 was defined to contain the following fragments:

Barnase [52-62] [69-77] [81-95] [96-101]

RNase.T1 [38-48] [54-62] [71-85] [87-92]

The first segment belongs to the nucleotide binding loop, other three segments belong to beta strands. The superposition of these segments (Barnase/RNase.T1) in block yields 2.0 Å rmsd for the C_α coordinates. The binding loops of both enzymes fit to within 0.95 Å rmsd. The coordinate transformation obtained from the superposition of the common core was then used to superimpose the entire structures of Barnase and RNase.T1, and putative residues involved in nucleotide binding and catalysis were defined by analogy with those supposed to play similar roles in RNase.T1, as follows: Ser 57 (Asn 43), Asn 58 (Asn 44), Arg 59 (Tyr 45), Glu 60 (Glu 46), Asp 54 (His 40), Glu 73 (Glu 58), Arg 87 (Arg77), His 102 (His 92), Phe 56 (Tyr 42), Tyr 103 (Phe 100), with residues in parenthesis belonging to RNase.T1. Using the same coordinate transformation the model of 2'GMP from the RNase.T1-2'GMP complex was positioned in the active site of Barnase. The interactions of the nucleotide with Barnase, van der Waals contacts and Hydrogen bonds, are found to be very similar to those formed in RNase.T1. A systematic exploration of conformational space of 2'GMP and 3'GMP performed as a function of 3 angles (N-C1', C2'-O2', O2'-P) in the presence of all protein atoms in Barnase and RNase.T1 shows that most favourable energies of the complex correspond to SYN conformation of the nucleotide portion. Moreoever, phosphate positions in the active site were found to be severely restricted in both enzymes.

3. Discussion

Using molecular graphics and simple computations, we have analysed the 2 Å resolution refined structure of Barnase.The complex of Barnase with 2'GMP and 3'GMP have been generated by model building based on detailed structural comparisons with RNase.T1. Analysis of inhibitor and substrate (3'GMP) interactions with the enzyme allows one to make a number of hypotheses concerning the catalytic mechanisms in these enzymes which will be tested using site directed mutagenesis. Model building of Barnase complex with other mononucleotides is in progress to assess the role of different active site residues in confering specificity in these enzymes.

[1] Delhaise, Ph., et al., J. of Molec. Graphics, 2, p.103 (1984)
[2] Brooks, B., et al., J. Comp. Chem., 4, p.187 (1983)
[3] Mauguen, Y. , et al., Nature, 297, p.162 (1982)
[4] Saenger, W., Free University, 1000 Berlin 33 (private communication)
[5] Chothia C. and Lesk A.M., EMBO J., 8, p.823 (1986)
[6] Van Cutsem, E., Université Libre de Bruxelles, cp160 (unpublished results)

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

-poster/presentation in Capri

-1987, paper in preparation.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

Two meetings have taken place between the partners.

1- Brussels, 21 January, 1987; present: Fersht (Imperial College), Janin (Orsay), Prevost &Wodak (ULB), Stanssens & Mathyssens (Plant Genetic Systems, Ghent).
Research goals and strategies were discussed. Agenda for information exchange and collaborations was determined.

2- Capri, May 21 ,1987; present: Fersht (Imperial College), Baudet (Orsay), Prevost & Wodak (ULB), Stanssens & Mathyssens (P.G.S. Ghent).
Recent progress on kinetic experiments done in the UK, on crystallisation attempts in France, and model building studies in Brussels, was discussed.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Imperial College of Science & Technology, London
Contract no.: BAP - 0050 - UK
Project leader: A.R. FERSHT
Scientific staff:

Address:
Imperial College of Science
and Technology
Department of Chemistry
South Kensington
UK - LONDON SW7 2AZ

Telephone no.: +44.1.5895111 EXT.4578

Telex no.: 261503 IMPCOL G

Other contractual partners in the joint project:

S. Wodak, U. L. B. (Bruxelles)
J. Janin, Université Paris-Sud (Orsay)
P. Stanssens, Plant Genetic Systems NV (Gent)

Title of the research activity:

Engineering of an extracellular ribonuclease by gene modification.

Key words:

Protein engineering, Enzymology, Barnase, Ribonuclease, Expression

Reporting period: January 1987 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

To develop rational approaches for engineering modified proteins using genetic engineering technology. A small ribonuclease, Barnase, from *Bacillus amyloliquefaciens* is being modified and its physical and kinetic properties analysed in combination with computer technology and X-ray crystallography.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Synthesis of Barnase gene and expression. Development of suitable assay systems for detailed kinetic work.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

METHODOLOGY AND RESULTS

Expression and Purification of Barnase

We synthesized the gene for barnase using six overlapping oligomers. During this period, however, the gene was cloned in *Escherichia coli* with its intracellular inhibitor by Paddon and Hartley (1987). They generously supplied the clone for this work. Mature Barnase is expressed using the *pho*A (alkaline phosphatase) promoter and signal sequence. The current work has involved optimizing the expression, purification and preliminary characterisation of Barnase from *E. coli*. Plasmid pMT410 carrying the genes for Barnase, Barstar and ampicillin resistance, was transformed into *E. coli* JM101. Single colonies were picked and used to inoculate 10 ml L-broth with 50μg/ml of ampicillin. The cultures were grown overnight at 37°C of which 2 ml was then used to inoculate 100 ml of MOPS low phosphate media in 500 ml Ehrlenmeyer flasks (Serpersu *et al.* 1986). The flasks were then grown overnight at 37°C with vigourous shaking; the absorbance at 600 nm was routinely 1.5-2.5.

Purification of the Barnase from *E.coli* carrying pMT410 has been optimised to require only two purification steps giving Barnase that is >95% pure. Barnase is partially secreted through the *E. coli* outer membrane (Hartley, personal communication) the remainder of which can be released from the periplasmic space with acetic acid.

Table 1: Purification of Barnase

Fraction	Volume (ml)	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Purifcn
Broth	500	1050	1.67×10^8	3.18×10^5	1
Phospho-cellulose	6	28	4.98×10^7	1.78×10^6	5.6
Desalting	7	23	4.65×10^7	2.02×10^6	6.4

Purified protein gave a single band on urea SDS-polyacrylamide gel electrophoresis of molecular weight 13,300 which is consistent with the Barnase M_R of 12,382 (Hartley and Barker, 1972). The final protein concentration as determined from the absorbance at 280nm, gave 23 mg/l of pure protein of specific activity 2.02×10^6 units/mg. The activity of the enzyme was determined using the precipitation assay of Rushizky *et al* (1963), and agrees with the specific activity of pure Barnase of 2×10^6 as has been previously determined. The optimal pH for this assay was between 8 and 9 which corroborates the earlier observations of Rushizky *et al* (1963) and confirms that the enzyme is Barnase.

The expression level of 23.2mg/l is significantly higher than the 2mg/l obtained by Hartley for this expression system and provides sufficient protein for NMR studies.

Assay Systems for Barnase

Several assay systems for Barnase have been tested to find a suitable system by which to characterise this ribonuclease. The original RNA precipitation assay can give variable results according to the quality of the

substrate RNA and does not allow for specific nucleotide interactions to be studied.

(A) *Adenosine deaminase coupled enzyme assay.* Using an adenosine deaminase coupled enzyme assay adapted from Ipata and Felicioli (1968), it is possible to follow Barnase activity by the release of adenosine from dinucleotides NpA according to scheme 1.

Scheme 1

$$GpA \xrightarrow{\text{Barnase}} GMP + \text{Adenosine}$$

$$\text{Adenosine} \xrightarrow[\text{Deaminase}]{\text{Adenosine}} \text{Inosine}$$

There is a spectral change on going from adenosine to inosine at 265nm that can be followed spectrophotometrically. The disadvantage of this system is that saturating concentrations of substrate cannot be reached due to the high background absorbance.

(B) *Direct observation of spectral change on hydrolysis of the dinucleotides GpN.* The small spectral change in absorbance at 280nm (~ 10%) can be followed spectrophotometrically. Due to the longer wavelength at which substrate hydrolysis is measured, higher substrate concentrations than in (A) can be used.

(C) *Release of proton.* Barnase is related to T_1 ribonuclease (Mauguen *et al*, 1983) and is therefore expected to hydrolyse RNA via a 2'3'cyclic intermediate. The hydrolysis of this intermediate releases a proton which can be followed using a pH stat. The advantage of this system is that substrate can be used at saturating concentrations and the hydrolysis of cyclic nucleotides can be followed.

DISCUSSION

We have established a rapid purification procedure for Barnase expressed from the cloned gene, have developed kinetic assay systems and have obtained preliminary kinetic results. The goals for year 1 have been accomplished six months ahead of schedule. The next report will describe the determination of the kinetic specificity of the enzyme and the construction and analysis of the first mutants.

References

Hartley R.W. and Barker E.A. (1972) Nature New Biology 235 15-16
Ipata P.L. and Felicioli E.A. (1968) FEBS Letters 1 29-31
Manuguen Y., Hartley R.W., Dodson E.J., Dodson G.G, Bricogne G., Chothia C. and Jack A. (1982) Nature 297 162-164
Paddon C.J. and Hartley R.W. (1987) Gene 53 11-19
Rushizky G.W., Greco A.E., Hartley R.W and Sober H.A. (1963) Biochemistry 2 787-792
Serpersu E.H., Shortle D. and Mildvan A.S. (1986) Biochemistry 25 68-77

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	No
Exchange of staff	No
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

A.R. Fersht has visited Brussels to meet with S. Wodak, J. Janin, their staff and that of Plant Genetic Systems. The strategy was planned at that meeting. We have been in regular contact by telephone to transmit our methods of assay, purification and first kinetic results. Crystallographic studies by Janin on inhibitor binding will be based on our kinetic data on specificity.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: C.N.R.S., Orsay

Contract no.: BAP - 0049 - F

Project leader: J. JANIN

Scientific staff:

Address:
Université Paris-Sud
Laboratoire de Biologie Physicochimie
UA 1131 du CNRS, Bât. 433
F - 91405 ORSAY

Telephone no.: +33.1.69417973

Telex no.: 692166 F

Other contractual partners in the joint project:

S. Wodak, U. L. B. (Bruxelles)
A.R. Fersht, Imperial College of Science & Technology (London)
P. Stanssens, Plant Genetic Systems NV (Gent)

Title of the research activity:

Engineering of an extracellular ribonuclease by gene modification.

Key words:

Protein engineering, Crystallography

Reporting period: January 1987 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Analysis of catalytic mechanism and substrate specificity of the bacterial ribonuclease Barnase by protein engineering.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Crystallographic analysis of modified Barnase and of complexes with inhibitors.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Barnase has been prepared from a gene cloned in E. coli. The purified protein has been crystallized in the presence of Zn^{++} ions as described for protein produced by B. amyloliquefaciens. Crystals of high quality have also been obtained without Zn^{++} in the presence of dinucleotide inhibitors.

Methods and Results

Barnase excreted by B. amyloliquefaciens has been crystallized and the X-ray structure has been solved to 2 Å resolution by Mauguen et. al. ( ). We attempted to crystallize the enzyme cloned in E. coli and its complexes with nucleotide inhibitors.

Plasmid pmt 410 carrying the genes for barnase and its natural inhibitor barstar was isolated by Dr. R. Hartley, who kindly made it available to us. The genes are under control of the pho promoter of E. coli and contain the sequence which determines excretion of the nuclease. Strain Jm 101 of E. coli was tranformed with the plasmid and grown in a 18 liter culture. Barnase excreted in the growth medium was adsorbed on phosphocellulose. Elution from the gel and further purification was performed as described by R. Hartley for the protein excreted by B. amyloliquefaciens. We obtained 3 mg of electrophoretically pure protein.

Crystals were grown in hanging drops containing 10 microliters of protein (10 mg/ml) in 0.85 M ammonium sulphat over pits containing 3 M ammonium sulphate. In the presence of 10 mM $Zn SO_4$, hexagonal prisms 0.4 mm in length and 0.1 mm in section were obtained. A crystal was mounted in a capillary tube and 12° precession photographs were taken. The precession patterns were compatible with the trigonal space group $P3_2$ of the B. amyloliquefaciens protein, though the cell parameters may not be identical to published ones (a = b = 59.0 Å, c = 81.6 Å).

Crystals of the same habit and size were also obtained in the absence of Zn^{++} when 2.5 mM of the deoxy-dinucleotide d(GC) was added to the protein solution. These crystals are not isomorphous to those of the B. amyloliquefaciens protein - Zn complex, as indicated by the cell parameters derived from precession photographs (a = b = 57.7 Å, c = 84.9 Å). Characterization of these crystals is under way

Discussion:

The X-ray analysis of barnase-inhibitor complexes should be possible in the near future on the new crystal form which we have obtained.

Acknowledgements:

We thank R. Hartley (Institutes of Diabetes, Digestive and Kidney Diseases, Bethesda, Maryland) for the gift of plasmid pmt 410 and for valuable information concerning purification and crystallization of barnase.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.2 M. Prévost, D. Van Belle, S. Wodak, P. Stanssens, G. Mathyssens, A.R. Fersht & J. Janin

Engineering of an Extracellular Ribonuclease by gene modification.

Abstract, BAP Meeting, Capri, May 1987

TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

Meeting of S. Wodak, J. Janin and A. Fersht
Brussels, January 21, 1987
Participation of S. Baudet to the BAP Meeting, Capri

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Novo Industri A/S, Bagsvaerd

Contract no.: BAP - 0155 - DK

Project leader: S.B. PETERSEN

Scientific staff:

Address:
Novo Industri A/S
Novo allé
DK - 2880 BAGSVAERD

Telephone no.: +45.2.982333

Telex no.: 37173a NOVO DK

Other contractual partners in the joint project:

G. Dodson, University of York

Title of the research activity:

Structure-function relationships in a peptide hormone and enzymes. The application of protein engineering.

Key words:

Protein engineering, Molecular modelling, Molecular dynamics, Genetic engineering

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

To study how alterations in the structure of proteins can change their functions.

The specific aim is to investigate the structure and functions of amylase enzymes.

The long range goal is to obtain the knowledge necessary for introducing appropriate changes in an enzyme molecule, which may lead to enzymes with enhanced properties.

In particular the posibilities for enhancing temperature stability will be studied.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

2.1 To clone the relevant genes and determine the sequences.
2.2 To develop analytical methods for characterizing the enzymes.
2.3 To clarify the mechanism of denaturation of the enzymes.
2.4 To find competitive inhibitors of the enzymes.
2.5 To develop molecular models and computing methods for the enzymes.

The work has been concentrated about studies of fungal and bacterial alpha-amylases.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

2.1 To clone the relevant genes and determine the sequences

Comparison of structural and functional properties of alpha-amylases from various sources may be suggestive in the choice of mutations in a given amylase to obtain a certain change. For that purpose one bacterial amylase and two fungal amylases have so far been cloned and partially sequenced, and a third fungal amylase is on its way. The genes are expressed in appropriate hot organisms for purification of the enzymes.

2.2 To develop analytical methods for characterizing the enzymes

With the aim of determining the stability of the fungal amylases an analytical method has been worked out.The method is a modification of an analytical method developed by Boehringer Mannheim for serum amylase using p-nitrophenyl-alpha-D--maltoheptaoside as substrate.

The substrate is decomposed by the alpha-amylase, and the formed decomposition products are further decomposed by the enzyme alpha-glucosidase, whereby a yellow colour is developed due to the liberation of p-nitrophenol. The change in absorbance as a function of time is measured in a spectrophotometer at 405 nm.

By changing the buffer system and introducing minor changes in the methodology an accurate and convenient method for analyzing fungal alpha-amylases have been obtained.

Further to be used as a screening test for potential amylase inhibitors an analytical method based on Pharmacias Phadebas method has been worked out. Again the main change consist in a change in buffer system. The developed method is well suited for screening purpose.

2.3 To clarify the mechanism of denaturation of the enzymes

As an introductory test in the work of clarifying the denaturation mechanism of amylases the heat stability of two alpha-amylases of commercial values have been determined.

The stability of a purified A. niger alpha-amylase in 0.1 M acetate buffer and the stability of TERMAMYL^{R} a commercial alpha-amylase from B. licheniformis in 0.1 M phosphate buffer have been determined.

Dilute solutions of the respective enzymes are kept at different temperatures and at certain time intervals the residual activities are measured. The denaturation constants kD are calculated from ln (residual activity)=-kD*t.

The results of the experiments are given in Table 1:

Table 1.

ENZYMES	DEGREE CELCIUS	DENATURATIONCONSTANT MIN^{-1}
	65	0.00382
Asp.	67.5	0.00719
niger	70	0.0269
	72.5	0.0737
	72.5	0.00925
Bacillus	75	0.0113
licheniformis	77.5	0.0293
	80	0.0484

Assuming normal reaction rate theory kD can be expressed as kD =K*exp(-E/RT) and on the basis of the results the activation energy of denaturation E can be calculated.

The activation energy for the Niger alpha-amylase has been found to be 96.9 kcal/mole and the activation energy for TERMAMYL^{R} has been found to be 57.9 kcal/mole.

2.4 To find competitive inhibitors of the alpha-amylases

With the purpose of supplying York with an inhibitor against Asp. niger alpha-amylase, which can be used in the structure determinations a screening work has been started.

Many glucose containing chemicals including the substrate analogs:
maltose, maltotriose, maltotetraose, maltopetaose, malohexsaose and maltoheptaose have been examined but no feasible inhibitors have been found.

The only inhibitor found has been Trastatin A from Hoffmann-La Roche, but the inhibition is to weak to be of practical value.

2.5 To develop molecular models and computing methods for the enzymes

It is the aim to aquire the sufficient knowledge to allow the researchers at Novo to predict the possible consequences of a induced mutation in a given enzyme based on knowledge of the 3-D structure of the native enzyme. In order to approach this declared goal a molecular modelling department has also been established at Novo. The hardware that this department encompasses includes a VAX 11/785, a PS 330 vector graphical terminal, a Silicon Graphics IRIS 3130 raster graphical terminal as well as a number of smaller terminals to the VAX. The software packages that are being used are "HYDRA", "CHARMM" and "PIR". "HYDRA" is a protein oriented modelling program, written by Dr. Rod Hubbard, University of York, that presently are running on both graphical systems. We are currently using "HYDRA" to evaluate possible mutationsites using the available Taka-amylase crystal structure data. It should be emphasized that the Taka amylase structure is a low resolution structure, which in addition is a mean structure between the unit cells three non-identical amylase molecules. Despite these limitations we believe that we have identified possible point mutations that can optimize certain physical chemical properties of amylases. We are also starting up activities in the area of molecular dynamics using the well known program "CHARMM", with the hope of being able to unravel some of the dynamic aspects of enzyme function. In order to avoid misinterpretation of the often very complex data sets that one can obtain from simulation protein dynamics, we have started focussing on smaller model proteins, such as a 72 residue long alpha helical poly alanin. These simulations showed quite

clearly that such an alpha helical structure will oscillate with a very low frequency when absorbing energy capable of exciting the amide band, which involves far higher frequencies than the ones characteristic for the helix oscillations. The amide band could typically be excited by an ATP to ADP conversion. The oscillation consists of a correlated accordion and twist motion. Parts of these results were presented at the "BAP" meeting on Capri in May 1987 (see reference 1). Finally a number of smaller utility computer programs have been written and are currently being debugged at Novo.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

The NOVO BAP contract research team has interacted strongly with the X-ray crystallographic and modelling team at the University of York, headed by Professor Guy Dodson. Key persons in both groups have visited the each other at frequent intervals, typically every 3 month. Purified enzyme material have been prepared at the premises of NOVO and send to York for crystallization and subsequent X-ray diffraction. Researchers from NOVO often spend time in Professor Dodsons laboratory to be introduced to new developments both in the area of X-ray structure determination proteins as well as in the area of modelling.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Univ. of York

Contract no.: BAP - 0154 - UK

Project leader: G. DODSON

Scientific staff: U. Derewenda, Z. Derenwenda, M. Harris, A. Raine

Address:
University of York
Department of Chemistry
Heslington
UK - YORK YO1 5DD

Telephone no.: +44.904.430000

Telex no.: 57933 YORKUL G

Other contractual partners in the joint project:

S.B. Petersen, Novo Industri A/S (Bagsvaerd)

Title of the research activity:

Structure-function relationships in a peptide hormone and enzymes. The application of protein engineering.

Key words:

Protein crystallography, Insulin, X-ray area detector, Protein engineering, Molecular graphics

Reporting period: June 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

To further the techniques of protein engineering and to establish how the separate techniques of X-ray analysis, of modelling, of functional studies and of molecular genetics link together effectively.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The Centre for Biomolecular Design has two objectives. First to establish rapid use of the Xentronics area detector in protein data collection. This includes the development of diagnostic tests which identify the systems behaviour and which will make it possible to optimise the accuracy of the data. Secondly to identify amino acids whose position and function in a protein are crucial to its functions and structure. From these studies proposals for substitutions likely to alter the proteins properties (e.g. substrate specificity and stability) in intended ways can be made.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Methodology

Protein Crystallography

The main experimental development has been the arrival of the Xentronics area detector. This has enabled us to collect accurate protein crystal data rapidly. Typically 2Å resolution data sets from crystals with axial lengths between 50-80Å can be collected in 2-4 days; larger crystal cells (greater than 150Å) at this resolution take longer, between 7-14 days. The internal agreement between equivalent reflections lies between 3-6% in intensities.

The enhancement of the HYDRA graphics systems which we used to display protein structure has enabled us to analyse and compare protein molecules effectively and rapidly. The HYDRA system has been implemented in the Silicon graphics (IRIS) and is being used and assessed by the investigators in their hormone and enzyme studies.

2. Results

The structure of a B12 site directed mutant human insulin has been determined in the R3 crystal. The mutation changes B12 val → ilu, a substitution that interferes with dimer formation. In the hexamer stabilised by the Zn coordination the steric pressure of the extra valine at the B12 ilu can be detected. There are small but distinct structural changes radiating out from the two B12 ilu in contact at the dimer centre illustrated in Figure 1. These changes are now being investigated through molecule dynamic simulation using CHARMM and the findings analysed with HYDRA.

(a) Molecule 1 (b) Molecule 2

Figure 1
The electron density for the mutant ile B12, whose bonds are represented by open lines. The dashed line represents the structure of native val B12. (a) molecule 1 - here there is evidence of disorder in the ile sidechain, (b) molecule 2 - here the ile conformation is well defined.

Crystals of a number of other site directed mutant human insulins have been prepared.

The enzyme X-ray analysis has gone well. The enzyme has been crystallised, space group C_{2221} with axial lengths a=80.6Å, b=98.2Å and c=139.4Å. There is one molecule per asymmetric unit, which contains about 50% solvent by volume. The best crystals were obtained at low pH which has limited heavy atom soaking reactions. However, a single site site Hg derivative has been prepared and further experiments are continuing.

Native and the Hg derivative data have been collected on the area detector. The Patterson function has been calculated with coefficients $||F_{HP}|-|F_P||^2$ where $|F_{HP}|$ is the heavy atom (Hg) and $|F_P|$ the native enzyme X-ray diffraction amplitude. The Patterson map shows clearly

the Hg has reacted as a single site derivative; see Figure 2.

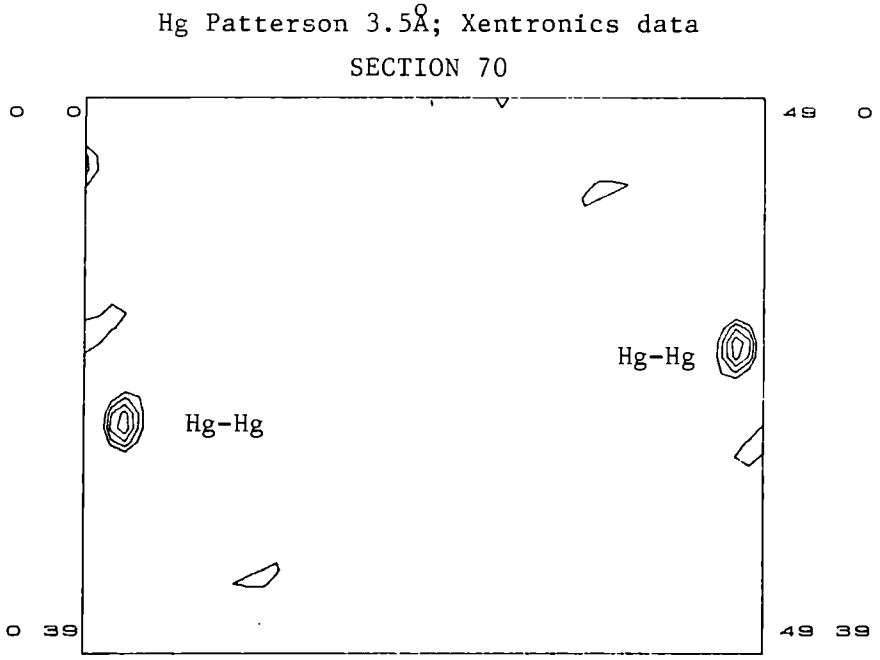


Figure 2
The Hg-Hg vector peak seen in the Harker section, w=½. Crystal symmetry generates two two fold related equivalent peaks. The data were collected on the Xentronics area detector.

3. Discussion

The area detector has altered our approach to protein crystal study. It can yield accurate data sets in 3-14 days and on this time scale the analysis of heavy atom derivatives can be carried out very rapidly. When the crystal under study is isomorphous with a known parent structure then a refined and accurate crystal structure can be available for study in a week. With more computer storage this stage could be much more rapid.

Thus the turn-round of X-ray structural conformation can be about the same as aquisition of n.m.r. spectra. Thus the potential for X-ray and structural analysis in favourable systems being an immediately useful tool for structural studies on mutated proteins is being realised.

It is also possible to use the Xentronics for surveying heavy atom derivatives. At present the system's architecture makes this a clumsy operation. We are anxious to modify the storage and computing arrangements to facilitate very rapid limited data collection.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

We have worked closely with the research group in Newcastle headed by Professor Roger Payne. The project we collaborate on is concerned with penicillin acylase. We have regular discussion meetings which particularly concentrate on identifying the active residues, designing inhibitors and heavy atom reagents for the manufacture of heavy atom derivatives.

The contacts with the NOVO research laboratories have been very close. Purified protein from NOVO (enzymes and hormones) have been crystallised and studied.

Staff from York have visited the NOVO laboratories to discuss all aspects of the programme and to carry out certain experiments. There have been regular (about 3 monthly) discussion meetings between the principal NOVO and York scientists. There have been "training" visits to the York laboratory in the areas of molecular computation, graphics and X-ray analysis.

BIOTECHNOLOGY

OF

INDUSTRIAL MICROORGANISMS

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Univ. College, Cork
Contract no.: BAP - 0008 - IRL

Project leader: C. DALY
Scientific staff: G.F. Fitzgerald, E. Caplice, F. Hayes, A. Harrington, A. Coffey, S. Lennon, J. Coveney, V. Costello, C. Hill

Address: University College
Dairy and Food Microbiology Department
Western Road
IRL - CORK

Telephone no.: +353.21.276871 EXT.2647

Telex no.: 26050 UNIC EI

Other contractual partners in the joint project:

M.J. Gasson, A. F. R. C. (Norwich)
M. Teuber, Bundesanstalt für Milchforschung (Kiel)
W.M. de Vos, N. I. Z. O. (Ede)
G. Venema, University of Groningen

Title of the research activity:
Genetic manipulation of lactic acid bacteria for improved dairy fermentations.

Key words:
Lactic streptococci, Genetic studies, Proteinase, Transposons, Bacteriophage resistance

Reporting period: April 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The principal goal of the joint project is to genetically manipulate lactic acid bacteria to yield improved cultures for dairy fermentations. The programme is directed towards the development of new vector plasmids, improvement of gene transfer systems and techniques that will facilitate easier manipulation of these bacteria. These innovations are being applied to the study of plasmid encoded metabolic traits (eg. lactose and protein metabolism), gene regulation functions (eg. promoter, termination sequences), characterization of bacteriophage:host interactions (eg. phage resistance) and the identification of chromosomally located functions (using transposon technology)

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The specific objectives of this laboratory are:
Proteinase activity: to characterize plasmid encoded prt genes from commercial strains of lactic streptococci; to clone selected prt genes; to improve and stabilize Prt activity in strains that are weakly proteolytic.
Bacteriophage-host interactions: detailed characterization of plasmid encoded phage resistance genes identified in this laboratory; cloning of these genes; examination of the mechanism involved; introduction of the phage resistance plasmids into commercially useful but phage sensitive strains.
Transposon technology: to exploit the properties of Tn919 for the genetic analysis of the lactic acid bacteria.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Methodology

The techniques and procedures used i.e. gene transfer protocols, chromosomal, plasmid and bacteriophage DNA isolation and purification, nick translation, Southern blot and DNA-DNA hybridization techniques, restriction mapping, gene cloning and electronmicroscopy of phage particles have been described in previous reports and publications (1,2,3,4,5) or were performed exactly as described by Maniatis et al. (6). The dideoxy DNA sequencing method of Sanger et al. (7) was used according to the method outlined in the Amersham (U.K.) M13 cloning and sequencing handbook (using M13 mp18 and mp19) to sequence putative promoter sequences from Tn919.

Results

Proteinase Studies

Proteinase plasmids of 50, 23 and 20 MDa were identified in S. cremoris strains UC317, UC205 and UC411, respectively. (These strains were previously designated S. cremoris 17, 047 and 077, respectively). Conjugative transfer of these plasmids to the plasmid-free S. lactis MG1363 could be observed only after mobilization by pAMB1. Analysis of Prt^+ transconjugants derived from strains UC317, UC205 and UC411 revealed the presence of novel recombinant plasmids some of which were pAMB1 cointegrates (See ref. 1).

A 40 MDa recombinant proteinase-pAMB1 plasmid, pCI210, derived from strain UC205 and deleted Prt^+ derivatives of recombinant plasmids from strains UC317 (pCI310; 15 MDa) and UC411 (pCI410; 11 Mda) were constructed and analysed. Detailed restriction maps of these three plasmids (Fig. 1) and the location of the prt genes on each has been determined using a proteinase probe (provided by G. Venema).

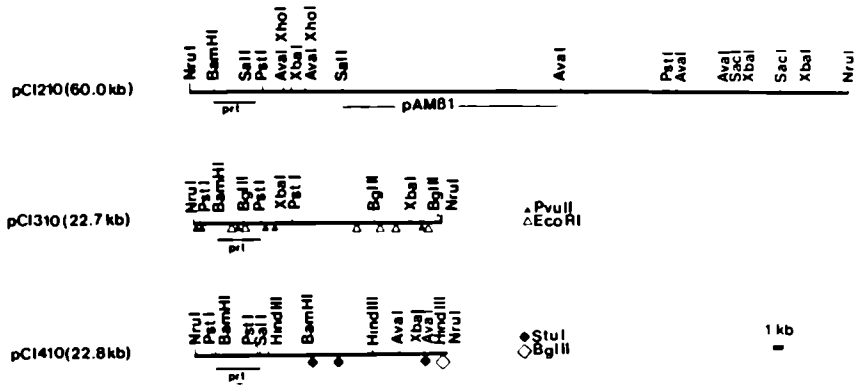


Fig. 1. Restriction maps of the proteinase plasmids pCI210, pCI310 and pCI410

An 8.1 kb Xba1-Nru1 fragment of pCI310, which completely spans the region of homology with the proteinase probe was cloned in S. lactis on the E. coli - Streptococcus - Bacillus shuttle vector pAM401 (8). A number of Cm resistant clones harboured recombinant plasmids containing an apparently intact 8.1 kb Xba1-Nru1 DNA insert. The proteolytic activity of these clones is being assessed.

The conjugative host range of the recombinant proteinase-pAMB1 plasmid, pCI210, was examined. Transfer to S. lactis recipients was demonstrated at a frequency of 8.0×10^{-3} and mobilization of the chromosomally located Tn919 was also observed. Transfer of both the pAMB1 associated Em marker and the Prt determinant to Leuconostoc recipients was also detected at frequencies of 3.3×10^{-7} and 1.3×10^{-8}, respectively.

Analysis of the plasmid profiles of the transconjugants showed the presence of a deleted derivative of pCI210 which still retained both it's Em resistance and *prt* genes based on Southern hybridization data. Weak expression of the Prt marker in the *Leuconostoc* transconjugants (which are naturally Prt-negative) was observed.

Bacteriophage-host interactions

We have previously reported two conjugative plasmids encoding bacteriophage resistance in the lactic streptococci - pCI750 (43 MDa, from *S. cremoris* UC653) and pCI829 (27 MDa, from *S. lactis* UC811) (1, 4). When present in *S. lactis* MG1363 following conjugative transfer from their respective donors, each plasmid confers total resistance to isometric-headed phage and partial resistance to prolate-headed phage based on a significantly reduced plaque size and a slight reduction in EOP. However, when both plasmids are present in the same strain, resistance to prolate-headed phage is significantly enhanced. The resistance mechanism(s) does not act at the level of phage adsorption and is not affected by exposure of the host to temperatures as high as 40°C. The presence of either pCI750 or pCI829 in strain MG1363 inhibits transfection of this host by phage 712 DNA but has only a marginal effect on transformation by plasmid DNA. These phage resistance plasmids have been transferred by conjugation to *S. lactis*, *S. lactis* subsp. *diacetylactis* and *S. cremoris* strains and resistance to phage homologous for the recipient is expressed. Preliminary restriction maps of pCI750 and pCI829 have been constructed and hybridization experiments using a biotin-labelled pCI750 probe showed that there was no homology between it and pCI829.

Transposon Studies

The conjugative transposon Tn*919* has a wide recipient host range among the lactic acid bacteria and insertion of the element, which can occur in chromosomal or plasmid DNA, has been shown to be random or site-specific depending on the recipient (2,3). A high-frequency delivery system for the transposon has also been developed (3) and this feature, combined with random insertion in *S. lactis* subsp. *diacetylactis* 18-16, makes it possible to attempt insertional inactivation of chromosomally located genes in this strain. Preliminary experiments have focused on a citritase (EC 4.1.3.6) negative mutant which harbours a single copy of Tn*919* in the chromosome. DNA flanking the element has been cloned in *E. coli* and is being characterized. The tetracycline resistance (*tet* M) gene of Tn*919* has been cloned and is expressed in *S. lactis*, *B. subtilis* and *E. coli*. Novel plasmid cloning vectors have been constructed based on this marker. Promoter containing DNA fragments from Tn*919* have been cloned in *B. subtilis* and sequenced and these closely resemble the canonical -35 and -10 regions described for *E. coli*, *B. subtilis* and *S. lactis*.

Discussion

The data generated from the restriction mapping of the Prt plasmids indicate that the regions encoding the *prt* genes are very similar. Although an Xba1-Nru1 fragment from pCI310 known to contain the entire *prt* gene has been cloned in *S. lactis* expression in this host was not observed. The possible reasons for this are being investigated.

The availability of restriction maps of the phage resistance plasmids will allow the identification and the cloning of the phage resistance genes on these plasmids. The actual mechanism(s) encoded by the plasmids is not yet clear but experiments are currently in progress to determine the steps in the phage infectious cycle that are inhibited by the presence of the plasmids.

The application of the conjugative transposon for the analysis of chromosomally located genes is a major advance in the genetic characterization of the lactic streptococci. The general techniques we have developed with Tn*919* will be used to target other chromosomally located genes of interest which, up to this point, were less accessible and consequently difficult to study.

Literature cited

1. Daly, C. 1986. Final Report of E.E.C. Biomolecular Engineering Programme on Genetic Engineering of Microorganisms Important for Agro-Food Industries, pp.453-464. Martinus Nijhoff Publishers.

2. Hill, C., Daly, C. and Fitzgerald, G.F. 1985. FEMS Microbiol. Lett. 35, 115-119.

3. Hill, C., Daly, C. and Fitzgerald, G.F. 1987. Appl. Environ. Microbiol. 53, 74-78.

4. Baumgartner, A., Murphy, M., Daly, C. and Fitzgerald, G.F. 1986. FEMS Microbiol. Lett. 35, 233-237.

5. Coveney, J., Fitzgerald, G.F. and Daly, C. 1987. Appl. Environ. Microbiol. (In Press, July).

6. Maniatis, T., Fritsch, E.F. and Sambrook, J. 1982. Molecular Cloning. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., U.S.A.

7. Sanger, F., Nicklen, S. and Coulson, A.R. 1977. Proc. Natl. Acad. Sci. USA, 74, 5463-5467.

8. Wirth, R., An, F.Y. and Clewell, D.B. 1986. J. Bacteriol. 165, 831-836.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1

Baumgartner, A., M. Murphy, C. Daly and G.F. Fitzgerald. 1986. Conjugative co-transfer of lactose and bacteriophage resistance plasmids from Streptococcus cremoris UC653. FEMS Microbiol. Lett. 35: 233-237.

Hill, C., C. Daly and G.F. Fitzgerald. 1987. Development of high-frequency delivery system for transposon Tn919: Random insertion in Streptococcus lactis subsp. diacetylactis 18-16. Appl. Environ. Microbiol. 53: 74-48.

Coveney, J., G.F. Fitzgerald and C. Daly. 1987. Detailed characterization and comparison of four lactic streptococcal bacteriophage based on morphology, restriction mapping, DNA homology, and structural protein analysis. Appl. Environ. Microbiol. 53: July.

Fitzgerald, G.F., C. Hill, E. Vaughan and C. Daly. 1987. Tn919 in lactic streptococci. In Streptococcal Genetics (Ferretti, J.J. and R. Curtiss III, eds.) American Society for Microbiology, Washington, D.C., U.S.A. (In press, June).

Daly, C. and G.F. Fitzgerald. 1987. Mechanisms of bacteriophage insensitivity in lactic streptococci. In Streptococcal Genetics (Ferretti, J.J. and R. Curtiss III, eds.) American Society for microbiology, Washington, D.C., U.S.A. (In press, June).

IV.2

Lennon, S., F. Hayes, P.O'Reilly, C. Daly and G.F. Fitzgerald. 1987. Genetic transformation of Streptococcus lactis MG1363. Ir. J. Fd. Sci. Technol. 10: 161.

Coveney, J., G.F. Fitzgerald and C. Daly. 1987. Characterization and comparison of lactic streptococcal bacteriophage. Ir. J. Fd. Sci. Technol. 10: 161.

Coffey, A., A. Baumgartner, M. Murphy, C. Daly and G.F. Fitzgerald. 1987. Identification of plasmids encoding bacteriophage insensitivity in lactic streptococci. Ir. J. FD. Sci. Technol. 10: 162.

Hayes, F., C. Daly and G.F. Fitzgerald. 1987. Analysis of lactose and proteinase plasmids in Streptococcus cremoris. Ir. J. Fd. Sci. Technol. 10: 162-163.

Daly, C., G.F. Fitzgerald, E. Mullins, et al. 1986. Exploitation of recombinant DNA technology to provide improved cultures for dairy fermentations. In Proceedings of Meeting on Genetic Engineering of Microorganisms Important in Agro-Food Industries - Meeting of Contractants in Biomolecular Engineering Programme, Cork, 1986. pp56-57. Commission of the European Communities.

Hayes, F. E. Caplice, A. Harrington, G. F. Fitzgerald and C. Daly. Conjugative mobilization of proteinase plasmids of Streptococcus cremoris strains: Analysis of recombinant plasmids. In Proceedings of Meeting on Culture Collections and Genetic Engineering of Microorganisms - Meeting of Contractants in Biotechnology Action Programme, Ioannina, 1987.pp. 38-39. Commission of the European Communities.

Daly, C. M. Murphy, A. Coffey and G.F. Fitzgerald. 1987. Phage resistance plasmids in Streptococcus cremoris and Streptococcus lactis. In Proceedings of Meeting on Culture Collections and Genetic Engineering of Microorganisms - Meeting of Contractants in Biotechnology Action Programme, Ioannina, 1987. pp.40-41. Commission of the European Communities.

The following papers were presented by members of this group at the CEC Meeting on Genetics of Lactic Streptococci and their Phages in Haren, The Netherlands, 23-24 September, 1986.

Coveney, J. Characterization and comparison of lactic streptococcal bacteriophage.

Fitzgerald, G. Plasmids encoding phage insensitivity mechanisms in lactic streptococci.

Hayes, F. Analysis of lactose and proteinase plasmids in Streptococcus cremoris 17.

Caplice, E. Proteinase plasmids in Streptococcus cremoris 047

Hill, C. Potential application for Tn919 in lactic streptococci.

IV.3

Patents deposited -- none

IV.4

Lennon, S. Genetic transformation of Streptococcus lactis MG1363. National University of Ireland. (University College, Cork). M.Sc. Degree awarded November, 1986.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff	Yes	
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

Our group maintains an extensive and highly productive degree of contact and cooperation with the laboratories of our partners collaborating in the joint project.

Exchange of materials
Bacterial strains, bacteriophage, plasmid and phage DNA are regularly and freely exchanged between this laboratory and the groups of G. Venema, W. deVos, M. Teuber and M. Gasson.

Exchange of staff
Graduate students from this laboratory have visited and worked in the laboratories of G. Venema (May 1986 -April 1987, under a Junior Level Training Contract in the Research Action Programme - Biotechnology) and M. Gasson (May 1987 - December 1987). These opportunities to work in a collaborating laboratory have proved to be very stimulating for the students and highly productive in terms of scientific achievement.

Joint meetings
The five collaborating laboratories in the joint project have regular meetings. During the present reporting period there was a meeting at The University of Groningen (September, 1986) entitled "Genetics of Lactic Streptococci and their Phages", hosted by G. Venema, where representatives from the five groups reported on their different projects, exchanged ideas and planned and discussed future experiments. Members of the five laboratories also met at the CEC meeting on Culture Collections and Genetic Engineering of Microorganisms (Ioannina, Greece, April 1987) where the progress achieved in the various projects was reported and discussed.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: A.F.R.C., Norwich

Contract no.: BAP - 0009 - UK

Project leader: M.J. GASSON

Scientific staff: H.M. Dodd, R. Davies, S.H.A. Hill, C. Shearman, H.M. Underwood, P. Anderson

Address: AFRC Institute of Food Research
Colney Lane
UK - NORWICH NR4 7UA

Telephone no.: +44.603.56122

Telex no.: 975453

Other contractual partners in the joint project:

C. Daly, University College (Cork)
M. Teuber, Bundesanstalt für Milchforschung
W.M. de Vos, N. I. Z. O. (Ede)
G. Venema, University of Groningen

Title of the research activity:

Genetic manipulation of lactic acid bacteria for improved dairy fermentations.

Key words:
Lactic streptococci, Genetic studies, Proteinase, Transposons, Bacteriophage resistance

Reporting period: April 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

1. Further development of cloning vectors on the basis of streptococcal plasmids.
2. Expression of homologous and heterologous genes in streptococci.
3. Improvement of gene transfer methods (e.g., transformation).
4. Stabilisation of fermentation properties by transfer of genes from plasmids into the chromosome.
5. Characterisation and construction of bacteriophage-resistant bacteria.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Clone and characterise a bacteriophage lysin gene.

Further characterise the proteinase gene of S. lactis 712.

Continue the development of vector systems and transformation protocols for the lactic streptococci.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Methods

Standard molecular biological techniques were used for DNA manipulation, cloning and sequencing. High titre bacteriophage lysates were prepared and concentrated by polyethylene glycol precipitation and purified on caesium chloride stepped gradients. Bacteriophage DNA was extracted by dialysis against 50% formamide followed by TE buffer. Further purification of DNA was sometimes performed on caesium chloride-ethidium bromide gradients.

Results and Discussion

Bacteriophage ØML3 lysin gene An E. coli bacteriophage lambda cloning strategy was used. Bacteriophage ØvML3 was partially digested with restriction endonuclease AluI to give random fragments sized between 0.5 and 4 kilobases. To the blunt-ended fragments, EcoRI linkers were added and the DNA was ligated to dephosphorylated, EcoRI-generated arms of lambda vector gt10. This vector was chosen because small fragments of

foreign DNA can be cloned and expressed under lambda cI control and because clones can be detected by their efficient plating on an E. coli hfl host. Clones expressing the bacteriophage ML3 lysin were sought by overlaying the lambda plaques with a concentrated suspension of S. cremoris 1196 cells in agarose. Zones of clearing in this overlay were detected for lysin expressing lambda clones. A collection of 50 clones were retained for further study and one with a 1.2 kilobase EcoRI insert was selected for detailed characterisation. The lysin gene on a 1.2 kilobase EcoRI fragment was sub-cloned into the EcoRI site of the E. coli plasmid vector pUC13 using dephosphorylated, EcoRI cleaved DNA. White ampicillin-resistant trans-formants of E. coli TB1 were selected on B agar containing "Bluo-gal" and IPTG. These were screened on DNA "miniprep." gels to find clones with a 1.2 kilobase EcoRI insert. One such clone, E. coli W3, was chosen for further characterisation. The strain was unusually lysis susceptible and had very poor viability. Cells with an atypical morphology and appearing to have inclusions were observed by phase contrast microscopy. Culture supernatants and cell-free extracts of E. coli W3 had strong lytic activity against S. cremoris 1196 and could be used to generate protoplasts within minutes.

Lactic streptococcal vectors have been developed which replicate in lactic acid bacteria, B. subtilis and E. coli. The lysin gene on a 1.2 kilobase EcoRI fragment was sub-cloned into the EcoRI site of two such vectors pC9 and pCK509 and in both cases expression of the lysin was detected. Plasmid pCK509 has a characterised lactic streptococcal promoter, "promoter A", adjacent to the EcoRI cloning site. For this vector clones which expressed lysin were of two types, some with expression levels similar to the pUC13 and pC9 clones and others with considerably elevated production of lysin. These observations suggest that the lysin gene is expressed from its own promoter but that read-through expression from an external lactic streptococcal promoter was also obtained.

A restriction map for the 1.2 kilobase EcoRI fragment was made and it was cloned intact and as a series of smaller inserts into the M13 vector M13mp18. The clones with a complete fragment expressed lysin enzyme. Using these clones, a sequencing strategy for the fragment has been devised and thus far 700 base pairs of continuous sequence from one fragment end have been determined.

Proteinase gene of S. lactis 712 The cloned proteinase gene of S. lactis 712 plasmid pLP712 has been further characterised. The promoter and amino terminus of the protein have been sub-cloned and sequenced. A 0.35Kb Cla-I DNA fragment was cloned into an M13mp18 vector. Only two authentic clones were found amongst 17 colourless M13 plaques analysed. T tracking showed that DNA from nine of the other plaques carried an identical M13 deletion of about 50 base pairs. The T tracks of the two authentic clones showed strong homology with the equivalent region of the S. cremoris Wg2 proteinase sequence (Kok et al., 1987). The DNA sequence that was determined showed a one-base difference from the equivalent S. cremoris Wg2 proteinase gene. Activity of this promoter region has been demonstrated in E. coli using promoter-probe vectors pKK175-6 and pKK232-8, which carry promoterless tetracycline and chloramphenicol resistance genes, respectively. The

0.35Kb ClaI fragment from the S. lactis 712 proteinase gene was excised and the single-stranded ends were filled in using Klenow enzyme. Cloning into pKK175-6 was achieved by blunt-end ligation into the SmaI site and tetracycline-resistant clones were selected. Sub-cloning of this same fragment into vector pKK232-8 gave chloramphenicol resistance. The authenticity of the inserts in these constructs was confirmed by cloning into M13mp19 and T-track analysis. This also showed that the proteinase promoter was correctly orientated with respect to the antibiotic-resistance-structural gene.

Further development of lactic streptococcal shuttle vectors The promoterless chloramphenicol resistance gene of the Bacillus plasmid pPL603 was excised as an EcoRI-BglII fragment and cloned between the same two sites of the lactic streptococcal vector pCK1. In this experiment the kanamycin-resistance gene was restored and was selected after Bacillus subtilis protoplast transformation. Vector pCK509 is typical of the kanamycin-resistant, chloramphenicol-sensitive clones obtained. Shotgun cloning of chromosomal, plasmid and bacteriophage DNA fragmented with EcoRI "star" digestion or by partial AluI digestion and linker addition was achieved using the EcoRI site of pCK509. Chloramphenicol-resistant transformants of S. lactis and B. subtilis were obtained with varying levels of chloramphenicol resistance. These putative promoter active fragments are being characterised further.

In the course of characterising a cloned DNA fragment that encoded the gene for phospho-β-D-galactosidase (Maeda & Gasson, 1986), a promoter active fragment was located on a small ClaI-HindIII fragment. This promoter A fragment efficiently expressed various gram negative genes in E. coli and a promoterless gram positive chloramphenicol resistance gene in both B. subtilis and S. lactis. Two expression vectors pCK536 and pCK965 have been constructed using the promoter A fragment adjacent to a polylinker derived from pUC18. Vector pCK965 also expresses a promoterless chloramphenicol resistance gene. Together with Dr A. Mercenier, Transgene, France, we have shown that both vectors efficiently express the Pseudomonas XylE gene in E. coli and B. subtilis but not in S. lactis.

High frequency conjugal transfer of the S. lactis plasmid pLP712 involves variant donor strains which exhibit cell aggregation (Gasson & Davies, 1980). This conjugal transfer system is able to mobilise the lactic streptococcal vector pCK1 at reasonably high requencies which are elevated when homology with the pLP712 plasmid is provided by gene cloning. Mobilisation of pCK vectors by the broad host range transmissible plasmid pAMβ1 has also been achieved.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

1. Gasson, M. J. (1987).
Genetics of Streptococcus lactis 712.
In Genetics of Industrial Microorganisms (ed. Alačevič, M., Hranueli, D. & Toman, Z., pp. 425-430.

2. Gasson, M. J. & Underwood, H. M. (1987).
Cloning and expression of a lactic streptococcal bacteriophage lysin gene.
Report of the First Meeting of Contractants in the CEC Biotechnology Action Programme, Ionnina, Greece.

3. Anderson, P. H., Rodriguez, A. & Gasson, M. J. (1987).
Further development of lactic streptococcal shuttle vectors.
Report of the First Meeting of Contractants in the CEC Biotechnology Action Programme, Ionnina, Greece.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Bacterial strains supplied to and received from the contractual partners.

Joint staff member shared with C. Daly (Ireland).

Joint experiments on lactose genes with W. De Vos (Netherlands).

Joint meeting held in Groningen, Netherlands, September, 1986.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Bundesanst. für Milchforschung, Kiel

Contract no.: BAP - 0010 - D

Project leader: M. TEUBER

Scientific staff: A. Geis, A. Wetzel (01.04.86-31.03.87), H. Neve, A. Budde-Niekiel (01.04.-31.10.86), V. Möller, U. Krusch, B. Luschei

Address: Bundesanstalt für Milchforschung
Institut für Mikrobiologie
Hermann-Weigmannstrasse 1
D - 2300 KIEL

Telephone no.: +49.431.609340

Telex no.: 292966 MIFO D

Other contractual partners in the joint project:

C. Daly, University College (Cork)
M.J. Gasson, A. F. R. C. (Norwich)
W.M. de Vos, N. I. Z. O. (Ede)
G. Venema, University of Groningen

Title of the research activity:
Genetic manipulation of lactic acid bacteria for improved dairy fermentations.

Key words:
Lactic acid streptococci, Bacteriophages, Phage resistance, Plasmids, Phage receptors

Reporting period: April 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

To apply genetic engineering techniques to the lactic acid streptococci with emphasis on the:

a. development of cloning of homologous and heterologous genes within the lactic acid streptococci on the basis of streptococcal plasmids,
b. expression of homologous and heterologous genes in the lactic acid streptococci,
c. improvement of gene transfer methods,
d. stabilization of fermentation properties by transfer of genes from plasmids into the chromosome,
e. characterization and construction of bacteriophage resistant bacteria.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

a. Collecting of virulent bacteriophages active against thermophilic lactic acid streptococci.
b. Investigation of phage resistance mechanisms (inhibition of phage adsorption, restriction/modification, immunization by lysogenization)
c. Biochemical analysis of phage receptors.
d. Isolation, characterization, cloning, and transfer of plasmid-borne phage-resistance genes.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

METHODOLOGY:

The isolation, purification and characterization of bacteriophages, and the isolation, characterization, curing, and conjugal transfer of plasmids have been described (1). Electronmicroscopic examination of phage adsorption has also been described (2). Preparation of cell walls was done according to E. Work (3). Standard gene cloning and molecular biological techniques were used.

RESULTS AND DISCUSSION:

a. PLASMID-DEPENDENT BACTERIOPHAGE RESISTANCE
A great number of strains from mesophilic lactic acid streptococci was screened by plasmid curing for plasmid-mediated phage resistance. In addition to the already described strains Streptococcus lactis 3085 and S.lactis subsp. diacetylactis Bul, carrying phage resistance plasmids of 15.3 Kb and 60 Kb (1) several new strains were isolated. S.cremoris 0/37 and S.cremoris 11/49 carry non-conjugative phage resistance plasmids of 19.5 Kb and 12.8 Kb, respectively. S.cremoris SD8, SD9, SD11 - slime producing (ropy) strains isolated from a Swe-

dish sour milk - also showed plasmid-dependent phage resistance, which was not merely caused by the presence of the slime polymer (4). Loss of the phage resistance of these strains was always accompanied by the loss of two plasmids, a 94 Kb protease- and a 26 Kb slime plasmid. Subsequent loss of a 68 Kb lactose plasmid rendered these phage-sensitive derivatives phage resistant again.

At the moment the phage-resistance plasmids are cloned in E.coli. To identify the genes responsible for phage resistance, subcloning into appropriate phage-sensitive lactic acid streptococci has been started.

b. BACTERIOPHAGE ADSORPTION STUDIES - PHAGE RECEPTORS

Electronmicroscopic studies on the adsorption of virulent bacteriophages of mesophilic lactic acid streptococci revealed two different modes: Uniform adsorption of phages over the whole cell surface or adsorption on specific 'receptor spots' on the cell surface (1). Adsorption of phages P008 and P127 to protoplasts or isolated cell walls of S.lactis subsp. diacetylactis F7/2 and S.cremoris WG2 occured in the same manner as on intact cells (uniform adsorption of phage P008 on strain F7/2 and adsorption of phage P127 on specific domains of S.cremoris WG2, Fig. 1). Trypsin digestion or extraction with Triton X-100 of isolated cell walls did not change phage adsorption. Adsorption experiments in the presence of different lectins as Concanavalin A or wheat germ agglutinin, e.g., were performed with several phage-host systems. None of the lectins had an influence on phage adsorption. Further adsorption studies with subfractions of cell walls from different strains are in progress and may reveal the nature of phage receptors of lactic acid streptocci.

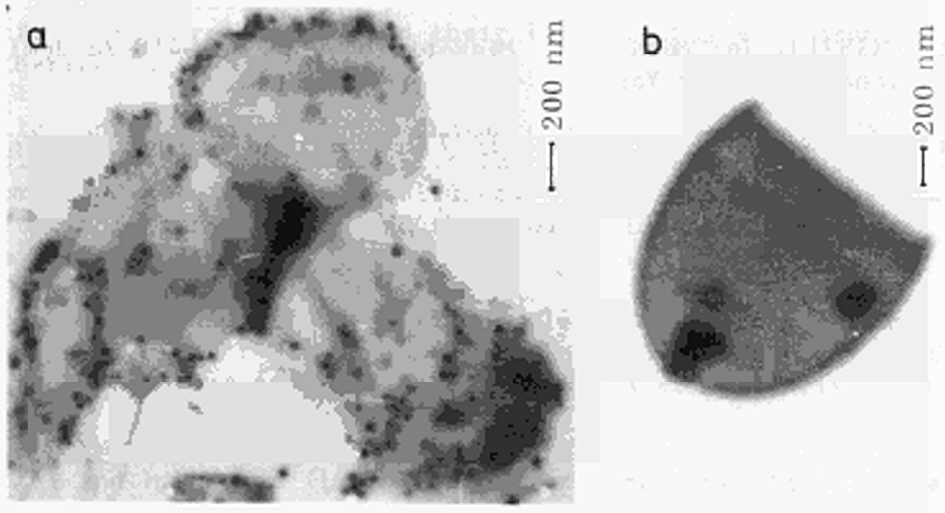


Fig. 1: Electron micrographs of phage P008 (a) and phage P127 (b) adsorbed on isolated cell walls of the host strains S.lactis subsp. diacetylactis F7/2 (a) and S.cremoris WG2 (b), respectively.

c. ISOLATION AND CHARACTERIZATION OF VIRULENT BACTERIOPHAGES OF STREPTOCOCCUS SALIVARIUS SUBSP. THERMOPHILUS (S.THERMOPHILUS)

97 virulent bacteriophages and more than 70 strains of S.salivarius subsp. thermophilus from various countries (Switzerland, France, F.R. Germany) were isolated and characterized according to their host ranges and by electron microscopy: The phages revealed distinct country-specific host ranges and - except for a few phage/host systems were unable to replicate on the host bacteria from other countries. All phages revealed isometric heads (45-60 nm) and long, non-contractile tails of various length (220-270 nm) and thickness (9-13 nm) with small base plates (Fig. 2). Therefore all phages belong into Bradley's group B.

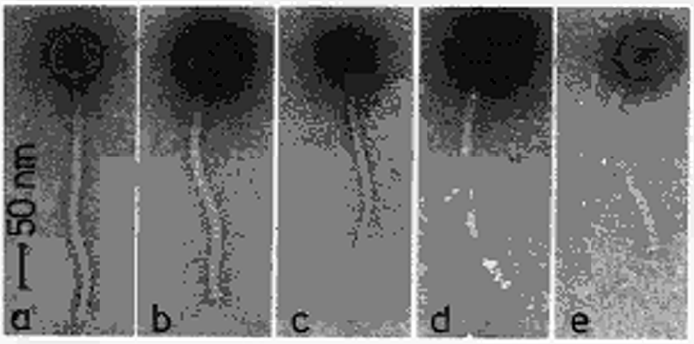


Fig. 2: Electron micrographs of negatively stained virulent bacteriophages active against S.salivarius subsp. thermophilus. (a) P50, (b) P54, (c) P76, (d) P103/40, (e) P13.

References:

(1) M. Teuber et al. (1986) CEC-BEP Final report, (E. Magnien, ed.) p. 539-546, Martinus Nijhoff Publishers, The Netherlands.

(2) A. Budde-Niekiel and M. Teuber (1987). Milchwissenschaft 47. In press.

(3) E. Work (1971). In: Methods of Microbiology, p. 361-418 Academic Press, London and New York.

(4) H. Neve, A. Geis and M. Teuber (1987). Submitted for publication.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

Publications:

V. Möller, A. Budde-Niekiel, J. Lembke and M. Teuber (1986) Charakterisierung von Bakteriophagen in einer Frischkäserei. Chem. Mikrobiol. Technol. Lebensm. 10: 90-92.

A. Wetzel, H. Neve, A. Geis and M. Teuber (1986) Transfer of plasmid-mediated phage resistance in lactic acid streptococci. Chem. Mikrobiol. Technol. Lebensm. 10: 86-89.

A. Budde-Niekiel and M. Teuber (1987) Electron microscopy of the adsorption of bacteriophages to lactic acid streptococci. Milchwiss. 47. In press.

H. Neve, A. Geis and M. Teuber (1987) Plasmid-encoded functions and phage-resistance of ropy lactic acid streptococci isolated from Scandinavian fermented milk. Submitted for publication.

M. Teuber (1987) The use of genetically-manipulated microorganisms in food: Opportunities and limitations. 4th Eur. Congr. Biotechnol. (ECB4). In press.

M. Teuber and M. Loof (1987) Genetic characterization of lactic streptococcal bacteriophages. In: J. J. Ferretti and R. Curtiss III (eds.). Streptococcal genetics. In press.

Short communications and internal reports:

H. Neve and M. Teuber (1986) Phage-resistance and plasmid-coded functions of ropy lactic acid streptococci. CEC-BAP meeting "Genetics of lactic acid streptococci and their bacteriophages", Haren, The Netherlands.

V. Möller and M. Teuber (1986) Characterization of highly bacteriophage resistant strains of mesophilic lactic acid streptococci isolated from mixed-strain starter cultures. CEC-BAP meeting "Genetics of lactic acid streptococci and their bacteriophages", Haren, The Netherlands.

A. Budde-Niekiel and M. Teuber (1986) Electronmicroscopic study on the adsorption of bacteriophages of lactic acid streptococci. CEC-BAP meeting "Genetics of lactic acid streptococci and their bacteriophages", Haren, The Netherlands.

A. Wetzel and M. Teuber (1986) Aspects of phage-resistance in lactic streptococci. CEC-BAP meeting "Genetics of lactic acid streptococci and their bacteriophages", Haren, The Netherlands.

H. Neve and M. Teuber (1986) Plasmid-coded functions of ropy lactic acid streptococci isolated from Finnish Vilii, Swedish sour milk and a German mixed-strain starter culture. ICODRL meeting on "Genetics of lactic acid bacteria", NIZO, Ede, The Netherlands.

A. Budde-Niekiel and M. Teuber (1986) Electronmicroscopic study on the adsorption of bacteriophages of lactic acid streptococci. ICODRL meeting on "Genetics of lactic acid bacteria", NIZO, Ede, The Netherlands.

V. Möller and M. Teuber (1986) Characterization of phage resistant strains isolated from mixed-strain starter cultures. ICODRL meeting on "Genetics of lactic acid bacteria", NIZO, Ede, The Netherlands.

H. Neve, A. Budde-Niekiel, A. Geis, and M. Teuber (1987) Bacteriophage receptors of lactic acid streptococci. Adsorption studies. CEC-BAP 1st Sectorial meeting on culture collection and genetic engineering of microorganisms, Ioannina, Greece.

A. Geis, H. Neve, and M. Teuber (1987) Plasmid dependent bacteriophage resistance in lactic acid streptococci: Isolation, characterization and cloning of plasmids. CEC-BAP 1st Sectorial meeting on culture collection and genetic engineering of microorganisms, Ioannina, Greece.

Ph.D.-thesis:

A. Budde-Niekiel (1987) Charakterisierung mesophiler Milchsäurestreptokokkenstämme eines Kulturenherstellers auf der Basis von Plasmidprofilen und Phagenresistenz. Universität Kiel.

Degree-thesis:

B. Luschei (1987) Elektronenoptische Charakterisierung virulenter Phagen von Streptococcus salivarius subsp. thermophilus. Universität Kiel

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff	Yes	
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

Exchange of materials:

Representative phages from our phage collection active against mesophilic lactic acid streptococci have been supplied to the University College, Cork, Ireland (C. Daly's group) for comparative study of phages from both countries.

Strains of lactic acid streptococci have been exchanged with the University of Groningen, The Netherlands (G. Venema's group) for cloning and transformation experiments.

G. Venema's group and W. de Vos' group (NIZO, Ede, The Netherlands) provided shuttle vectors for cloning in lactic acid streptococci, bacilli, and E. coli.

Exchange of staff:

A. Geis worked as a host scientist at the Institute of Molecular Genetics of the University of Groningen, The Netherlands (G. Venema's group) for training in cloning techniques with E. coli, bacilli, and lactic acid streptococci (CEC-training grant, senior level; 01.05.86 - 01.11.1986).

Drs. M. van Belcum from the University of Groningen, The Netherlands (G. Venema's group) visited Kiel to learn our technique for the preparative isolation of high molecular plasmid-DNA (November 1986).

Joint meetings:

23rd - 24th September, 1986. CEC-BAP Meeting in Haren, The Netherlands: "Genetics of lactic acid streptococci and their phages".

23rd - 25th April, 1987. CEC-BAP 1st Sectorial Meeting in Ioannina, Greece: "Culture collection and genetic engineering of microorganisms".

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: N. I. Z. O., Ede

Contract no.: BAP - 0011 - NL

Project leader: W.M. DE VOS

Scientific staff: P.A.J. Vos, I.J. Boerrigter, W.M. de Vos

Address: Netherlands Institute for Dairy Research (NIZO)
P.O. BOX 20
NL - 6710 BA EDE

Telephone no.: +31.8380.19013

Telex no.: 37205

Other contractual partners in the joint project:

C. Daly, University College (Cork)
M.J. Gasson, A. F. R. C. (Norwich)
M. Teuber, Bundesanstalt für Milchforschung
G. Venema, University of Groningen

Title of the research activity:

Genetic manipulation of lactic acid bacteria for improved dairy fermentations.

Key words:

Proteinase, Secretion, Sequence, Processing, Expression

Reporting period: April 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

1. Development of cloning vectors on the basis of streptococcal plasmids.
2. Expression of homologous and heterologous genes in streptococci.
3. Improvement of gene transfer methods.
4. Stabilization of fermentation properties.
5. Characterization and construction of bacteriophage resistant bacteria.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

1. Determination of the nucleotide sequence of the S. cremoris SK11 proteinase (prt) gene.
2. Study of the expression of the SK11 prt gene in lactic streptococci.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

INTRODUCTION

Proteinases of lactic streptococci are key enzymes in the proteolytic degradation of casein which occurs during growth in milk and the subsequent ripening of the fermented milk product, for the manufacture of which the organisms are used as starter.
Two main groups of lactic streptococcal proteinases may be distinguished on the basis of their caseinolytic specificities: (i) the PI proteinase present in strains S. cremoris HP and Wg2, and (ii) the PIII proteinase only found in S. cremoris AM1 and SK11 (1). The S. cremoris genes encoding both types of proteinase, derived from the bitter strain Wg2 and from the non-bitter strain SK11, have recently been cloned and expressed in S. lactis (2, 3).
In order to provide the basis for a further analysis of the specificity of the proteinases and their regulation, we determined the complete nucleotide sequence of the SK11 proteinase gene. In addition the possibilities of producing the non-bitter SK11 proteinase on a large scale were evaluated.

METHODOLOGY

DNA fragments of the S. cremoris SK11 proteinase (prt) gene were isolated from recombinant lambda EMBL3 phages expressing the proteinase (3), and subcloned into M13 vectors. Single stranded DNA was isolated from recombinant M13 phages and the nucleotide sequence of the DNA fragments was determined using a cascade sequencing

strategy employing oligonucleotide primers. Remaining gaps in the nucleotide sequence (e.g. at the border of two fragments) were sequenced directly on double-stranded DNA using specific oligonucleotide primers. The complete nucleotide sequence of the prt gene was deduced from the sequence of the various fragments and compared with the sequence of the prt gene of S. cremoris WG2 (4)

RESULTS AND DISCUSSION

NUCLEOTIDE SEQUENCE OF THE SK11 PROTEINASE GENE

The nucleotide sequence of the prt gene reveals the presence of a long open reading frame of 5886 nucleotides resulting in a polypeptide of 1962 amino acids with a molecular weight of 220 kilodalton (Figure 1). This open reading frame is preceeded by a consensus Shine-Dalgarno sequence and a highly d(A-T) rich promoter sequence containing several potential "-35" and "-10" domains. These transcription and translation signals are also functional in E. coli and B. subtilis.

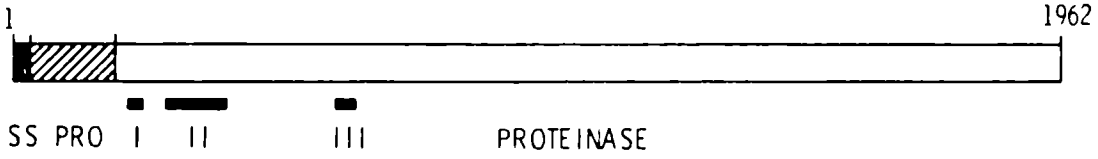


Figure 1: Organization of the SK11 proteinase (see text).

The proteinase contains a signal peptide-like N-terminal amino acid sequence with several possible processing sites for the release of a signal sequence. In addition, the N-terminal amino acid sequence of the isolated proteinase has been determined and compared with the proteinase amino acid sequence. The results provide evidence for the presence of a "pro" sequence preceeding the mature SK11 proteinase (see Fig. 1).
The proteinase exhibits extensive amino acid sequence homology with serine proteinases of the subtilisin family around the asparagine, histidine and serine residues found in the active centre of these proteinases (see domains I, II and III in Figure 1). These proteinases are also synthesized as pre-pro-proteins.
As expected the prt genes of S. cremoris SK11 and WG2 were found to be very similar. Apart from a small duplicated sequence of 60 amino acids found near the C-terminus of the SK11 proteinase the two enzymes differ only in a number of point mutations. There is an overall homology of 98,8 % on the nucleotide level and 97,8 % on the amino acid level in the coding sequence of both genes. Some of these mutations are conservative but others result in completely different amino acids and therefore may be involved in the different caseinolytic properties of both enzymes. Compared to the WG2 proteinase the SK11 enzyme contains 6 % more basic amino acids.
Obviously the differences in amino acid sequence of the SK11 and WG2 proteinases must be responsible for the different caseinolytic properties of both enzymes. The data obtained however, give no direct indication which regions are important with respect to cleavage

specificity. A logical way to proceed would be to investigate the cleavage specificity of hybrid proteinases derived from SK11 and WG2. This approach will be pursued in collaboration with BAP contractor G. Venema (Groningen, NL) in whose group the S. cremoris WG2 prt gene has been cloned and sequenced (2, 4).

EXPRESSION OF THE SK11 PROTEINASE GENE: SECRETION OF THE PROTEINASE ALLOWS LARGE SCALE PRODUCTION AND RECOVERY

Previously (3) we showed that S. lactis MG1363 harbouring the SK11 prt gene expressing plasmid pNZ511 synthesized a proteinase that (i) contained the same caseinolytic specificity as the proteinase isolated from S. cremoris SK11, and (ii) appeared to be secreted when the construct was grown in milk containing 0.5 % glucose. Although secretion of the non-bitter proteinase would allow its isolation and use on a large scale, two major barriers were encountered: (i) the lactose-deficiency of the host prevented growth in milk based media, and (ii) the isolation of the proteinase required a multistep procedure. Therefore, we first constructed a proteinase synthesizing strains capable of fermenting lactose by introducing the prt plasmid pNZ511 into S. lactis MG1820 (5), containing the small, stable lactose plasmid pMG820. The final construct MG1820 harbouring pNZ511 was able to grow in milk and milk based media without loss of pNZ511, even in the absence of selective pressure. Secondly, we tested the growth of the new construct and the production and secretion of the proteinase in a number of media. In a simple, clear whey-based medium strain MG1820 harbouring pNZ511 appeared to grow with a generation time of 60 min at 30 °C to high densities with the concommitant secretion of the proteinase, which could be isolated in a one step procedure.

CONCLUSION

In conclusion, we have characterized the SK11 proteinase gene on the nucleotide level and studied the processing of the gene product. Furthermore, we have constructed a strain which secretes the SK11 proteinase when grown on a cheap, industrially used medium. This allows the large scale production and isolation of the non-bitter proteinase in order to evaluate its use in the acceleration of cheese ripening and in the formation of natural flavours from the milk protein casein.

REFERENCES

(1) Visser, S. et al. (1986) Appl. Environ. Microbiol. 52, 1162-1166.
(2) Kok, J. et al. (1985) Appl. Environ. Micoribiol. 50, 94-101.
(3) De Vos (1986) in Biomolecular Engineering in the European Community. Final Report (E. Magnien, Ed.) pp 465-472.
(4) Kok, J. (1987) PhD Thesis, RU Groningen.
(5) Maeda & Gasson (1986) J. Gen. Microbiol. 132, 331-340.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

(1) Simons, A.F.M. & De Vos, W.M.
Gene Expressin in Lactic Streptococci
Proc. 4th European Congress on Biotechol. 1987, Volume I (Neyssel, Van der Meer and Luyben Eds.) pp 458-460.

(2) De Vos, W.M.
Gene Cloning and Expression in Lactic Streptococci
FEMS Microbiol. Rev. (1987) in press.

(3) De Vos, W.M.
Gene Cloning in Lactic Streptococci
Neth. Milk Dairy J. 40(1986)141-154.

(4) De Vos, W.M.
in Biomolecular Engineering in the European Community. Final Report, 1986 (E. Magnien, Ed.) pp 465-472.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	No
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Exchange of materials

Various plasmids and constructs containing the S. Lactis P-b-Gal gene with the BAP contractor M.J. Gasson, UK.

Joint experiments

- Sequence determination of the S. lactis P-b-Gal gene in collaboration with BAP contractor M.J. Gasson, UK.
- Construction of fusion proteins using the S. cremoris SK11 and WG2 proteinase genes in collaboration with contractor G. Venema, NL (projected).

Joint meetings

- September 1986: Contractors Meeting in Haren, NL, organized by contractor G. Venema.
- April 1987: Contractors Meeting Ioanninna, GR.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: University of Groningen

Contract no.: BAP - 0012 - NL

Project leader: G. VENEMA

Scientific staff: D. van der Lelie, A.J. Haandrikman, J.M.B.M. van der Vossen

Address:
University of Groningen
Institute of Genetics
Kerklaan 30
NL - 9751 NN HAREN

Telephone no.: +31.50.632993 or +31.50.632992

Telex no.:

Other contractual partners in the joint project:

C. Daly, University College (Cork)
M.J. Gasson, A. F. R. C. (Norwich)
M. Teuber, Bundesanstalt für Milchforschung (Kiel)
W.M. de Vos, N. I. Z. O. (Ede)

Title of the research activity:
Genetic manipulation of lactic acid bacteria for improved dairy fermentations.

Key words:
Lactic acid streptococci, Plasmid vectors, Proteinase, Gene cloning systems, Expression signals

Reporting period: August 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The objectives of the joint project are to improve mesophilic dairy starter cultures by means of genetic systems and recombinant DNA technologies.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The specific objectives for this period were to examine:
(a) the enzyme specificity of the proteinase specified by the gene of Streptococcus cremoris Wg2 carried by S. lactis, and to examine the coding specificity of the 6.5 kB HindIII fragment encoding the proteolytic system of S. cremoris Wg2 (1), (b) whether pAMβ1 can be used to mobilize the transfer of recombinant plasmids from Bacillus subtilis to lactic streptococci and whether protoplast fusions are suitable for plasmid transfer, (c) whether plasmid DNA can be made to integrate in the chromosome of lactic streptococci and (d) whether the B. subtilis serine protease can be expressed in S. lactis.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY

(a) The specificity of the proteinase produced by S. cremoris Wg2 and by S. lactis, carrying pGK500, which contains the 6.5 kB HindIII fragment, was determined from their casein digestion patterns. An internal, several 3' deletions and a frame-shift mutation were made in the 6.5 kB HindIII fragment. The effects of these mutations were examined in a cell free transcription-translation system and in B. subtilis by means of crossed immuno-electrophoresis (CIE).

(b) pAMβ1 was transferred from S. lactis MG3020 to B. subtilis by a plate mating procedure. Protoplasts were fused by means of PEG.

(c) Chromosomal DNA fragments were cloned in pHP12 (Em^R), unable to replicate in S. lactis, yielding pGI30 and pGI31, and in pLB2 (Cm^R), equally unable to replicate in S. lactis, yielding pGI6 and pGI7.

(d) The B. subtilis serine protease was cloned in pMGH10 (Km^R) in B. subtilis, and subsequently transferred to a proteinase deficient S. lactis strain. The S. lactis Km^R transformants were screened for rapid growth.

2. RESULTS

(a) The S. cremoris Wg2 proteinase gene, cloned in S. lactis had the same specificity as the proteinase obtained from the original host (Fig. 1). Deletion of the C-terminal 343 amino acids from the proteinase did not affect the specificity. In vitro transcription-translation studies showed that even 771 amino acids can be deleted from the proteinase without loss of proteolytic activity, but that a frame-shift mutation introduced in the 9th codon abolished the formation of the proteinase. CIE showed that the proteins A and B as identified in the proteolytic system of S. cremoris Wg2 (1) are both encoded by the proteinase gene.

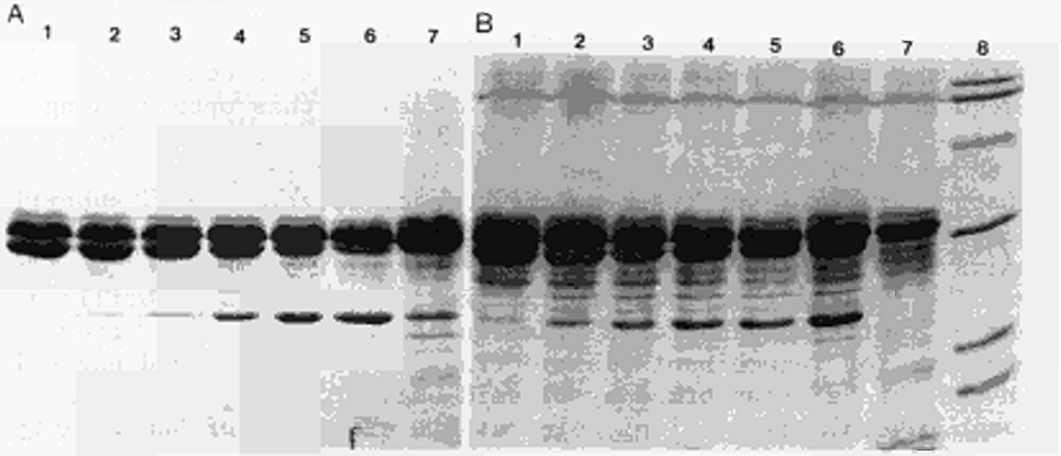


Figure 1. Substrate specificity of the proteinase activity released from intact cells of S. cremoris Wg2 (Prt^+) (panel A) and S. lactis (pGKV500) (panel B). Lanes 1, 2, 3, 4, 5, 6, and 7: 0h, 1h, 2h, 4h, 6h, 8h, and 24h of incubation, respectively. Lane 8: molecular weight standard: 12,300 (cytochrome C); 17,200 (myoglobin); 30,000 (carbonic anhydrase); 45,000 (ovalbumin); 66,250 (bovine serum albumin); 76-78,000 (ovotransferrin). Reaction products of whole casein were analysed on a 15% polyacrylamide gel.

(b) pAMβ1 was transferred from S. lactis to B. subtilis. Neither the transformed B. subtilis, nor S. lactis in which the plasmid, which had been cycled through B. subtilis was reintroduced, was capable of conjugally donating the plasmid to S. lactis. Apparently, transfer functions had been lost during the stay of the plasmid in B. subtilis. Support for this was obtained from restriction and Southern blot analyses, showing that pAMβ1 had suffered a major specific 10.6 kB deletion, and several minor, but also specific deletions in B. subtilis. Hybridization studies showed that the 10.6 kB segment of pAMβ1 had not been transposed to the B. subtilis chromosome. As an alternative for rapid plasmid transfer from B. subtilis to lactic acid bacteria, protoplast fusions were examined. Except for some S. cremoris strains, including Wg2, the plasmids were readily transferable from B. subtilis to S. lactis, S. lactis subsp. diacetylactis, Lactobacillus casei and S. cremoris F16.

(c) Both pGI30 and pGI31, carrying a fragment of chromosomal DNA from S. lactis IL1403 yielded Em^R transformants when transformed into S. lactis IL1403. The same applied to the production of Cm^R transformants when pGI6 and pGI7, carrying fragments of S. lactis MG1363 DNA, were transformed into S. lactis MG1363. No transformants were observed when the recipients were exposed to the parental vectors.
In minipreparations of DNA obtained from the transformants no free plasmid DNA could be detected. Southern blots analysis and hybrid-

ization of the chromosomal DNA from the transformed recipients showed a strong signal at the position of chromosomal DNA, much stronger than that of the non-transformed recipients.
These results suggest a Campbell-type integration of the plasmids and subsequent amplification of the integrated vector DNA.

(d) S. lactis containing the pre-pro serine protease B. subtilis gene showed no increased growth as compared to a protease-deficient S. lactis.

3. DISCUSSION

(a) Since the introduction of a frame-shift in the extreme 5' part of the proteinase gene abolished the synthesis of the proteinase, only one open reading frame specifies the proteinase. This is consistent with the sequence data of the gene (2). Nevertheless this open reading frame specifies two immunological distinct proteins. It is suggested that these result from autodigestion of the proteinase.

(b) The fact that pAMβ1 suffers a specific major deletion in B. subtilis, renders this plasmid unsuitable to conjugally transfer recombinant plasmids from B. subtilis to lactic streptococci by means of mobilization. The finding that in protoplast fusion between B. subtilis and a number of lactic acid bacteria plasmids can be transferred offers an alternative for recombinant plasmid transfer to lactic acid bacteria which can not be transformed by plasmid DNA.

(c) Although several observations point at the existance of Campbell-type integration of plasmid DNA into the chromosome of S. lactis, definite proof for this has to be derived from Southern blot analysis and hybridization analyses of restricted chromosomal DNA of the transformants. This may be difficult to achieve, because the chromosomal DNA of S. lactis seems to be particularly resistant to the digestion with a great number of restriction enzymes recognizing hexanucleotides.

(d) So far the expression of the B. subtilis serine protease in S. lactis was judged on only one criterium: rapid growth of the transformants on casein containing plates. The protease gene is preceded by a σ^{37} promoter which may not, or only weakly recognized by the S. lactis transcription system. Whether the protease is formed in S. lactis under this promoter requires more sensitive tests. If not produced, this promoter will be replaced by a lactic streptococcal-specific promoter, several of which are available (3, 4).

References

1. J. Kok, J.M. van Dijl, J.M.B.M. van der Vossen, G. Venema. Cloning and expression of a Streptococcus cremoris proteinase in Bacillus subtilis and Streptococcus lactis. Appl.Envir.Microbiol.50, 94-101 (1985).
2. J. Kok, C.J. Leenhouts, A.J. Haandrikman, A.M. Ledeboer and G. Venema. Nucleotide sequence of the gene for cell wall bound proteinase of Streptococcus cremoris Wg2. Appl.Environ.Microbiol. (submitted for publication).
3. J.M.B.M. van der Vossen, J. Kok and G. Venema. Construction of cloning, promoter-screening, and terminator-screening shuttle vectors for Bacillus subtilis and Streptococcus lactis. Appl.Environ.Microbiol.50, 540-542 (1985).
4. J.M.B.M. van der Vossen, D. van der Lelie and G. Venema. Isolation and characterization of Streptococcus cremoris Wg2-specific promoters. Appl.Environ.Microbiol. (accepted for publication).

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1. Publications in scientific journals

- G. Venema, J. Kok. Improving dairy starter cultures. Trends in Biotechn. 5, 144-148 (1987).
- J. Kok, C.J. Leenhouts, A.J. Haandrikman, A.M. Ledeboer, G. Venema. Nucleotide sequence of the gene for cell wall proteinase of Streptococcus cremoris Wg2. Appl.Environ.Microbiol. (submitted for publication).
- J. Kok, D. Hill, A.J. Haandrikman, M.J.B. de Reuver, H. Laan, G. Venema. The proteinases of lactic streptococci: a genetic approach. Appl. Environ.Microbiol. (submitted for publication).
- J.M.B.M. van der Vossen, D. van der Lelie, G. Venema. Isolation and characterization of Streptococcus cremoris-specific promoters. Appl.Environ.Microbiol. (accepted for publication).
- D. van der Lelie, G. Venema. Bacillus subtilis generates a major, specific deletion in pAMβ1. Appl.Environ.Microbiol. (accepted for publication).
- J. Hugenholtz, D. van Sinderen, J. Kok, W.N. Konings. Cell wall-associated proteases of Streptococcus cremoris Wg2. Appl.Environ.Microbiol.53, 853-859 (1987).

IV.2. Short communications

- J.M.B.M. van der Vossen, J. Kok, G. Venema. Isolation and characterization of lactic acid streptococcal promoters. Book of Abstracts, ICODRL Meeting on Genetics of lactic acid bacteria, Ede, September 25-26, 1986.
- J. Kok, J.M.B.M. van der Vossen, G. Venema. Development of a host vector system for lactic acid streptococci. Book of Abstracts, ICODRL Meeting on Genetics of lactic acid bacteria, Ede, September 25-26, 1986.
- S. Bron, P. Haima, L. Jannière, S.D. Ehrlich. Restriction and modification in Bacillus subtilis 168M. Target sites and effects on plasmid transformation. Book of Abstracts, CEC Contractants Meeting, BAP, Ioannina, April 23-25, 1987.
- J.M.B.M. van der Vossen, D. van der Lelie, G. Venema. Isolation and characterization of gene expression signals of Streptococcus cremoris. Book of Abstracts. CEC Contractants Meeting, BAP, Ioannina, April 23-25, 1987.
- J. Kok, C.J. Leenhouts, A.J. Haandrikman, A.M. Ledeboer, G. Venema. Nucleotide sequence of the gene for cell wall-bound proteinase of Streptococcus cremoris Wg2. Book of Abstracts, CEC Contractants Meeting, BAP, Ioannina, April 23-25, 1987.
- J. Kok, J.M.B.M. van der Vossen, C.J. Leenhouts, A.J. Haandrikman, A.M. Ledeboer, G. Venema. Development and use of a gene cloning system for lactic acid streptococci. Proc.4th Eur.Congr.Biotechn., Amsterdam, June 14-19, 1987, p 433-435.

IV.3. Doctorate thesis (Ph.D)

J. Kok. Development and use of a gene cloning system for lactic streptococci. University of Groningen (1987).

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Materials: Several plasmids and strains were both distributed to and received from the other four groups in the joint project.

Staff: Dr. A. Geis (Bundesanstalt für Milchforschung, Kiel) worked for 6 months in the Groningen Department of Genetics on the cloning of a proteinase gene in B. subtilis and Mr. C. Hill (University College Cork) for one year on the cloning and sequencing of promoters of a lactic streptococcal-specific transposon. Both periods were made possible by grants in the framework of BAP. Mr. M. van Belkum from the Groningen group was instructed in Kiel to isolate a bacteriocin-encoding plasmid. Prof. C. Daly (University College Cork) was a member of the PhD examining committee of Dr. J. Kok's thesis.

Experiments: The cooperation between the Kiel and Cork groups with the Groningen group was continued.

Meetings: A spontaneous contractants meeting of the five cooperating groups was held at Haren, University of Groningen, September 23-24, 1986.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Univ. degli Studi di Roma

Contract no.: BAP - 0061 - I

Project leader: L. FRONTALI

Scientific staff: C. Palleschi, C. Falcone, L. Fabiani, S. Francisci, M. Bianchi

Address: University of Rome
Dept. of Cell and Developmental Biology
Piazzale A. Moro
I - 00185 ROMA

Telephone no.: +39.6.4953950

Telex no.:

Other contractual partners in the joint project:

H. Fukuhara, Institut Curie (Orsay)
C.P. Hollenberg, Inst. für Mikrobiologie (Düsseldorf)

Title of the research activity:
Development of host-vector systems in dairy yeasts.

Key words:
Transformation, Gene vectors, Plasmids, Kluyveromyces, Dairy yeast

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Development of host-vector systems in industrial yeasts.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Development of efficient transformation systems for K.lactis and related species. Construction of expression vectors and cloning of heterologous genes.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

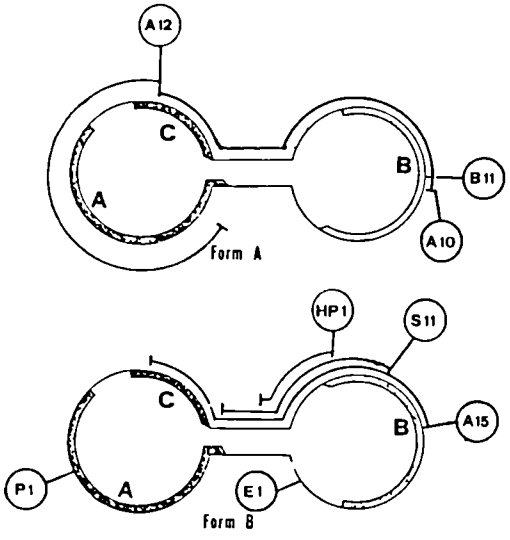
METHODOLOGY- Transformation of K.lactis was essentially as reported by Hinnen et al.(1) with the minor modifications described in reference (2). Construction of chimaeric plasmids was performed following standard procedures. Stability of plasmids in non selective media was measured by comparing the percentage of ura^+ cells in the population before and after growth for 7-8 generations in complete medium. Recombination between transforming chimaeric plasmids and resident plasmids was analyzed by transforming E.coli with DNA from single transformed K.lactis clones. Minipreparations of DNA from single bacterial transformants were then digested with an appropriate restriction enzyme and analyzed by gel electrophoresis.
For the isolation of alcohol dehydrogenase genes from K.lactis, a genomic library was constructed in the λL47 vector and screened with the ADH1 gene from S.cerevisiae.
RESULTS- 1) Transformation systems for K.lactis.
Efficient transformation of K.lactis has been achieved with various vectors.
ARS based vectors. Efficient transformation of both K.lactis and S.cerevi-

siae has been achieved with vectors containing the yeast integrative plasmid YIp5, ligated with sequences of K.lactis chromosomal DNA selected for capability of autonomous replication in S.cerevisiae.
We have found that some(but not all)of these sequences have an ARS function in both species and might therefore be used as shuttle vectors. Stability is similar to that expected for ARS based vectors (15%, after 6 generations in non selective media. These ARS elements are different from those previously reported by Das and Hollenberg (3). Sequencing and functional analysis are in progress to identify the sequences responsible for ARS function in the two yeast species.
pKD1 based vectors. The pKD1 plasmid, originally isolated from K.drosophilarum (4,5) has a genome organization analogous to that of the 2μ circle, although the two plasmids share little sequence homology and have different host specificities. The pKD1 plasmid was transferred to K.lactis where it can replicate stably. pKD1 based vectors were constructed by ligating either the entire plasmid sequence, or the region with the origin of replication, to the YIp5 plasmid containing the bacterial plasmid pBR322 and the yeast selectable marker URA 3. Vectors containing the bacterial transposon Tn 903 (conferring kanamycin resistance) as an alternative yeast selectable marker to URA 3 were also satisfactory.
2) Stability of transformants. Some of the vectors containing the entire pKD1 sequence (in which no genes had been disrupted) were highly stable. On the contrary, when the B and C genes were distrupted or absent, stability was very low; the stability of these vectors increased when a resident pKD1 plasmid was present.

A detailed analysis of stability in cir^+ and cir° strains has been performed,along with an analysis of recombination events involving transforming chimaeric plasmids and resident circles. Copy number has also been determined.
The main results are summarized in Table I.

TABLE I-Stability, recombination and structure of chimaeric plasmids

Plasmids	Stability(1) cir°	Stability(1) cir^+	Recombined plasmids/ number of analyzed molecules (2)
E1	97	99	2/25
P1	58	61	6/48
B11	18	82	5/51
A15	29	67	20/48
S11	37	60	17/88
HP1	39	44	0/96
A10	-	69	32/34
A12	-	93	25/26



(1) Stability was measured after 7-8 generations in non-selective medium and is expressed as the ratio between the final and the initial percentage of ura$^+$ cells.
(2) Number of plasmid molecules undergoing recombination with resident pKD1: plasmid DNAs were prepared from single transformed K.lactis clones and analyzed after amplification in E.coli.

3) Expression vectors

Expression of heterologous genes in K.lactis has been found to originate from several S.cerevisiae promoters. Expression of the URA 3 gene from S.cerevisiae present in the vectors mentioned above occurs from its own promoter. The promoter of the alcohol dehydrogenase gene from S.cerevisiae has been succesfully used for the expression of the bacterial Kanamycin resistance gene. Satisfactory expression of the same gene can also be driven by sequences upstream from the A,B and C genes of pKD1.

We have subsequently undertaken a search for a strong and regulated promoter of K.lactis. Two K.lactis genes coding for alcohol dehydrogenase were identified, cloned in E.coli and partially sequenced. Analysis of the alcohol dehydrogenase isozymes from K.lactis showed that in this species as in S.cerevisiae, they are modulated by the presence of glucose and of ethanol. Sequencing and analysis of the upstream sequences are in progress.

CONCLUSIONS AND PERSPECTIVES

Efficient transformation of K.lactis has been achieved with ARS based vectors and pKD1 based vectors. However,the latter constitute by far the most efficient and practical transformation system found to date for K.lactis. The potential industrial use of genetically engineered K.lactis will require the stability of transformants in non-selective media, high copy number of plasmids, and construction of good expression vectors. To this end, our present research is mainly focused on problems involving plasmid stability, such as identification of the stability locus, analysis of partitioning at cell division, copy number control,and the effects of recombination with resident plasmids.

On the other hand in the industrial utilization of genetically engineered microorganisms, the control of the time of expression of foreign genes is often desirable. Therefore, the isolation of a strong homologous and regulated promoter can prove very useful for the industrial utilization of transformed K.lactis. Our present research is aimed at the detailed investigation of the regulation of the K.lactis alcohol dehydrogenase system and the utilization of control and promoter regions of alcohol dehydrogenase genes for the construction of regulated expression vectors.

REFERENCES

1) Hinnen,A. et al. 1978 Proc.Nat.Acad.Sci.U.S.A. 75,1929-1933.
2) Bianchi,M.M. et al. 1987 Curr.Genetics 11 (in press).
3) Das,S. and Hollenberg,H. 1982 Curr.Genetics 6, 123-128.
4) Falcone,C. et al. 1986 Plasmid 15, 248-252.
5) Chen,X.J. et al. 1986 Nucl.Ac.Res. 14, 4471-4481.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.I - C.Falcone,M.Saliola,X.J.Chen,L.Frontali and H.Fukuhara: Analysis of a 1.6 um circular plasmid from the Yeast Kluyveromyces drosophilarum:structure and Molecular Dimorphism.Plasmid 15, 248-251, 1986.

- X.J.Chen,M.Saliola,C.Falcone,M.Bianchi and H.Fukuhara: Sequence organization of the circular plasmid pKD1 from the yeast Kluyveromyces drosophilarum.Nucleic Acid Research 14, 4471-4481, 1986.
- M.Bianchi,C.Falcone,X.J.Chen,M.Wesolowski-Louvel,L.Frontali and H.Fukuhara: Transformation of the yeast Kluyveromyces lactis by new vectors derived from the 1.6 um circular plasmid pKD1. Current Genetics 11,1987(in press).

IV.2- M.M.Bianchi, C.Falcone,X.J.Chen, M.Wesolowski-Louvel, L.Frontali and H.Fukuhara. A transformation system for the yeast K.lactis: new vectors derived from a 1.6 um circular plasmid. XIII Conference on yeast genetics and molecular biology. Banff 1986. Yeast 2, 26.

- Bianchi,M.M., Chen,X.J., Fabiani,L., Saliola,M., Falcone,C., Fukuhara,H. and Frontali,L.: pKD1 derived vectors and transformation of Kluyveromyces lactis. Commission of the European Communities, First BAP Meeting. Culture Collections and Genetic Engineering of Microorganisms, Ioannina 23-25 april 1987.

IV.3 - Vettori di clonazione e di espressione di geni eterologhi in lieviti e lieviti trasformati con tali vettori: Italian patent (27 march 1986) n. 47830A86.
Extension has been asked for USA, Japan and European countries.

IV.4 - Caratterizzazione di un plasmide isolato da Kluyveromyces drosophilarum e sua utilizzazione per la trasformazione di lieviti appartenenti a questo genere. Dr. Dhurata Frasheri, 1986 Degree thesis.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Exchange of plasmids and strains has been a constant practice among the laboratories involved in the joint proposal.

Dr.Michele Bianchi from Rome has been working in Orsay with a fellowship from Rome University.Joint experiments have also been constantly performed, e.g. sequencing of pKD1 plasmid was performed part in Rome part in Orsay. Collaboratorion with the laboratory in Düsseldorf began with a meeting last December and meetings between Rome and Orsay take place regularly three or four times per year.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Institut Curie, Paris Contract no.: BAP - 0026 - F

Project leader: H. FUKUHARA
Scientific staff: X.J. Chen, C. Wilson, M. Wésolowski-Louvel, C. Tanguy, A. Ragnini

Address:
Institut Curie
Section de Biologie
Bâtiment 110
Centre universitaire
F - 91405 ORSAY

Telephone no.: +33.1.69076467

Telex no.: FACORS 692166

Other contractual partners in the joint project:

L. Frontali, University of Roma
C.P. Hollenberg, Institut für Mikrobiologie (Düsseldorf)

Title of the research activity:
Development of host-vector systems in dairy yeasts.

Key words:

Yeast, Kluyveromyces lactis, Plasmid, pKD1, Transformation

Reporting period:
July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

1. Establishment of efficient transformation systems for Kluyveromyces in view of valorisation of the yeast species potentially useful in dairy industries.

2. Molecular and genetic characterization of the vector system derived from the plasmid pKD1.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

1. Development of pKD1-derived vectors carrying various yeast selection markers.
2. Search for new transcription promotors for the expression of foreign genes in Kluyveromyces lactis.
3. Development of gene fusion systems for the study of gene regulation in Kluyveromyces lactis.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Methodology

1. K.lactis promotors

Equivalent genes from S.cerevisiae and from K.lactis are often regulated quite differently. In order to allow expression of foreign genes in K.lactis, several K.lactis promotors have been studied. Two promotor-less genes, the lacZ'gene of E.coli and our Kan' gene from Tn903, were placed next to several putative promotor sequences isolated from K.lactis. These sequences were (i) the inverted terminal repeat of the kl killer DNA, (ii) the 5' leader sequences of the plasmid pKD1 genes and (iii) a few known promotors from S.cerevisiae. All of them were found to be functional when placed in appropriate configuration. In particular, the inverted terminal repeat (about 200 bp) was very active in both orientations.

2. Gene fusion systems for K.lactis.

In order to search for regulated or strong promotors in K.lactis, N-terminus-deleted lacZ' or Kan' were placed under a polylinker in three reading phases, so that a bank DNA carrying a promotor and a 5' coding sequence could be cloned by positive selection. Preliminary tests have shown that these systems were operational and could be used for the study of gene regulation.

Results and Discussion

1. Transformation system

The two technical improvements discussed in the methodology section are now considered to be almost completed. Combined with these promotors, k1 toxin N-terminal presequence is being introduced into the vectors in order to obtain secretion vectors.

2. Actual gene cloning using the pKD1-derived transformation system.

The killer toxin of K.lactis is produced by the linear plasmids k1 and k2. The expression of the killer system is under the control of nuclear genes. The mutation of one of such genes, KEX1, leads to a non-killer phenotype despite the presence of intact killer plasmids. In order to understand the function of the KEX1 gene, we attempted to isolate it by functional complementation using K.lactis DNA bank. The bank was constructed by inserting K.lactis DNA fragments into a pKD1-derived plasmid KEp6. When the kex1 mutant was mixed with the bank DNA, a few transformants were obtained which showed killer phenotype. The analysis of the cloned DNA revealed that KEX1 gene was functionally closely related with the KEX 2 endopeptidase gene of S.cerevisiae. It thus appears that the KEX1 product is required for the correcct processing of the toxin protein precursor. This result demonstrated that pKD1-based vectors can be practically used for gene cloning in K.lactis.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

Publications in scienific journals

Analysis of a new circular plasmid, pKD1, from Kluyveromyces drosophilarum: Structure and molecular dimorphism. Falcone,C., Saliola,M., Chen,X.J., Bianchi,M.M., Frontali,L. and Fukuhara,H. Plasmid, 15, 248-252 (1986)

Sequence organization of the circular plasmid pKD1 from the yeast Kluyveromyces drosophilarum. Chen,X.J., Saliola,M., Falcone,C., Bianchi,M.M. and Fukuhara,H. Nucl.Acids Res. 14, 4471-4481 (1986)

Transformation of the yeast Kluyveromyces lactis. Bianchi,M.M., Falcone,C., Chen,X.J., Wésolowski-Louvel,M., Frontali,L. and Fukuhara,H. Current Genetics 11, (1987) in the press.

Short communications

A transformation system for the yeast Kluyveromyces lactis: New vectors derived from a 1.6 um circular plasmid. Bianchi,M.M., Falcone,C., Chen,X.J., Wésolowski-Louvel,M., Frontali,L. and Fukuhara,H. 13th International Conference on Yeast Genetics and Molecular Biology, August 31-September 5, 1986, Banff, Canada. Yeast 2, S27 (1986) special issue.

A transformation system for the yeast Kluyveromyces lactis: New vectors derived from a 1.6 um circular plasmid. Bianchi,M.M., Falcone,C., Chen,X.J., Wésolowski-Louvel,M., Frontali,L. and Fukuhara,H. In: "Physiological and Genetic Modulation of Product Formation". European Federation of Biotechnology, 8-10 May 1986, Como, Italy. Proceedings.

pKD1-derived vectors and transformation of Kluyveromyces lactis. Bianchi,M.M., Chen,X.J., Fabiani,L., Saliola,M., Falcone,C., Fukuhara,H. and Frontali,L. 1st Sectorial Meeting on Culture Collection and Genetic

Engineering of Microorganisms. Commission of the European Communities. Ioannina, 23-26 April 1987.

Gene cloning in Kluyveromyces lactis by pKD1-derived vectors: Isolation of a chromosomal gene required for the production of the linear DNA-coded killer toxin. Wésolowski-Louvel,M., Tanguy,C. and Fukuhara,H. 1st Sectorial Meeting on Culture collection and Genetic Enginering of Microorganisms. Commission of the European Communities. Ioannina, 23-26 April 1987.

Patent application

Vecteur de clonage et d'expression d'un gène hétérologue dans la levure. Falcone,C., Frontali,L. and Fukuhara,H. Deposit for italian patent under 47830A86, March 26 1986, and extension to Europe, USA and Japan, March 1987.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Exchange with the University of Rome

Dr.M.Bianchi of the University of Rome has been working in our laboratory for two years for the previous BEP program. After this post-doctral training, he returned to Rome to continue to work on K.lactis transformation in the framework of the present BAP program. Another collaborator of Pr.Frontali, Dr.A.Ragnini now joined our group to work also on Kluyveromyces lactis. The project leaders meet regularly to coordinate the program. All biological materials are used in common.

Exchange with the University of Düsseldorf

The Düsseldorf group led by Dr.Hollenberg joined the project only recently. To start our collaboration, the pKD1 vector system has been sent to Düsseldorf to be exploited in the study of gene expression in K.lactis.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: John Innes Inst., Norwich

Contract no.: BAP - 0063 - UK

Project leader: R. CASEY

Scientific staff: P. Ealing

Address: John Innes Institute
Colney Lane
UK - NORWICH NR4 7UH

Telephone no.: +45.603.52571

Telex no.: 975122 JIINOR G

Other contractual partners in the joint project:

D. von Wettstein, Carlsberg Laboratory (Copenhagen)
A. Petersen, Danish Distilleries Ltd (Copenhagen)

Title of the research activity:

Construction of a baker's yeast secreting legume lipoxygenase during production of bread dough.

Key words:

Lipoxygenase, Pisum sativum, Bread-making, Baker's yeast, Legume

Reporting period: December 1986 - July 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The overall aim of the project is the genetic engineering of an industrial microorganism; specifically it is to construct strains of baker's yeast that express and secrete the enzyme lipoxygenase (E.C.1.13.11.2), using sequences derived from the pea (Pisum sativum). The intention is that such strains would be used in bread-making to impart favourable rheological properties to the dough; it is also expected that the resulting bread would have a very white crumb, as a consequence of the bleaching of the pigments of wheat flour by the enzyme.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Isolation of a full-length pea seed lipoxygenase cDNA and its characterization by restriction mapping and terminal sequence determination prior to complete sequencing and introduction of the sequence into bakers' yeast.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

see separate sheet:

1. METHODOLOGY:

PolyA+ RNA was prepared from developing seeds of P. sativum cv. 'Birte', and size fractionated on denaturing sucrose gradients (Casey et al., 1985); lipoxygenase mRNAs were identified by cell-free translation/immunoprecipitation (Fig. 1).

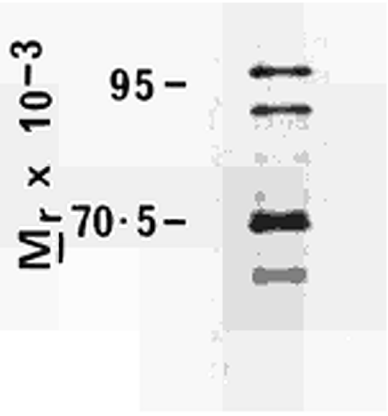


Fig. 1 SDS-gel analysis of the cell-free translation products of size-fractionated poly A^+ RNA from P. sativum cv. 'Birte'. The two upper bands, of M_r ~90,000-95,000, are immunoprecipitable with antiserum to soybean lipoxygenase (Casey et al., 1985).

Fractions containing RNA of approx. 2-3kb were converted to ds cDNA (Gubler and Hoffman, 1983) and cloned directly into the SmaI site of pUC19. Recombinants containing inserts of lipoxygenase cDNA were selected by colony hybridization using the cDNA insert from the partial lipoxygenase cDNA clone pCD45 (Casey et al., 1985). Full-length clones were analyzed by restriction mapping, and restriction fragments at the 5' and 3' ends were sequenced by the dideoxy termination method (Sanger et al., 1977). Orientation relative to the message sense DNA strand was determined by supercoiled plasmid sequencing (Chen and Seeburg, 1985). Hybrid selection and in vitro translation in rabbit reticulocyte lysates were carried out as previously described (Domoney and Casey, 1983).

2. RESULTS

Fifty lipoxygenase positive cDNA clones were screened from a library of 1300 clones; homology with pCD45 was assessed by varying the hybridization stringency. Two clones hybridized at a lower stringency (55°C/2xSSC) than the majority (65°C/0.1xSSC); fourteen of these clones had inserts of 2.5-3.0kb, which is close to the expected size of the mRNA corresponding to pCD45. Three cDNA clones selected at the higher

stringency (pPE67, pPE551 and pPE1036) and one at the lower stringency (pPE923), were all >2.9kb, and were further analyzed by restriction mapping and sequencing. The clone pPE67 has 100bp less 5' sequence than pPE551 or pPE1036, which appear to be identical; pPE923 is also ~100bp shorter at the 5' end, but has a distinctly different restriction map (Fig. 2), although the positions of certain restriction sites remain unaltered.

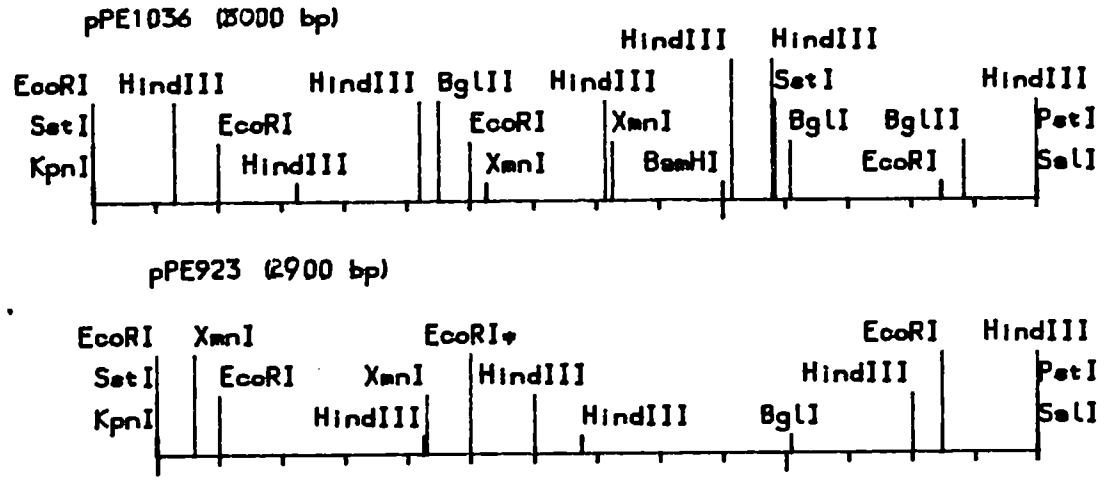


Fig. 2. Restriction maps of full length cDNA clones pPE923 and pPE1036. The orientation of the cDNA inserts within these pUC19 clones was determined by plasmid sequencing and is shown in Fig. 3.

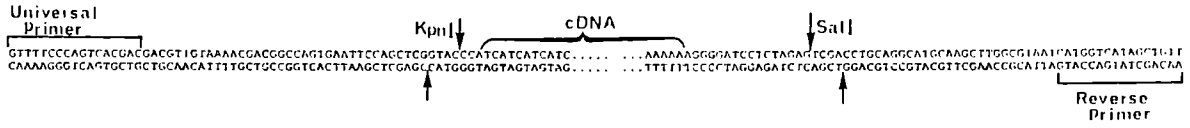


Fig. 3. Orientation of the cDNA insert of pPE1036 in the polylinker of pUC19.

The 5' sequence of both pPE923 and pPE1036 was obtained; pPE1036 starts with an unusual repetitive sequence, then is followed by two in frame methionine codons (ATG). The sequence of pPE923 has a deduced amino acid sequence that is 73% homologous with the translated open reading frame of pPE1036 (Fig. 4).

```
                .         .         .         .         .
39 VVLMRKNVLDIN.....SLTTVGGVIGQQGFDILGSTVDNLTAFLGRSVSL 83
   ********** *          * ***  *  * *** * ***********
 1 VVLMRKNVLDFNTIVSIGGGNVHGVIDSGINIIGSTLDGLTAFLGRSVSL 50
                .         .         .
84 QLISATKPDATGKGKLGKATFLEGIISSLPTLGA 117
   ******* *  **** ** *****   *******
51 QLISATKSDRNGKGKEGKDTFLEGVLASLPTLGA 84
```

Fig. 4. Alignment of the translated open reading frames at the 5' ends of the cDNA clones pPE923 and pPE1036.

The 3' ends of each of these clones have also been sequenced but display no homology. There are however several potential polyadenylation signals in each sequence. Experiments using pPE923 and pPE1036 for mRNA hybrid selection followed by in vitro translation indicate that pPE923 selects the mRNA corresponding to the lower molecular weight lipoxygenase band in Fig. 1, whereas pPE1036 selects the mRNA for the higher molecular weight band.

3. DISCUSSION

Pea lipoxygenase cDNA clones, very close to full length by the criterion of insert size, were obtained from fractionated, developing seed mRNA. There appear to be two classes of cDNA on the basis of probe homology and hybrid-selection/in vitro translation experiments. In view of the suggested absence of type 1 lipoxygenase from pea seeds (Yoon and Klein, 1979, Vernooy-Gerritsen et al., 1984), the two lipoxygenase polypeptides that are immunoprecipitated (Fig. 1) may correspond to lipoxygenases 2 and 3, although not necessarily in that order. Preliminary DNA sequencing of the two classes of cDNA clones have shown that they are indeed homologous variants of each other. Further sequencing will be undertaken, and genomic clones sought. One of the cDNA clones (pPE1036) has been given to our collaborators at the Carlsberg Laboratories in Copenhagen, where they are currently attempting to express the sequence in a yeast vector construct, under the direction of the maize ADH1 promotor.

References

Casey, R., Domoney, C. and N. C. Nielsen. (1985) Biochem. J., 232, 79-85.

Domoney, C. and R. Casey. (1983) Planta, 159, 446-453.

Gubler, U. and B.J. Hoffmann (1983) Gene, 25, 263-269.

Chen, E.Y. and P.H. Seeburg (1985) DNA, 4, 165-170.

Sanger, F., Nicklen, S., and A. R. Coulson (1977) Proc. Nat. Acad. Sci. USA. 74, 5463-5467.

Vernooy-Gerritsen, M., Leunissen, J.L.M., Veldink, G.A. and J.F.G. Vliegenthart (1984) Plant Physiol, 76, 1070-1079.

Yoon, S. and B.P. Klein (1979) J. Agric. Food Chem. 27, 955-962.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

1. None
2. Contractors' meeting report, Ioannina, 1987
3. None
4. None

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

The full length cDNA clones have been given to our collaborators at the Carlsberg laboratory for introduction into bakers' yeast.
Dr. Kristine Keiding from the Carlsberg lab. has visited the John Innes and Dr. Paul Ealing from Norwich has visited Copenhagen each for several days, during which time extensive discussion meetings took place. The nature of the project is that of a continuing joint experiment between John Innes and Carlsberg labs.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Carlsberg Lab. Copenhagen
Contract no.: BAP - 0025 - DK

Project leader: D. VON WETTSTEIN
Scientific staff: Cand. scient. Kristine Keiding
cand. scient. Bjørn Eggert Christensen

Address: Carlsberg Laboratory
Department of Physiology
Gamle Carlsberg Vej 10
DK - 2500 COPENHAGEN Valby

Telephone no.: +45.1.221022 EXT.5225

Telex no.: 15434

Other contractual partners in the joint project:

R. Casey, John Innes Institute (Norwich)
A. Petersen, Danish Distilleries Ltd (Copenhagen)

Title of the research activity:
Construction of a baker's yeast secreting legume lipoxygenase during production of bread dough.

Key words:
Baker's yeast, Lipoxygenase, Wheat flour, Dough

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

It is the aim of the present project to construct by genetic engineering yeast strains which during the production of dough secrete the enzyme lipoxygenase (E.C.1.13.11.12) from Pisum sativum (pea) and thereby bleach the yellow pigments of the wheat flour to give a bread with very white crumb, simultaneously improving the mixing properties of the dough ingredients and the strength of the dough. The yeast strains to be constructed will allow to test the correctness of the current hypotheses as to how lipoxygenases from added soybean flour influence the characteristics and flavour of the dough.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Results obtained during the period:

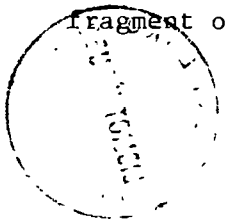
The project requires the isolation of full length cDNA clones for the three isozymes of lipoxygenase from pea. This work is in progress under a separate BAP contract with Dr. R. Casey, John Innes Institute, Norwich. It requires further the construction of yeast-E.coli shuttle vectors for genetic transformation of the production strain DDSF102 of the Danish Distilleries Ltd., a tetraploid strain which has been genetically characterized by conventional tetrad analysis in combination with Southern type molecular hybridizations using available probes from genetic standard strains.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Two transformation systems have been set up with the aim to introduce and integrate into the chromosomes of the baking strain DDSF102 the genes encoding lipoxygenase from pea. In the first system auxotrophic mutants are used, while the second system can be used on wild type strains. A gene encoding α-amylase from the mouse is used as a reporter gene and will in due time be replaced with genes encoding lipoxygenase. Laboratory yeast strains transformed with plasmids containing the α-amylase gene, transcribe the gene, translate it into the enzyme and excrete the enzyme into the medium. Using 0.4% soluble starch in the medium and potassium iodide as a stain permits detection of α-amylase secretion from colonies, in that these are surrounded by a clear zone of degraded starch in the dark iodine stained background.

(1) Selection of transformants by acquired prototrophy. Transformants are directly selectable if the recipient strain contains an auxotrophic recessive mutation and the plasmid contains the wild type dominant allele, resulting in prototrophy upon transformation. In addition to the dominant S.cerevisiae marker the vector may carry an origin of replication from yeast and a bacterial part.

One of the plasmids employed in this project, pMS12 (Thomsen 1983), is shown in Figure 1. TRP1 is the selectable gene and the 2 kb EcoRI-PstI fragment of the 2 micron (2μ) plasmid from yeast (in b form) contains an



origin of replication. The reporter cDNA gene for α-amylase from mouse is inserted behind the yeast promoter ADH1 (alcohol dehydrogenase I). In the plasmid pMS12(ΔSphI) the small SphI-SphI fragment of pMS12 is deleted.

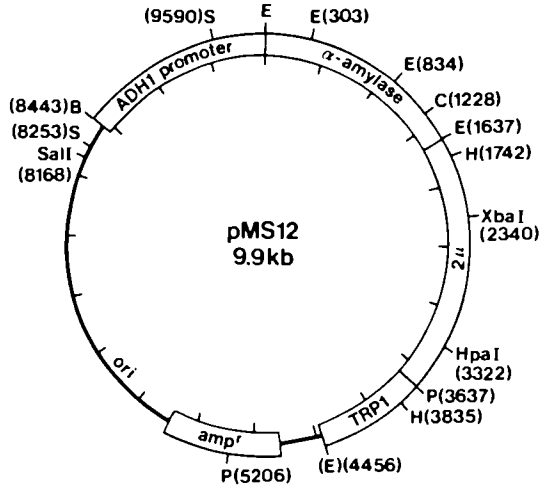


Figure 1. Restriction endonuclease site map of the plasmid pMS12 (Thomsen 1983). ADH1 promoter, 2μ and TRP1 are all from S. cerevisiae. The line including amp^r (ampicillin resistance) and ori (origin of replication) is from pBR322. B - BamHI, C - ClaI, E - EcoRI, H - HindIII, P - PstI, S - SphI, () - former site, destroyed during construction. Positions of sites are shown in bp. Marks are drawn every 500 bp.

The transformation was performed by the procedure of Ito et al. (1983) using LiOAc: A loopful of cells was inoculated in 10 ml YPD and incubated at 30°C over-night. The culture was washed in water, afterwards in TE + 0.1 M LiOAc before resuspending the cells in TE + 0.1 M LiOAc to a final concentration of 10^8 cells x ml^{-1}. (10 x TE stock solution: 0.1 M Tris; 0.01 M EDTA; pH 8.0.) 5 μl of salmon sperm DNA (10 mg x ml^{-1}), 10 μg of plasmid DNA and 0.7 ml 40% PEG (polyethylene glycol) were added to 0.1 ml of this suspension and mixed gently. Incubation was performed with careful but gentle shaking for 60 min at 30°C. After 5 min at 42°C, the mixture was centrifuged for about 3 sec in the Eppendorf centrifuge and the supernatant removed with a pipette. Cells resuspended in water were spread on selective medium, i.e. plates without tryptophan. From strain KK7, a stable trp1 mutant of $A9_1$ (a spore-derived clone of strain DDSF102), 16 transformants per μg of pMS12 were obtained, whereas the laboratory strain DB 746 (MATα his3Δ1 leu2-3 leu2-111 ura3-52 trp1-289) gave 100 transformants per μg of pMS12.

After 3 to 7 days of growth, transformants were picked up and screened for the ability to secrete active amylase to the surrounding medium. Consistently more amylase was secreted from transformants of the industrial yeast derivative KK7 than from those of the laboratory strain. The reason for this important observation is not yet known.

The stability of tryptophan prototrophy was tested by growth for 17 to 20 generations in non-selective medium, i.e. YPD, followed by plating on YPD and replica plating onto SD and YPD + 0.4% starch. For transformants with pMS12, about 20% of the colonies have lost both tryptophan prototrophy and amylase secretion. The corresponding value for pMS12(ΔSphI) is 7%. In other transformants of industrial yeast strains and their derivatives, plasmid loss frequencies of 50% have been reported under similar conditions.

In attempts to obtain higher stability through integration of the plasmid in the genome of KK7, pMS12 was linearized through partial digestion with restriction endonuclease HindIII before transformation. The free ends obtained in the TRP1 gene are expected to stimulate integration of pMS12 into the trp1 gene of KK7 (Orr-Weaver et al. 1983). By this procedure the frequency of transformants was lowered by a factor of 2. Stability tests revealed out of 12 transformants one strain, e7, to have no loss of tryptophan prototrophy or amylase secretion after 60 generations in non-selective medium. Upon gel electrophoresis of restricted DNA, Southern transfer and

hybridization to a radioactive probe (1.6 kb PstI fragment of pMS12) it was demonstrated that e7 has pMS12 integrated in the trp1 gene.

Through integration of a plasmid containing a sequence homologous to one in the transformed strain partial stabilization is obtained. By subsequent excision complete stabilization for the homologous sequence can be obtained. Also chromosome breakage and chromosome loss has been reported (Falco et al. 1982, Holmberg at al. 1982) due to unequal crossing-over between chromatids containing 2μ sequences during mitosis. This will result in lower viability, just as crossing-over between integrated and unintegrated 2μ may do.

In e7, some of the isolated clones show long-term stability in both tryptophan prototrophy and amylase secretion. Other clones are stable in tryptophan prototrophy, unstable in amylase secretion and exhibit a reduced growth rate compared to KK7, whether grown in selective or unselective conditions. A plasmid with a dominant yeast gene has thus been introduced into the meiotic progeny of the industrial yeast strain after induction of the relevant auxotrophic mutation. The resulting transformants should be crossed to other meiotic segregants to reestablish characteristics close to that of the original industrial strain. Hybridization between e7 and several spore-derived strains with mating type a has therefore been performed. The hybrids obtained so far were not stable and displayed a variety of amylase expression levels. Since the hybrids are heterozygous for the integrated plasmid this apparently leads to viable products with deletion of the amylase gene in some cells as a result of chromosome breakage.

(2) Use of integration vector with drug resistance as selective marker.
While selection of antibiotic-resistant transformants is commonplace in bacterial transformation systems, such selection has only recently become available for yeast. S. cerevisiae is sensitive to geneticin (G418 sulphate, Jimenez and Davies 1980) which is an aminoglycoside antibiotic structurally related to kanamycin and gentamycin. The transposon Tn903 codes for resistance to G418.

The integration vector pDY3 (Figure 2) was kindly provided by R. Yocum and R. Daves, Biotechnica International, Inc., Cambridge, MA, USA. It is constructed to introduce a desired gene in yeast and jettison all vector sequences resulting in stable transformants.

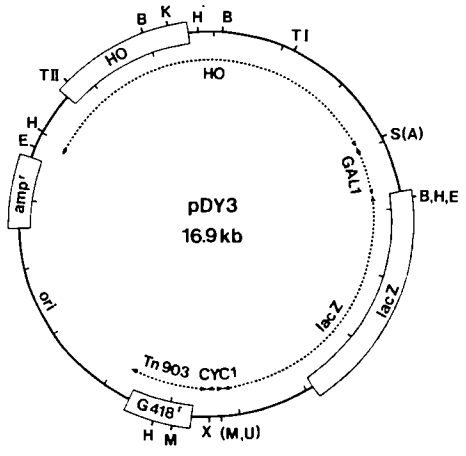


Figure 2. Partial restriction endonuclease site map of the plasmid pDY3 (Yocum 1984). The HO region, GAL1 (the galactose 1 promoter) and CYC1 (iso-1-cytochrome c promoter) are all from S. cerevisiae. The lacZ (β-galactosidase) is from E.coli, Tn903 is the gene bacterial transposon 903 coding for resistance to G418. The rest of the plasmid is from pBR322. A - XbaI, B - BamHI, E - EcoRI, H - HindIII, K - KpnI, M - SmaI, S - SalI, TI - SstI, TII - SstII, U - StuI, X - XhoI, () - former site, destroyed during construction. The marks on the plasmid indicate 1000 bp.

The transposon Tn903 is combined with amp^r and ori from pBR322 and lacZ (from E. coli) which is placed under yeast GAL control. Furthermore, the vector contains a sequence which is homologous with a sequence (a "target" sequence) of chromosome IV of S. cerevisiae, namely the HO gene and adjacent

regions. By insertion of the desired gene in the HO region followed by linearization with KpnI and transformation, transformants appearing on G418 containing medium will contain the desired gene as well as the rest of the vector integrated in chromosome IV, see Figure 3. In addition to endowing the transformants with resistance to G418, introduction of the vector also results in production of β-galactosidase, when the yeast is grown with galactose as carbon source. On medium containing X-gal this results in blue colonies. With a certain frequency, crossing over between homologous sequences on the modified chromosome IV results in looping out of vector sequences, leaving behind only the desired gene. By growing transformants for 20 - 60 generations in unselective medium and subsequent plating on X-gal medium, several crossing-over events will reveal themselves as white colonies. Screening of these for the presence of the desired gene and the absence of vector sequences will reveal the clones wanted.

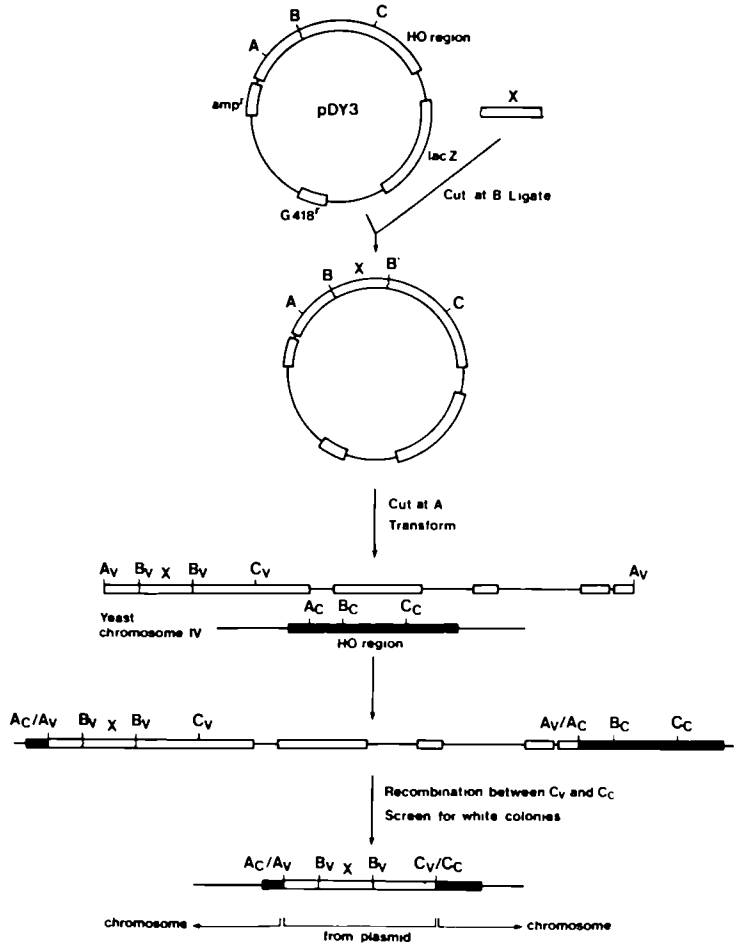


Figure 3. Strategy for integration of a desired gene, X, in chromosome IV of S. cerevisiae by the vector pDY3.

In order to determine the transformability of several strains relevant to this project pDY3 was linearized with KpnI prior to the transformation to facilitate integration into the HO gene of the recipient strain. After transformation the cells were suspended in 400 µl YPD and shaken at 30°C for 3 hours before plating on the selective medium (YPD containing G418). During incubation in YPD, the plasmid could be integrated and Tn903 could be expressed. The transformation frequencies are shown in Table 1. It should be mentioned that addition of 1% $(NH_4)_2SO_4$, 0.2% $CaCl_2$ or 0.2% $MgSO_4$ to the medium results in resistance of yeast to G418 up to 2 mg x ml^{-1}. Thus, if the use of synthetic media is needed, the nitrogen source should be one or several amino acids.

Colonies with expression and secretion of β-galactosidase appeared as blue colonies on X-gal medium due to the conversion of 5-bromo-4-chloro-3-indoxyl-β-D-galactoside (X-gal, colourless, Sigma No. B4252) to D-galactose and 5-bromo-4-chloro-indole-3-ol. Differences in the behaviour were found for transformants of different strains as well as transformants of the same strain in the intensity of the blue colour. This could be due to differences in the number of β-galactosidase genes integrated. Experiments are now in progress to integrate the α-amylase gene into the HO region of chromosome IV. In principle we expect to stably integrate 1 copy of the amylase gene in each of the four chromosomes IV of the industrial yeast. This should result in a high and stable enzyme production.

Table 1. Transformation frequency upon transformation with pDY3.

Strain transformed	Concentration of G418 for selection of transformants mg x ml^{-1}	Frequency of transformants No. x μg^{-1} DNA
DDSF102	0.5	200
A_{91}[a]	1.0	10
KK7	0.5	120
A_6[a]	1.0	50
KK194[b]	1.0	150

[a] meiotic progeny of strain DDSF102; [b] diploid laboratory strain

Constructions by which the ADH1 promoter and other strong promoters for expression of the lipoxygenase gene are inserted into the pDY3 vector are in progress.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

Keiding, K. Characterization and transformation of a distiller's yeast. Abstr. 13th Int. Meeting on Yeast Genetics & Molecular Biology. Aug. 31 - Sept. 6, 1986. Banff, Canada.

Keiding, K. Genetic analysis and transformation of distiller's yeast. In: H.F. Linskens and J.F. Jackson (eds.) Modern Methods of Plant Analysis. Springer Verlag, Berlin, Heidelberg, New York (in press).

Christensen, B.E. and Eriksen, H. Screening and testing new distillers' yeasts for their potential in molasses ethanol fermentation. In: H.F. Linskens and J.F. Jackson (eds.) Modern Methods of Plant Analysis. Springer Verlag, Berlin, Heidelberg, New York (in press).

Keiding, K., von Wettstein, D. and Petersen, Aa. Construction of a baker's yeast secreting legume lipoxygenase during production of bread dough. Abstract BAP Meeting, Ioannina, Greece.

TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

A cDNA clone for lipoxygenase from pea has now been obtained in Dr. Casey's laboratory by Mr. Paul M. Ealing, who will visit the Carlsberg Laboratory in the week of July 6 to exchange material and discuss joint experiments.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: University of St. Andrews

Contract no.: BAP - 0039 - UK

Project leader: J.R. KINGHORN

Scientific staff: S.E. Unkles (postgraduate associate)
E. Campbell (technician)

Address: University of St. Andrews
Dept. of Biochemistry and Microbiology
Irvine Building
North Street
UK - ST.ANDREWS, FIFE KY16 9AL

Telephone no.: +44.334.76161 EXT.296

Telex no.:

Other contractual partners in the joint project:

P.H. Pouwels, T. N. O. (Rijswijk)
W. Fiers, Laboratory of Molecular Biology (Gent)

Title of the research activity:

Development of a gene mediated transfer and selection system for filamentous fungi.

Key words:

Filamentous fungi, Transformation systems, Nitrate assimilation, Heterologous expression, Fungal gene structure

Reporting period: October 1986 - July 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The objectives of the research programme are

(1) to develop gene mediated transformation systems for industrial filamentous fungi.

(2) to optimise transformation frequency.

(3) to express mammalian genes of commercial interest in filamentous fungi.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The objectives are to utilise the nitrate reductase pathway for the development of transformation. Specifically nitrate reductase structural gene mutants were to be isolated from several fungi particularly Aspergillus oryzae but also Penicillium chrysogenum, Cephalosporium acremonium and Aspergillus niger. Secondly, expression of the already cloned Aspergillus nidulans nitrate reductase gene in these fungi was to be attempted. Thirdly attempts to isolate the corresponding nitrate reductase gene from these fungi were to be made.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Methodology

(a) Mutant isolation Spontaneous mutants were generated on the basis of chlorate resistance. A large number were isolated for each fungus. Growth tests were performed to differentiate between nitrate reductase structural gene mutants (niaD) and other mutants which are chlorate resistant such as the cofactor (cnx), control (nirA, areA) and transport (crnA) genes.

(b) The identification of corresponding genes by cross hybridisation using the A. nidulans niaD gene as a heterologous probe. Genomic DNA was isolated from the test organisms,

restricted and subjected to electrophoresis followed by Southern Blotting. Hybridisation was carried out under relaxed conditions and cross hybridisation detected by autoradiography.

(c) The isolation of corresponding niaD genes. This was was performed by colony hybridisation.

(d) Transformation in filamentous fungi. This was carried out basically using the conventional protocol developed for A. nidulans.

2 Results

(a) Mutant isolation Nitrate reductase structural gene mutants were successfully isolated from P. chrysogenum, A.oryzae and A.niger. However C. acremonium proved to be more difficult since the wild type is unable to grow on hypoxanthine as a sole source of nitrogen and hence it is not possible to distinguish between niaD and cnx co-factor mutants by this screening technique. Notwithstanding these difficulties, we developed other approaches for identification which included the assay of cytochrome c reductase, in-vitro complementation and assay of hypoxanthine dehydrogenase activity in non-denaturing polyacrylamide gels. Using a combination of these approaches, C. acremonium gene mutants were isolated.

A second series of experiments was carried out to establish if any of these mutants were non-reverting. For all test fungi, we have isolated niaD mutants which appear to be non-reverting (below 1 in 10^{10}). These have been used subsequently as recipients in transformation experiments.

(b) Isolation of corresponding genes. Using a 2.7 Kb XbaI fragment of the A. nidulans gene, signals were seen in genomic blots of all fungi. The sizes of the bands are presented in Table 1.

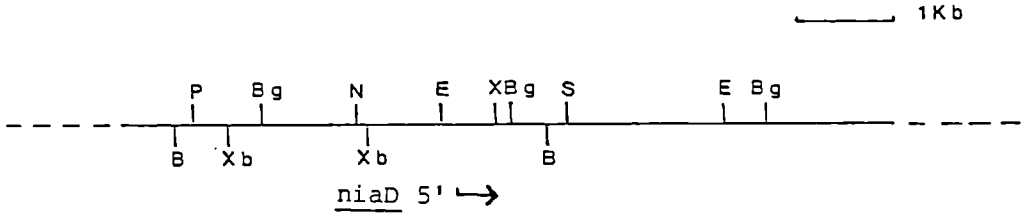
Table 1. Summary of Southern blot hybridisations.

Organism	Restriction enzyme	Size of band (Kb)
A. nidulans	Bam H1	15
A. niger	Eco R1	2.9; 2.0
	Bam H1	4.0
A. oryzae	Hind III	6.0
	Bam H1	3.0
P. chrysogenum	Hind III	9.0; 7.4
	Eco R1	7.0
	Bam H1	10.6
C. acremonium	Hind III	8.9; 6.5
	Eco R1	9.4

Since the strength of the A. niger band was considerably greater, we decided to clone this gene first. A plasmid bank was sent to us [Dr R Contreras, Belgium] in the form of various pools. We hybridised the A. nidulans Xba fragment against these, identifying a single pool which contained the gene.

High density colony blots were analysed and from these we identified three colonies which hybridised strongly. DNA from these was isolated, a preliminary restriction map made and the approximate position of the niaD gene on the clone determined by Southern blotting (Figure 1).

Figure 1 Restriction map of the A.niger niaD gene



The genomic signal from C. acremonium is rather weak. Notwithstanding this we have isolated a putative clone from a C. acremonium λ bank and this is being investigated at the present time.

(c) Transformation We have obtained evidence for heterologous expression of the A. nidulans gene in A. niger, P. chrysogenum and A. oryzae. The transformation frequency is low. Our present experiments are aimed at increasing this frequency as well as characterising the transformants by Southern blotting. Homologous expression of the A. niger gene in A. niger has been obtained at a few transformants per μg DNA. These are being examined further.

3. Conclusions

The A. niger gene has been isolated and returned into an A. niger niaD mutant to give reversal of the niaD phenotype, i.e. growth on nitrate. The frequency is low but we can improve on this. Secondly cross-hybridisation signals are seen on genomic blots of the other fungi. This is very encouraging and we expect to isolate these genes in the coming year.

In summary we now have the basis of a good transformation system in all four fungi.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

S. Gurr, S. Unkles and J.R. Kinghorn (1987) 'Gene structure and organisation in filamentous fungi'. In 'The gene structure in microbial enkaryotes. Editor J. R. Kinghorn IRL Press, Oxford.

S.E. Unkles 'Transformation in filamentous fungi using the nitrate assimilation system' (1987). In 'The genetics and molecular biology of nitrate assimilation'. Editor J.L. Wray and J.R. Kinghorn, Oxford Press.

S.E. Unkles 'Transformation in filamentous fungi' (1987). Second International Meeting on Nitrate Assimmilation St. Andrews, U.K.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)	Yes	
Joint meeting(s)	Yes	

Descriptive information for the above data.

(a) Materials. An Aspergillus niger gene bank has been made available to us by the Ghent group. This has saved us a great deal of time and effort. Additionally the A. niger glucoamylase gene has been sent to us to use as a probe for cloning the corresponding A. oryzae gene. This is to generate secretion signals for A. oryzae.

We have sent various niaD mutants and the A. nidulans clone to the group in the Netherlands. They are sending us an A. oryzae bank to isolate the niaD gene from this organism.

(b) Exchange of Staff. None in the first year. We felt that we should get on with our various tasks to lay a foundation. Exchanges have been arranged for the second year.

(c) Joint experiments.

(1) The isolation of the A. niger niaD gene (Belgium)

(2) The isolation of the A. oryzae gene (Netherlands)

(3) Transformation of A. oryzae (Netherlands)

(4) Isolation of the A. oryzae glucoamylase gene (Netherlands and Belgium).

(d) Joint meeting. J.R. Kinghorn, E. Campbell and S. Unkles had several detailed discussions with Dr Peter Pouwels (Netherlands) and Dr. Roland Contreras (Belgium) at Ioannina, Greece. We discussed the direction of the project, joint experiments and exchange of staff. All three groups plan to meet together later (September 1987) in the Netherlands.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: T.N.O., Rijswijk

Contract no.: BAP - 0064 - NL

Project leader: P.H. POUWELS

Scientific staff: I.E. Mattern, P.J. Punt, H. Hessing, C.A.M.J.J. van den Hondel

Address: TNO
Medical Biological Laboratory
P.O. BOX 45
NL - 2280 AA RIJSWIJK

Telephone no.: +31.15.138777

Telex no.: 38034 PMTNO NL

Other contractual partners in the joint project:

J.R. Kinghorn, University of St. Andrews
W. Fiers, Laboratory of Molecular Biology (Gent)

Title of the research activity:
Development of a gene mediated transfer and selection system for filamentous fungi.

Key words:
Fungal transformation, Aspergillus oryzae, Nitrate reductase, Filamentous fungi, Recombinant DNA

Reporting period: January 1987 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The objective of our research is the development of a (homologous) cloning system for commercially important filamentous fungi. This system would permit the isolation, identification and analysis of fungal genes of industrial importance. As a first step a gene mediated transfer system for Aspergillus oryzae will be developed.
Subsequently expression vectors will be constructed which permit expression of chimaeric genes under nitrate control utilising the nia promoter and upstream control region. Additionally it is planned to extend this study to more distantly related industrial filamentous fungi.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Development of a gene-transfer system for A.oryzae, based on
1) the A.nidulans amdS gene, coding for aceetamidase (1)
2) the A.niger pyrG gene, coding for orotidine-5'-phosphate decarboxylase (2) This approach requires the isolation of pyrG mutants of A.oryzae.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

METHODOLOGY

1). Isolation of mutants:

PyrG mutants were isolated from an A.oryzae IMI 44242 nia strain, by UV-irradiation and subsequent selection with 5-fluoro-orotic acid (5-FOA) as described for A.niger (2). A suspension of conidiospores ($1-10 \times 10^6$/ml) was irradiated with UV-light (600-750 J/m^2) to a survival of 5-30%. Since conidiospores of A.oryzae are multinucleated samples of the irradiated suspension of spores were plated onto uridine containing agar plates to allow segregation of mutated nuclei. After incubation at 34^{0}C for 72-96h, sporulating colonies had developed. The spores were collected and plated onto agar plates containing 10mM uridine and 1mg/ml 5-FOA,

2). Transformation:

Protoplasts were obtained by treatment with Novozym 234 and helicase, according to the method of Yelton et al. (3), except that spores were inoculated at a density of 4×10^6/ml and incubated at 25^{0}C for 16-18 h in minimal medium, supplemented with 0,05% casamino acids and 10mM uridine. Per gram of mycelium $0,7-3 \times 10^8$ protoplasts were obtained, 10-30% of which were viable. Transformation of these protoplasts was performed as described for A.niger (2).

RESULTS

ad1) Transformation of A.oryzae with the A.nidulans amdS gene.
A.oryzae does not grow on 10mM aceetamide supplemented with 15mM CsCl. so in principle, the amdS selection can be used. However, in various experiments with vector p3SR2 carrying the amdS gene, no clearcut AmdS$^+$ transformants were obtained: a few colonies appeared, more or less sporulating, but the same number also appeared on the control plates using the vector without the amdS gene.

ad2) Transformation of A.oryzae with an A.niger pyrG gene and the equivalent N.crassa pyr4 gene.
Several independently isolated uridine requiring (Uri$^-$) mutants were obtained. Four of them were used for transformation with either pAB4-1 or pDJB3 carrying pyrG or pyr4 respectively. Sporulating Pyr$^+$ colonies were obtained after transformation of three Uri$^-$ mutants with pAB4-1, at a frequency of up to 16 transformants per µg of DNA. These transformants are mitotically stable. In addition at least ten times as many abortive colonies were found. Southern blot analysis of DNA from some transformants of one pyrG mutant (AO 4.1) showed that vector DNA was present in the chromosomal DNA, in various copy numbers and presumably at different sites. This indicates 1) that the Pyr$^+$ colonies were indeed pAB4-1 transformants, 2) that the A.niger expression signals are recognized in A.oryzae and that the A.niger enzyme is functional in A.oryzae.

In contrast to the results obtained with pAB4-1, Pyr$^+$ colonies were not obtained with pDJB3, although pyrG mutants of A.nidulans and A.niger can be transformed with this vector (2, 4). This suggests that either the transformation frequency is much lower, that N.crassa expression signals are not efficiently recognized, or that the N.crassa pyr4 gene product is not functional in A.oryzae.

The possibility to introduce unselected genes into A.oryzae by cotransformation with pAB4-1 was investigated with the vector pAN5-41B, which contains the F.coli lacZ gene flanked at both sides by A.nidulans expression signals (5). After treatment with equimolar amounts of both plasmids, up to 80% cotransformation of pAN5-41B was observed. From this result we conclude that also A.nidulans expression signals are recognized in A.oryzae, and that efficient cotransformation of unselectable genes is possible.

DISCUSSION AND FUTURE PLANS

A transformation system for A.oryzae has been developed, based on the pyrG gene of A.niger. Up to 16 transformants per µg of DNA were obtained. This provides the basis for studies on the regulation of gene expression and protein secretion at the molecular level in this organism. Efficient cotransformation of non-selectable genes has also been performed.

Presently we are isolating the homologous pyrG gene from an A.oryzae gene bank, which has been constructed recently, in order to establish a homologous transformation system. This is necessary to direct vector integration to a specific site in the genome, which is required for a quantitative analysis of gene expression signals (5). In addition, it will probably allow us to increase the transformation frequency (2). Furthermore, we are developing a transformation system based on the dominant selectable gene for phleomycin resistance (6).

References

1. Kelly, M.K. and Hynes, H.J. (1985), EMBO J. 4, 475-479.
2. Van Hartingsveldt, W., Mattern, I.E., van Zeijl, C.M.J., Pouwels, P.H. and van den Hondel, C.A.M.J.J. (1987), Mol. Gen. Genet. 206, 71-75.
3. Yelton, M.M., Hamer, J.E. and Timberlake, W.E. (1984), Proc. Natl. Acad. Sci. USA 81, 1470-1474.
4. Ballance, D.J. and Turner, G. (1985), Gene 36, 321-331.
5. Van Gorcom, R.F.M., Punt, P.J., Pouwels, P.H. and van den Hondel, C.A.M.J.J. (1986), Gene 48, 221-217.
6. Gatignol, A., Baron, M. and Tiraby, G. (1987), Mol. Gen. Genet. 207, 342-348.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1 Mattern, I.E., Pouwels, P.H. and van den Hondel, C.A.M.J.J.. Transformation of Aspergillus oryzae using the A.niger pyrG gene, Mol.Gen. Genet., in press.

IV.2 Mattern, I.E., Punt, P.J., Dingemanse, M.A., Pouwels, P.H. and van den Hondel, C.A.M.J.J. (1987). Development of a gene mediated transfer and selection system for filamentous fungi, Poster and abstract for EEG meeting (Greece), May 1987.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes*
Exchange of staff	No
Joint experiment(s)	No
Joint meeting(s)	No

Descriptive information for the above data.

Exchange of strains and DNA with Dr. J.R. Kinghorn, St. Andrews.
Exchange of staff and joint meetings with Dr. Kinghorn and his staff are being planned.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: University of Ioannina

Contract no.: BAP - 0153 - GR

Project leader: C. DRAINAS

Scientific staff:

Address: University of Ioannina
Department of Chemistry
Division of Organic Chemistry/
Biochemistry
GR - 45332 IOANNINA

Telephone no.: +30.651.92210 or +30.651.37208

Telex no.: 032216

Other contractual partners in the joint project:

M.A. Typas, University of Athens
H. Sahm, Kernforschungsanlage Jülich

Title of the research activity:
Genetic manipulation of the anaerobe Zymomonas mobilis for fermentation of fruit juices.

Key words:
Zymomonas mobilis, Ethanol production, Transformation, Plasmids, Zymomonas genetics

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The genetic improvement of the anaerobic bacterium *Zymomonas mobilis* by means of formal genetics, as well as genetic engineering for ethanol production by fermentation of agricultural products with high carbohydrate content. The construction of high expression vectors and the development of a gene transfer system in *Z.mobilis* by transformation, conjugation, transduction or protoplast fusion. The long-term aim is to transfer in *Z.mobilis* genes coding for enzymes which reduce to glucose sugars non-fermentable by *Z.mobilis*. The genetically improved strains are expected to ferment various agricultural products to ethanol more efficiently.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

A. Determination of kinetic parameters for DNA uptake by *Z.mobilis*, towards the development of a transformation system.

B. Fusions between *Z.mobilis* and *E.coli* protoplasts, to contruct hybrids with high efficiency in fermenting various sugars (sorbitol, or maltose, or raffinose, or cellobiose) to ethanol.

C. Screening of *Z.mobilis* strains for growth on various fruit juices. This was made in collaboration with the Athens group.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

METHODOLOGY

Towards the development of a transformation system in *Z.mobilis*, efforts were made to determine the kinetic parameters for optimum DNA uptake by *Z.mobilis* cells. For this purpose, the recombinant plasmid pDS212, which can replicate and express stably in *Z.mobilis* when transferred by helped conjugation [1,2], was labelled with ^{32}P-dATP and used to determine the optimum conditions for cell competence and uptake of DNA. The stractural form of uptaken DNA was followed by immediate disrruption of the cells after the uptake experiment and autoradiography.

Protoplast fusion is applied to overcome possible difficulties of expression of genes coding for reduction to glucose of sugars, like sorbitol, or maltose, or raffinose, or cellobiose in *Z.mobilis* cells. A first approach to this task is the fusion of *Z.mobilis* and *E.coli* protoplasts. A mutant *Z.mobilis* strain resistant to rifampicin (CP4-Rif5),

isolated for this purpose, and *E.coli* strain RR1 carrying the recombinant plasmid pDS212 [1,2] were protoplasted by glycin treatment, following a modification of the method described by Yanase et al [3]. The effect of refampicin and tetracycline on the regeneration of protoplasts was studied in order to establish selection conditions for the isolation of hybrids which follow the fusion of the protoplasts.

In collaboration with the Athens University group, a first sreening of the *Z.mobilis* strains ATCC 10988, CP4 and NCIB 11163 for growth on ectracts of watermelon, orange, peach, apple and sugarbeet was performed. Parameters like doubling time, production of CO2, ethanol productivity and yield along with growth were determined.

RESULTS

A. Determination of kinetic parameters of DNA uptake for the development of a transformation system for *Z.mobilis*.

To estimate the accumulation of ^{32}P-pDS212, *Z.mobilis* cells were grown on liquid complete or minimal medium [4]. Aliquotes of cells were taken at various growth times throughout the entire growth of the cultures, pretreated with $CaCl_2$ or $MgCl_2$ and incubated with the labelled DNA, as described for *E.coli* transformation methods [5,6]. The higher accumulation of radioactivity was observed in cells from the early exponential phase. This accumulation was greater than the one observed in the *E.coli* competent cells. Cells from this phase were used to isolate *Z.mobilis* transformants expressing the gene markers of pDS212. Although all the methods established for *E.coli* [5,6], as well as recently reported methods for *Z.mobilis* transformation [7,8] were employed, the results were negative as yet.

B. Protoplast fusions.

Z.mobilis CP4-Rif5 and *E.coli* RR1/pDS212 protoplasts were constructed with high yields and regeneration frequencies (over 95%). First efforts to isolate products of protoplast fusions, selecting for double resistance on 10 μg/ml rifampicin (marker of CP4-Rif5) and 20 μg/ml tetracycline (marker of RR1/pDS212) were not successful. It was found that refampicin and tetracycline reduce dramatically the regeneration of CP4-Rif5 and RR1/pDS212 protoplasts respectively. This was overcome by reducing the concentration of refampicin and tetracycline to 4 μg/ml and 10 μg/ml

respectively. Selection of fusion products on these conditions is in progress at present.

C. Screening of *Z.mobilis* strains for growth and ethanol production on various fruit juices.

The strain NCIB 11163 did not grow on sugar beet extract. All other strains grew well on all the extracts tested without the addition of any nutrients. Ethanol production was best (≈ 5-6 % w/v) using strain ATCC 10988 and CP4 on apple extract. On the other fruit extracts ethanol production was lower due mainly to the low fermentable sugar content by *Z.mobilis* . On sugarbeet extract, which is rich in sucrose and raffinose, only CP4 gave significant ethanol productivity (≈ 5 % w/v).

DISCUSSION

Z.mobilis cells can take up radiolabelled DNA as efficiently as *E.coli* competent cells. It is likely that competence on DNA uptake appears on the early exponential phase. However, efforts to transform cells from this phase were not successful. No radioactivity with the form of plasmid DNA was detected by autoradiography. This result indicates that plasmid DNA may loose its integrity upon entrance to *Z.mobilis* cells and therefore it fails to replicate. Detection of endonuclease activity, which degrades covalently closed circular pDS212 DNA, supports this hypothesis. Experiments are in progress to reveal the form of uptaken DNA, which will throw more insight towards the development of a transformation system in *Z.mobilis*.

Protoplast fusion is another approach for engineering *Z.mobilis*. Preliminary results have clarified the protoplasting and selection conditions for the isolation of hybrids between *Z.mobilis* and *E.coli* cells, a process which is in progress at present. This approach may be a bypass towards the more efficient fermentation by *Z.mobilis* of agricultural products like sugarbeets and starchy products.

REFERENCES

1. Afendra, A.S. & Drainas, C. (1987) J. Gen. Microbiol. 133: 127-134.
2. Scordaki, A & Drainas, C. (1987) J. Gen. Microbiol. 133: in press.
3. Yanase, H. et. al. (1985). Agr. Biol. Chem. Tokyo. 49: 133-140.
4. Galani, I. et. al. (1985). Biotechnol. Lett. 7: 673-678.
5. Cohen, S.N. et. al. (1972). Proc. Natl. Acad. Sci. USA. 69: 2110-2114.
6. Maniatis, T. et. al. (1982) Molecular Cloning. A Laboratory Manual CSH.
7. Buchholz, S.E. & Eveleigh, D.E. (1986) Appl. Environm. Microbiol. 52: 365-370
8. Yanase, H. et. al. (1986) Agr. Biol. Chem. Tokyo. 50: 3139-3144.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

EXCHANGE OF MATERIALS

Exchange of *Z. mobilis* strains and recombinant plasmids was made with our collaborating research group headed by Prof. H. Sahm at Julich, towards the joint effort in constructing a high expression vector and developing a transformation system for *Z. mobilis*.

In addition plasmid pJRD215 was received from Dr. J. Davison (Belgium) to check for expression in *Z. mobilis*. The *Z. mobilis* strain ATCC 10988 was sent to Dr. J. Davison to test the λ expression system.

JOINT MEETINGS

The leaders of the three collaborating groups (Prof. Sahm, Julich, Dr. Typas, Athens, and Dr. Drainas, Ioannina) met together in Athens on October 1986 and programmed the materialization of the collaboration, as well as the joint experiments which will be performed upon a forthcoming visit of Dr. Typas and Dr. Drainas to Julich on July 1987.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: University of Athens Contract no.: BAP - 0152 - GR

Project leader: M.A. TYPAS
Scientific staff: E. Alexandri, I. Galani, B.J. Mouzak

Address: University of Athens
Panepistemiopolis
Kouponia
GR - ATHENS T.K. 15701

Telephone no.: +30.1.7240091 EXT.638

Telex no.: 223815

Other contractual partners in the joint project:

C. Drainas, University of Ioannina
H. Sahm, Kernforschungsanlage Jülich

Title of the research activity:

Genetic manipulation of the anaerobe Zymomonas mobilis for fermentation of fruit juices.

Key words:

Zymomonas mobilis, Ethanol production, Ethanol tolerance, Glutamine uptake, Zymomonas strain construction

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The genetic improvement of the anaerobic bacterium Zymomonas mobilis by means of formal genetics , as well as genetic engineering for ethanol production by fermentation of agricultural products with high carbohydrate content . The development of a gene transfer system in Z.mobilis by transformation , conjugation , transduction or protoplast fusion and the construction of high expression vectors . The long term aim is to extent the spectrum of substrates utilized by this organism by gene transfer (including genetic manipulation) .

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

A. Strain construction by mutagenesis and attempts to produce high ethanol tolerant mutants .

B. Membrane permeability and the uptake of nitrogen metabolites by Z.mobilis .

C. Screening of Z.mobilis strains for growth on various fruit juices (in collaboration with the Ioannina group) .

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Several antibiotic resistance markers were found to be rather unstable in some Z.mobilis strains or to revert back to wild-type at high frequencies. To avoid such instability problems in the construction of suitable strains for conjugation , a combination of auxotrophic (unable to grow on the chemically defined minimal medium , Galani et.al. 1985) and stable resistant markers was made . The viability of all Z.mobilis strains was tested on various ethanol concentrations (2-15% v/v). Following various types of mutagenesis (NTG , EMS , U.V., transposon) attempts to isolate ethanol tolerant mutants were made at ethanol concentrations which were totally inhibitory for the wild-type . Independent colonies showing growth at such high ethanol concentrations were further tested for ethanol tolerance in both solid and liquid media . Those appearring to be tolerant were further mutated and checked at higher ethanol concentrations .

Initial experiments with Z.mobilis have shown that the organism can

utilize glutamine , glutamate , asparagine and aspartate as sole nitrogen sources. Therefore, the uptake of glutamine by Z.mobilis was examined following the increase of radioactivity in cells , at various time intervals of incubation with ^{14}C-glutamine . The effect of physiological conditions on the uptake , specificity , energy dependence and localization of mediator(s) were investigated. Sphaeroplasts were prepared by glycine treatment following a modification of the Yanase et.al.,1985 method and cold-shocked cells were prepared by the Fujimura et.al.,1983 and Masters & Hong ,1981 techniques .

In collaboration with the Ioannina University group , a first screening of the Z.mobilis strains ATCC 10988 , CP4 and NCIB 11163 for growth on extracts of watermelon , orange , peach , apple and sugarbeet was performed. Several parameters , such as doubling time , production of CO_2 , ethanol productivity and yield along with growth were determined .

RESULTS

A. Auxotrophic mutants of all Z.mobilis strains were further mutated with chemical mutagens to acquire antibiotic resistance . Only non-reverting antibiotic resistance markers were used . Ethanol concentration of 6% (v/v) had no effect on either growth or viability (examined after 24h) for all Z.mobilis strains tested . Concentrations of 10% (v/v) ethanol restricted growth (50-75%) but viability was not affected . At 12% (v/v) ethanol neither growth nor viability was observed . The above concentration was used for selection of tolerant mutants amongst NTG- and U.V.-treated cells . Several isolates tolerating this concentration were obtained . However , further mutagenic treatment of these isolates resulted in unstable mutants which were unable to maintain higher ethanol tolerance (14%, v/v) when subcultured. The reasons for this instability are under investigation now .

B. Growth and pre-incubation media were found to play a significant role on glutamine uptake . Cells grown on MM instead of CM showed the best uptake and similarly the same medium was the best pre-incubation medium . Pre-incubation temperature also affected markedly the uptake. The Km value was 0.04×10^{-5}M gln and the Vmax was 10 mμ mols gln.mg^{-1}.min^{-1} . The specificity of glutamine uptake was examined by comparing the effect of the analogue L-glutamic acid-γ-monohydroxamate (GH), as well as that of a number of amino acids . From the amino acids tested glutamate , asparagine and GH were competitors when either MM or CM were used as incubation media , as their effect on glutamine uptake could be reversed by increasing the glutamine concentration . Arginine was competing only on MM whereas alanine failed to

compete on either . Arsenate , 2,4-dinitrophenol and merthiolate inhibited glutamine uptake by 44% , 72% and 46% , at low concentrations (0.5mM) and by 84%, 99% and 96% at high concentrations (5mM) respectively . All inhibitors are toxic to Z.mobilis even at the lower concentrations used . Finally, when viable sphaeroplasts were used no uptake was observed and the corresponding uptake from osmotically shocked cells was drastically reduced .
C. The strain NCIB 11163 did not grow on sugar beet extracts . All other strains grew well on all extracts tested without the addition of any nutrients . Ethanol production was best (5-6% w/v) using strain ATCC 10988 and CP4 on apple extract . On the other fruit extracts ethanol production was lower due mainly to their lower fermentable sugar content . On sugarbeet extract , which is rich in sucrose and raffinose , only CP4 gave significant ethanol productivity (5% w/v) .

DISCUSSION

Mutliply marked strains suitable for conjugation experiments including auxotrophies and stable resistant antibiotic markers of all Z.mobilis strains were constructed . Although some tolerant to ethanol mutants were produced at 12% (v/v) , others tolerant to higher ethanol concentrations (14-15% v/v) were very unstable , indicating that a multiple-step mutagenesis in continuous cultures is required . The glutamine uptake of the organism has been studied and it was found to be energy-dependent,possibly due to a membrane bound carrier protein , as shown from the sphaeroplasts and osmotically-shocked cells uptake . The glutamine transport system is not specific only for glutamine as other amino acids and analogues (glutamate, asparagine , arginine and GH) can compete with glutamine . The study of the nitrogen metabolite uptake system may help to understand problems of differential membrane permeability of the various Z.mobilis strains.

REFERENCES

1. Galani,I et.al.(1985): Biotechnol.Lett. 7 : 673-678
2. Fujimura ,T. et.al.(1983): Biochemistry 22 : 1954-1959
3. Masters P.S & Hong J-S (1981) : J.Bacteriol. 147 : 805-819
4. Yanase ,H et.al.(1986) : Agr.Biol.Chem. Tokyo 50 : 3139-3144

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

a)	Exchange of material(s)	Yes
b)	Exchange of staff	Yes
c)	Joint experiment(s)	Yes
d)	Joint meeting(s)	Yes

Descriptive information for the above data.

a) Exchange of Z.mobilis strains and recombinant plasmids was made with both the other two collaborating groups towards the establishment of suitable strains for conjugation experiments .

b) The leaders of the three collaborating groups (Prof.Sahm , Jülich , Dr.Drainas , Ioannina and Dr.Typas , Athens) met together in Athens on October 1986 and programmed the materalization of the collaboration.

c) Dr.Drainas and Dr.Typas will visit Jülich in July 1987 to carry out some joint experiments on transposon mutagenesis and recombination system of Z.mobilis .

d) A member of the Jülich group (M.Reynen) participated to the BAP-meeting in Ioannina , April this year and discussed the work for the near future with Dr.Drainas and Dr.Typas .

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: K.F.A., Jülich Contract no.: BAP - 0200 - D

Project leader: H. SAHM
Scientific staff: M. Reynen, G. Sprenger

Address: Kernforschungsanlage Jülich
Institut für Biotechnologie 1
Postfach 1913
D - 5170 JÜLICH

Telephone no.: +49.2461.613294

Telex no.: 833556 KFA D

Other contractual partners in the joint project:

C. Drainas, University of Ioannina
M.A. Typas, University of Athens

Title of the research activity:

Genetic manipulation of the anaerobe Zymomonas mobilis for fermentation of fruit juices.

Key words:

Zymomonas mobilis, Ethanol production, Pyruvate decarboxylase gene, Expression vector

Reporting period: January 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The fermentative gram negative bacterium **Z.mobilis** has a considerable potential for industrial ethanol production as ethanol is formed 5 to 6 times faster than in yeast. However, this bacterium has a very limited substrate spectrum, only glucose, fructose and sucrose can be utilized, but not starch, pentoses or other renewable carbon sources. Therefore, it is of great interest to extent the substrate spectrum of this organism by genetic manipulation.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

In the first period the main object in our laboratory was to establish gene cloning techniques for **Z.mobilis**, especially the construction of an expression vector for this bacterium. Since it has been shown that foreign genes are often expressed very poorly in **Z.mobilis**, we intent to use the promoter of the cloned pyruvate decarboxylase (pdc) gene of this organism for construction of an efficient expression vector.

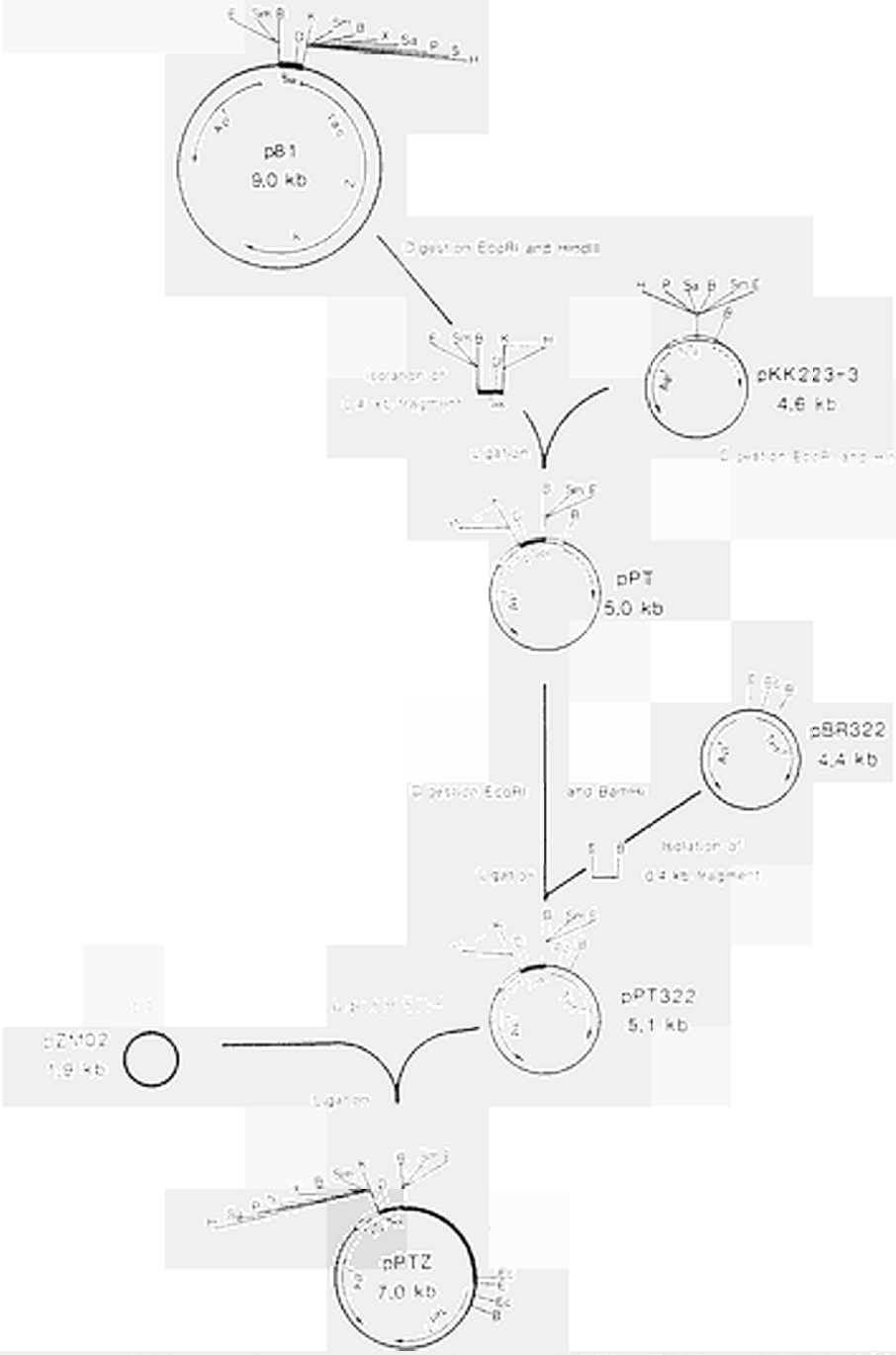
III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Localisation of the pdc gene promoter

The cloned pdc gene from **Z.mobilis** ZM6 (Bräu and Sahm, 1986) was subcloned as a 3.2 kb BstEII-SphI fragment, a restriction map was made, and the gene was sequenced completely by the methods of Maxam-Gilbert and Sanger. To localize the promoter of the pdc gene we also used the cloned **Z.mobilis** DNA fragment for a shotgun cloning of small restriction fragments into promoter test vectors pNM480-482 (Minton, 1984). The screening for DNA fragments with promoter activity in **E.coli** MC1061 was done on agar plates containing 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (BCIG). Clones with a promoter fragment could be recognized by their ability to hydrolyse the colourless compound BCIG to a blue coloured compound. Promoter strengh was investigated more recently by measuring the ß-galactosidase activity of the clones. Some fragments with strong promoters in **E.coli** MC1061 were sequenced and compared with the sequence of the pdc gene. By this way the pdc gene promoter was localized and could be subcloned on the plasmid p81

within a fragment from -315 bp to +21 bp. Furthermore, this promoter was fused for further constructions to a polylinker with restriction sites of KpnI, SmaI, BamHI, XbaI, SalI, PstI, SphI and HindIII (Fig. 1).

Figure 1: Construction of an expression vector for Z.mobilis



Ap^R, ampicillin resistance gene from pBR322, Tet^R, tetracycline resistance gene from pBR322, lacZ, lacY, ß-galactosidase gene from *E.coli*, P_{pdc} promoter of the *pdc* gene, T_1T_2, *rrnB* transcriptional termination signals ; kb, kilobasepairs, B, *Bam*HI, D, *Dra*I, E, *Eco*RI, Ec, *Eco*RV, H, *Hind*III, K, *Kpn*I, P, *Pst*I, S, *Sph*I, Sa, *Sal*I, Sm, *Sma*I, X, *Xba*I

2. Construction of an expression vector for Z.mobilis
Recent results indicated that fusions of the pdc promoter to structural genes with no downstrean termination signal are unstable. Since it is known that cloning of strong promoters often require the downstream placement of strong transciption termination signals (Gentz et al., 1981), we fused the transcriptional terminators rrnB T1T2 (Brosium et al., 1981) to the 3'-end of the polylinker behind the pdc gene promoter. This was done by cloning the EcoRI-HindIII fragment of plasmid p81, carrying the pdc promoter, into the vector pKK223-3 (Fig. 1).

Into the new plasmid a selectable marker for **Z.mobilis** had to be introduced. As **Z.mobilis** has a natural resistance to ampicillin the EcoRI-BamHI fragment of pPT, harbouring the tac promoter was exchanged against the EcoRI-BamHI fragment of pBR322, carrying the 5'-region of the tet^R gene, missing on pKK223-3 (Fig. 1). After this the vector pPT322 was fused with the cryptic **Z.mobilis** plasmid pZM 02 (Afendra, 1987) by EcoRI digestion and ligation of the two linearized plasmids (Fig. 1). The resulting vector pPTZ was transformed into **Z.mobilis** using the method described by Yanase (Yanase et al., 1986), stability is under investigation. First results show a 5 % loss of tetracycline resistence per generation under nonselective conditions.

References
A.S.Afendran et al., J.Gen.Microbiol. (1987) **133**:127-134
B.Bräu and H.Sahm, Arch.Microbiol. (1986) **144**: 296-301
J.Brosius et al., Plasmid (1981) **6**: 112 ff
J.Brosius et al., J.Mol.Biol. (1981) **148**: 107 ff
T.Conway et al., J.Bact. (1987) **169**/3: 949-954
R. Gentz et al., PNAS (1981) **78**/8: 4936-4940
A.D.Neale et al., Nucl.Acids Res. (1987) **15**/4: 1753-1761
H.Yanase et al., Agric.Biol.Chem. (1986) **50**/12:3139-3144

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

a)	Exchange of material(s)	Yes
b)	Exchange of staff	Yes
c)	Joint experiment(s)	Yes
d)	Joint meeting(s)	Yes

Descriptive information for the above data.

a) Plasmids and bacterial strains have been exchanged between our group and the groups of Dr. Drainas and Dr. Typas. Dr. Drainas' group provided two plasmids stable in **Z.mobilis** (pDS191 and pDS212), Dr. Typas' group provided the **Z.mobilis** strain CP4, and we provided the cloned pdc-promoter on the vector p81 including all sequence data.

b) and c) Dr. Drainas and Dr. Typas will visit us in Jülich in July this year to carry out some joint experiments on phages for **Z.mobilis** and transposon mutagenesis.

d) A member of our group has joint the BAP-meeting in Ioannina, April this year and discussed the work for the near future with Dr. Drainas and Dr. Typas.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: I. C. P., Brussels
Contract no.: BAP - 0048 - B

Project leader: J. DAVISON
Scientific staff: F. Brunel, N. Chevalier

Address: International Institute of
Cellular and Molecular Pathology
Unit of Molecular Biology
Avenue Hippocrate 75
B - 1200 BRUSSELS

Telephone no.: +32.2.7647463

Telex no.: 23722 UCLWOL B

Other contractual partners in the joint project:

B. Witholt, Groningen Biotechnology Centre

Title of the research activity:
Engineering of gram negative bacteria with industrial potential.

Key words:
Pseudomonas, Methylotroph, Lignin, Vanillate, λ Repressor

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Application of gene cloning techniques to Gram-negative bacteria of industrial potential. Particular attention is paid to these bacteria able to utilize methanol and lignin degradation products, to degrade pollutants such as substituted aromatic and aliphatic hydrocarbons, to catalize industrially interesting bioconversions.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

1) Development of wide host cloning vectors based on plasmid RSF1010 or on small cryptic plasmids of Pseudomonas.
2) Development of wide host range expression systems based on the repressor and the rightword operator and promoter of phage λ.
3) Analysis of the structure, function and regulation of specific genes (vanillate degradation ; SDS degradation).

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

A 'phase-shift' fusion system for the regulation of foreign gene expression by λ repressor in Gram-negative bacteria.

A 'phase-shift' translation fusion vector was constructed in which mutually compatible restriction sites BamHI, BclI and BglII are positioned each in a different reading frame, immediately following the ATG initiator codon and ribosome binding site of the λ cro gene. The λ cro gene is expressed from promoter P_R and controlled by λ repressor (which is also carried by the plasmid). The usefulness of the expression vector was demonstrated using a galK gene lacking the ATG initiator codon and fusing this to the λ cro initiator ATG codon gene, resulting in λcI_{857} regulated expression of galactokinase. The vector is of general application for foreign gene expression in E. coli

The λcI_{857}-P_R-cro-galK cassette was cloned into pJRD215, a wide host range plasmid and transferred by conjugation to a variety of Gram-negative bacteria. In all cases, thermosensitive regulation of galactokinase

could be demonstrated (Table 1). These results show that the powerful λ P_R promoter and the efficient λ repressor can be used to regulate expression of foreign genes in Gram-negative bacteria other than E. coli.

a) pJRD187

```
      900        910        920        930        940        950
GGTTGTATGG ATCCGTCTAT GATCAGATCT CGAGCTCGCG AAAGCTTGCA TGCCTGCAGG
       B          B      B   X   S   N      H      S      P  B   S
       A          C      G   H   A   R      I      P      S  S   A
       M          L      L   O   C   U      N      H      T  P   L
       1          1      2   1   1   1      3      1      1  1   1
```

b) pJRD187K

```
      890        900        910        920        930
GTACTAAGGA GGTTGTATGG ATCCGTCTAT GATCTCGAGG AGTCTGAAAG
                  B                  X
                  A                  H
                  M                  O
                  1                  1
```

Fig 1 : a) Phase shift vector pJRD187. The ATG codon is that of the cro gene of phage λ preceeded by its natural Shine and Dalgarno site and the pR promoter of λ. The BamHI, BclI and BglII sites are each out of phase by one nucleotide to enable fusion in all the reading frames.
Fig. 1 b) Galactokinase expression vector pJRD187K. The AGT codon at position 921 is the 2nd codon of the galK gene.

Table 1 : λ repressor regulated expression of galactokinase in various bacteria

Host	Plasmid	Units galK 32°C	42°C
Escherichia coli C600 galK⁻	pJRD187K	2	1935
Escherichia coli C600 galK⁻	pJRD215K	6	841
Pseudomonas insueta ATCC 21276	"	3	466
Pseudomonas aeroginosa PAO 1162	"	5	425
Klebsiella pneumoniae	"	6	406
Salmonella typhimurium	"	11	383
Pseudomonas sp.ATCC 1951	"	4	320
Enterobacter aerogenes	"	7	311
Serratia marcescens	"	9	301
Providencia stuartii	"	0	159
Acinetobacter anitratum	"	20	142
Erwinia carotovora	"	1	100
Methylotrophus methylophilus	"	3	76
Pseudomonas putida	"	1	17

Cloning and sequencing of Pseudomonas genes involved in the demethylation of the lignin degradation derivative, vanillate.

A 2.5 kb SalI-HincII DNA fragment from Pseudomonas sp. has been cloned and sequenced, which contains the genetic information necessary for the demethylation of vanillate (3-methoxy-4-hydroxybenzoate). Complementation data and insertional inactivation experiments show that two genes at least (A and B) are involved in vanillate catabolism and that transcription proceeds from SalI to HincII in a polycistronic manner. Secondary transcription of the B gene may also occur from an internal promoter. Nucleotide sequencing confirms these data. Two open reading frames may be found in the "transcribed orientation" from bases 258 to 1248 and from 1248 to 2192 which are characteristic of Pseudomonas coding sequences (>90% GC in the third base of the triplet) and would give proteins of 36543 and 33679 daltons respectively. The sizes of the polypeptides synthesized from the recombinant DNA in the E. coli T7 expression system are in good agreement with those predicted. Upstream from the A and B genes are two sequences of 17 bp which fit very well the consensus sequences for inducible promoters of Pseudomonas chromosomals genes and Klebsiella pneumoniae nif genes. In fact, the P_1 promoter at position 208-224 is identical to that of the nif H gene. P_1 fusion to galK and subsequent measurement of galactokinase activity show that P_1 does not function in E. coli and that it is inducible (30 fold) in Pseudomonas in the presence of vanillate. 57 bp upstream of P_1 is a DNA sequence resembling sites where regulatory protein bind to DNA.

Further work will involve the precise characterisation of this potential "activator sequence" and the overexpression and characterization of the proteins involved in vanillate demethylation.

Cloning and sequencing of the genes for SDS degradation

The genes of Pseudomonas sp. ATCC19151 for the degradation of SDS have been cloned by complementation of a mutant host. Subcloning and characterization are in progress.

A dual replicon vector

A dual replicon mobilizable cosmid has been constructed from pKC7 and a small cryptic plasmid of Pseudomonas sp. Work is in progress to develop a resident complementing vector compatible with wide host range vector pJRD215.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV. 1. Publications in scientific journals and monographs.

1. Biotechnology with Gram-negative bacteria and yeast. Davison, J., Brunel, F., Heusterspreute, M., Oberto, J., Ursi, D. and Prozzi, D. Genetic Engineering in Belgium : research and perspective of industrial applications. P. Crooy (Ed.), Division de biotechnologie de la Société Chimique Belge, 1987, 44-50.

2. Vectors with restriction site banks. V. pJRD215, a wide host range cosmid vector with multiple cloning sites. Davison, J., Heusterspreute, M., Chevalier, N., Ha Thi, V. and Brunel, F. Gene 51 (1987), 275-280.

3. Restriction site bank vector for cloning in gram negative bacteria and yeast. Davison, J., Heusterspreute, M. and Brunel, F. Methods in Enzymology, Academic Press, Recombinant DNA Part D. (Ed. R. Wu). (1987), 153, in press.

4. Davison, J., Heusterspreute, M., Chevalier, N. and Brunel, F. A 'phase-shift' fusion system for the regulation of foreign gene expression by λ repressor in Gram-negative bacteria. Gene (1987) manuscript submitted.

5. Davison, J. Plant beneficial bacteria (a review). Bio/technology (1987) manuscript submitted.

6. Brunel, F., Chevalier, N. and Davison, J. Cloning and sequencing of Pseudomonas genes encoding vanillate demethylation in Pseudomonas sp. J. Bacteriol. Manuscript to be submitted.

IV. 2. Short communication and internal reports.

1. Improved wide host range cosmid vectors: Cloning of the vanillate degradation genes of Pseudomonas. Brunel, F., Pilaete, M. F., Davison, J. Abstract of the E.M.B.O. Workshop on "The genetic manipulation of Pseudomonads - Applications in biotechnology and medicine", Geneva (Switzerland) p 30-31, September 1986.

2. The use of new wide host range cosmid vectors to clone the vanillate degradation genes of Pseudomonas. Brunel, F., Pilaete M. F. and Davison, J. Abstract of the 5th International Symposium on the Genetics of Industrial Microorganisms Split (Yugoslavia) p 58, September 1986.

3. A plasmid for the regulated expression of foreign genes in Pseudomonas. Davison, J., Heusterspreute, M., Chevalier, N. and Brunel, F. Abstract of the Commission of European Communities (B.A.P.) meeting, Ioannina (Greece) p80-81, April 1987.

4. Cloning and sequencing of Pseudomonas genes involved in the demethylation of the lignin degradation derivative, vanillate. Brunel, F., Pilaete, M. F., Chevalier, N. and Davison, J. Abstract of the Commission of European Communities (B.A.P.) meeting, Ioannina (Greece), p 78-79, April 1987.

5. Brunel, F., Davison, J. and Heusterspreute, M. Annual Scientific Report of the International Institute of Cellular and Molecular Pathology (1986), in press.

6. Brunel, F., Chevalier, N. and Davison, J. Cloning and sequencing of Pseudomonas genes involved in the demethylation of the lignin degradation derivative, vanillate. Conference on Reducing risks from environmental chemicals through biotechnology, Seattle (USA) July 1987.

7. Brunel, F., Chevalier, N. and Davison, J.. Cloning and sequencing of Pseudomonas genes involved in the demethylation of the lignin degradation derivative, vanillate. Forum for Applied Biotechlology, Gent, October 7, 1987.

IV. 3. Patents

None

IV. 4. Thesis.

van der Linden, M. Characterization of two small Pseudomonas plasmids in an attempt to develop a dual replicon cloning vector.
Minor thesis as part of study for a degree in Biochemistry at the State University of Groningen (research preformed for a six month period in the laboratory of Dr. J. Davison in Brussels). Submitted May 1987.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Exchange of Materials

There has been frequent exchange of plasmids, bacterial strains and computer programmes between the Brussels and Groningen collaborating laboratories. In addition, vectors developed in the Brussels laboratory have been distributed to several laboratories within the BEP and BAP programmes.

Exchange of staff and joint experiments

Mr. Mark van der Linden (from Prof. B. Witholts latoratory ; University of Groningen) spent six months in Brussels working on a joint project to develop alternative vector systems based upon two small cryptic plasmids previously isolated in Brussels under the BEP programme (GBI-3-016-B). This visit was partially financed (travel and accomodation) by the BAP contract (BAP-0048-B) to the Brussels laboratory.

Joint meetings

Over the last 3 years, the laboratories of Dr. J. Davison (Brussels) and Prof. B. Witholt (Groningen) have regularly held joint meetings in the form of visits and seminars, as well as encounters at scientific conferences.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Rijksuniversiteit Groningen
Contract no.: BAP - 0047 - NL

Project leader: B. WITHOLT
Scientific staff: M. Kok, M. Wubbolts

Address: Groningen Biotechnology Centre
Nijenborgh 16
NL - 9747 AG GRONINGEN

Telephone no.: +31.50.634165

Telex no.:

Other contractual partners in the joint project:

J. Davison, I. I. C. M. P. (Brussels)

Title of the research activity:
Engineering of gram negative bacteria with industrial potential.

Key words:
Pseudomonas, Alkane oxidation, Multiphase catalysis, Hydroxylase, Cell engineering

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The aim of the joint project "genetic engineering of Gram-negative bacteria with industrial potential" is the construction and application of Gram-negative bacteria with altered catalytic activities for the production of fine chemicals. To achieve this, vector systems need to be developed that ensure properly regulated stable expression of the genetic material of interest, preferably cloned on plasmids. The genetic stability of the engineered bacteria under fermentation conditions is considered to be the main bottleneck in the application of Gram-negative bacteria in industrial processes.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

We have isolated and characterized the inducible *alkBAC* promoter, and we have identified the sequences involved in regulator recognition and RNA polymerase binding. The *alk* promoter is very suitable for application in a broad host range expression vector since it functions equally well in *Pseudomonas* and *E. coli*, has a very high induction ratio, and requires a very cheap inducer.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

INTRODUCTION

The *alkBAC* operon encodes several components of the alkane degradation pathway in *Pseudomonas putida*. The *alk* system is of biotechnological interest since it encodes a terminal monoxygenase which specifically oxidizes aliphatic hydrocarbons at the terminal carbon atom only, and because it can epoxidize terminally unsaturated hydrocarbons stereospecifically.

Both terminally oxidized aliphatic hydrocarbons and epoxides are interesting products which can potentially be made with a biotechnological process at competitive prices (K. de Vries 1982).

The *alkBAC* operon is induced 500 fold when a few drops of inducer (i.e. n-octane) are added to a culture of *P. oleovorans*. The induction ratio and kinetics are identical for *P. putida* and *E. coli*, making the *alk*

promoter very suitable for applications in various biotechnological processes.

The *alk* promoter was initially identified on a 524 bp DNA fragment, derived from a genomic clone, between a PstI site and the initiation site of transcription. It has been cloned and characterized using a transcriptional fusion to the chloramphenicol transferase (cat) gene.

CLONING AND CHARACTERIZATION OF THE *alk* PROMOTER

The *alk* promoter has been cloned in the pBR322 derivative pKK232.8, which carries a promoterless chloramphenicol acetyl transferase gene flanked by terminators. The initial clone pGMK1106 (figure 1) carried a 1.1 kb insert that included the first two transcribed bases (GA) of the *alkBAC* mRNA.

This plasmid generated *alkR* and inducer dependent CAT activity in *E. coli* which could be measured in cell free extracts (table 1). Subsequently deletions were generated in this plasmid enabling the mapping of the sequences involved in promoter activation in *E. coli*.

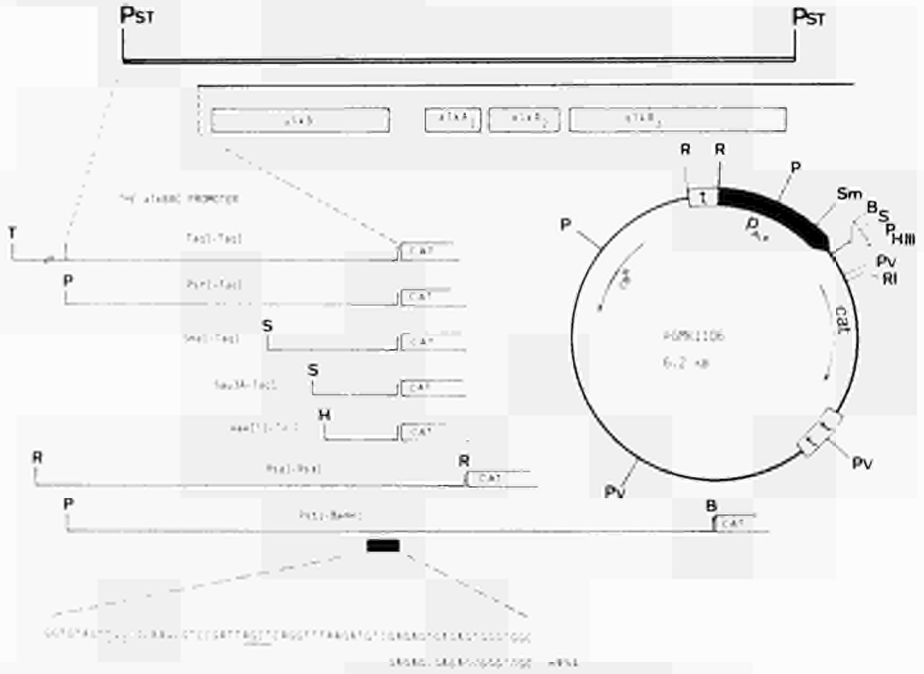


Figure 1. Detailed structure of the alk promoter

An alk-cat fusion was constructed using the promoter probe vector pKK232.8 (Brosius 1984). Expression of the CAT gene on pGMK1106 could be activated by the addition of octane to E. coli GEc73, which carries alkR on a separate plasmid. The promoter-regulation region could be narrowed down to a 94 bp sequence. A sequence homologous to several other Pseudomonas promoters was found close to the mRNA initiation site. This sequence is believed to be the RNA polymerase binding site.

RESULTS AND DISCUSSION

Part of the DNA sequence of the *alkBAC* promoter is shown in figure 1.

The promoter does not resemble the *E. coli* consensus promoter, although it was shown to be very efficient in *E. coli*. It is, however, similar to other promoters isolated from *Pseudomonads*, and to the nitrogen regulated promoters of *E. coli* and *Klebsiella pneumoniae*. Thus far, the *alk* promoter is the only promoter isolated from *Pseudomonas* that is equally efficient in *E. coli*. Another well characterized promoter from *Pseudomonas putida*, the *xylCAB* promoter (Inouye 1984), requires the *rpoN* gene product, a sigma factor involved in the activation of nitrogen regulated promoters, for activation in *E. coli* (Dixon 1986). This is not true for the *alk* promoter as was shown by the activation of the CAT gene in an *E. coli rpoN* mutant.

table 1 CAT expression as regulated by the alk promoter in *E. coli*. (clone 6 taken as 100%)

Clone	Promoter fragment*	transcribed alk DNA	CAT activity (%) induced	not induced
1.	-1100 / +2	GA	0.7	51
2.	-526 / +2	GA	n.d.	n.d.
3.	-180 / +2	GA	1.0	66
4.	-112 / +2	GA	1.0	30
5.	-90 / +2	GA	1.2	34
6.	-650 / +136	leader+13 codons	3.6	100
7.	-524 / +549	leader+151 codons	3.0	22

*The amount of alk DNA fused to the CAT gene, with initiation at G taken as +1.

We have narrowed down the promoter-operator region to 94 bp, allowing ample room for the interaction of the positive regulator (AlkR) with the promoter DNA. In clones 1-5, the 3' end of the promoter fragment coincided with the second transcribed nucleotide. A 3' extension (clone 6) yielded two fold higher expression both in induced and in uninduced state.

We believe this reflects the mRNA stability of the hybrid *alk-cat* mRNA.

We are presently investigating the role of mRNA stability in the expression of the alk peptides, and the requirements for transcription in *E. coli* and *Pseudomonas*.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.2 Short commenucation and internal reports

Cell Engineering of Gram-negative Bacteria: the alk regulon of Pseudomonas oleovorans M. Kok, R. Lageveen, G. Eggink and B. Witholt Abstract of the C.E.C.- B.A.P. meeting at Ioannina p82-83 (1987)

IV.4 Thesis

M van der Linden
Characterization of two small Pseudomonas plasmids in an attempt to develop a dual replicon cloning vector
Minor thesis for Masters Degree at the University of Groningen (Biochemistry). The research was performed at the lab of Dr J. Davison in Brussels).

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

1. Exchance of materials.

During the last years of cooperation between the Brussels and the Groningen group there has been a considerable exchange of biological materials (such as plasmids, strains) as well as computer software. The exchange has intensified during the B.A.P. contract.

2. Exchange of staff, and joint experiments (3).

Mr. Mark vander Linden spent half a year at John Davisons lab in an exchange of personel. In this period he performed his second research project for his masters degree in Biochemistry (Groningen University).

The exchange was partially financed by the BAP contract (BAP-0048-B) to the Brussels lab of Dr. J. Davison.

Mark van der Linden will, in future, join the staff of the Biochemistry lab of Groningen University.

4. Meetings with the Brussels group.

The cooperation between the groups at the ICP in Brussels and the University of Groningen has been stimulated by the joint meetings that were helt over the last years.

These meetings involve giving seminars, informal lectures and speaking to researchers from the other group as well as with other researchers from the institute.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: P.H.L.S., Salisbury

Contract no.: BAP - 0046 - UK

Project leader: N.P. MINTON

Scientific staff: J.D. Oultram, T.J. Swinfield, J.K. Brehm, D.E. Thompson, D.A. Barstow, A.F. Sharman, S. Prior

Address: Microbial Technology Laboratory
PHLS Centre for Applied
Microbiology and Research
UK - PORTON DOWN, SALISBURY SP4 OJG

Telephone no.: +44.980.610391 EXT.367

Telex no.: 47683 PHCAMR G

Other contractual partners in the joint project:

W.L. Staudenbauer, Technische Universität München
M. Young, University College of Wales (Aberystwyth)

Title of the research activity:

Development of host/vector systems in clostridia of industrial and agricultural importance.

Key words:

Clostridium acetobutylicum, ferredoxin, Plasmid pAMB1, Replicon, Cointegration

Reporting period: October 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Saccharolytic clostridia are widely recognised as organisms of biotechnological importance. Potentially, they may be employed in the large scale conversion of agricultural wastes and surpluses into fuels and solvents, and to produce chiral compounds by stereospecific reductions that are difficult to accomplish chemically. It is the objective of this project to develop reliable means of achieving genetic exchange in Clostridium spp., thereby allowing realisation of their biotechnological potential. The availability of such a genetic system will allow the amplification and controlled expression of genes concerned with substrate utilisation and product formation.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The objectives of the first 9 months of the programme have been:-

i) the construction of a clostridial expression cartridge based on the ferrodoxin (Fd) gene of C. pasteurianum.

ii) isolation and examination of "clostridial" replicons.

iii) exploitation of the plasmid pAMB1 as a conjugal delivery systems for transferring replicons and cloned genes into C. acetobutylicum.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY

Expression cartridge construction: E. coli clones carrying the Fd gene were identified in a C. pasteurianum plasmid gene bank using an oligonucleotide probe. A 650 bp Sau3A fragment carrying Fd was converted into an EcoRI fragment, by subcloning into the BamHI site of M13mp7, and HindIII and SstI sites created within the Fd coding region using site-directed mutagenesis. The DNA between these two sites was then substituted with a polylinker region carrying 20 different unique cloning sites.

Vector construction: Potential E. coli/Clostridium shuttle vectors were constructed, based on the pMTL series of E. coli cloning vectors. They carry extensive polylinker regions (20 cloning sites) within a lacZ' gene, allowing for "blue/white" selection of cloned DNA fragments. Plasmid pMTL20E carries the pAMB1 Em^r gene (the entire sequence of which was

determined during the course of this work), pMTL20C carries the Cm^r pC194 gene, while pMTL21EC possesses both of these Gram $^+$ve resistance genes. An analogous E. coli/Bacillus shuttle vector, pMTL33, was also made by combining pMTL21 with pC194.

2. RESULTS

Replicon Analysis

The complete nucleotide sequences of the C. butyricum plasmids pCB101 (6.03 kb) and pCB102 (8.05 kb) were determined. Both plasmids lacked any features (at the DNA or protein level) normally associated with DNA replication. The possibility that clostridial plasmid replication regions could be identified by comparisons to other characterised plasmids replicons therefore appears untenable. The 3.45 kb Sau3A subfragment of pCB101, shown to enable replication of replicon deficient plasmids in B. subtilis, contained two ORF's capable of encoding proteins of M_r 27,134 and 43,039. Removal of 9 codons from the C-terminus of the larger ORF destroyed the replicative ability of this fragment.

Two cryptic C. paraputrificum plasmids (pCP1, 4.05 kb; pCP2, 6.22 kb) were restriction mapped and numerous subfragments cloned into pMTL20E and 20C. None of the recombinant plasmids replicated in B. subtilis. In contrast, a 5.05 kb EcoRI fragment derived from pAMB1 conferred replicative ability on these plasmid vectors in B. subtilis.

Gene Transfer by Conjugal Cointegrate Transfer

The feasibility of using the conjugal R factor pAMB1 to introduce cloned genes into C. acetobutylicum has been tested. A DNA fragment encoding the leuBC genes of C. pasteurianum was cloned into pMTL21EC and the recombinant plasmid transformed into B. subtilis carrying pAMB1, selecting for both Em^r and Cm^r. As pMTL21EC cannot replicate in B. subtilis, Em^r-Cm^r transformants were composed of cells in which pMTL21ECLeu$^+$ had cointegrated with pAMB1 by virtue of homologous Em^r regions. The plasmid cointegrates were conjugated into 2 different Leu$^-$ auxotrophic C. acetobutylicum mutants. In the one case, the Em^r-Cm^r transconjugants obtained became phototrophic. Subsequent growth in the absence of antibiotics resulted in cells which became sensitive to both Em and Cm concommitant with reversion to Leu$^-$ auxotrophy.

Fd expression Cartridge

To test the Fd expression cartridge we have initially used the Pseudomonas xylE gene. Nucleotide sequencing of a 2.0 kb XhoI fragment carrying this gene enabled its isolation as a 1.3 kb ScaI-KpnI fragment, where the xylE ribosome binding site was located 50 bp downstream from the ScaI site. This fragment was inserted into the Fd cartridge, such that expression would be under the transcriptional control of the Fd promoter, and the "recombinant" cartridge inserted into pMTL21EC and pMTL33. In the latter vector expression levels equivalent to 15% of the cells soluble protein were obtained in E. coli (compared to 25% using the lac promoter), while in B. subtilis much lower levels were observed (0.1%). A pAMB1::pMTL21EC cointegrate carrying Fd+xylE has subsequently been conjugated into C. acetobutylicum, and the presence of the gene demonstrated by in situ chromogenic assay.

3. DISCUSSION

Data obtained suggests that clostridial plasmid replicons bear no resemblance to equivalent regions of other characterised bacterial plasmids. This apparent dissimilarity may explain why the fragments of 4 of the 5 clostridial plasmids examined were incapable of replicating in B. subtilis. Indeed, even in the case of recombinant plasmids carrying the putative pCB101 replicon, the presence of extrachromosomal DNA in B. subtilis could only be demonstrated by Southern blot techniques. Clostridial plasmids may be functionally distinct from known Gram $^{+}$ve plasmids.

Experiments have demonstrated that pAMB1 may be used to effect the transfer of cloned genes into C. acetobutylicum. The present strategy, however, is unsatisfactory as the "mobilised" cloning vector is uanble to replicate upon resolution of the cointegrate. We are therefore currently working on modified conjugal cointegrate transfer strategies where replication of the resolved vector is ensured. Greater emphasis, however, will now be placed on developing a protoplast transformation system using pMTL20E/C carrying the cloned pAMB1 replicon.

At present our data suggests that while the Fd promoter functions extremely efficiently in E. coli, it is relatively ineffective in B. subtilis. At present no quantitative data is available for C. acetobutylicum. Future work will be concerned with utilising a clostridial gene.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

1. PUBLICATIONS IN SCIENTIFIC JOURNALS

Brehm, J.K., Salmon, G.P.C. and Minton, N.P. (1987 Sequence of the adenine methylase gene of the Streptococcus faecalis plasmid pAMB1. Nucl. Acids Res. 15:3177.

2. SHORT COMMUNICATIONS

Brehm, J.K., Barstow, D.A., Pennock, A., Young, M. and Minton, N.P. (1986) Characterisation of a replication origin from a cryptic plasmid of Clostridium butyricum. Abstracts XIV Int. Cong. Microbiol. p.132.

Young, M. and Minton, N.P. (1987) Development of host-vector systems for saccharolytic clostridia. Abstracts of 4th Int. Conference on Genets. and Biotechnol. of Bacilli. p.47.

Brehm, J.K., Oultram, J.D., Thompson, D.E., Swinfield, T.J. and Minton, N.P. (1987) Construction of plasmid vectors for gene transfer experiments in Clostridium acetobutylicum. Abstracts of 4th Int. Conference on Genets. and Biotechnol. of Bacilli. p.50.

Barstow, D.A., Brehm, J.K., Minton, N.P., Pennock, A. and Young, M. (1987) Analysis of the replication origin of plasmid pCB101 from Clostridium butyricum. Abstract CEC-BAP Meeting, Ioannina, p.54.

Brehm, J.K., Oultram, J.D., Thompson, D.E., Swinfield, T.J. and Minton, N.P. (1987) Transfer of homologous and heterologous genes into Clostridium acetobutylicum. Abstract CEC-BAP Meeting, Ioannina, p.50.

Oultram, J.D., Brehm, J.K., Swinfield, T.J., Thompson, D.E., Barstow, D.A. and Minton, N.P. (1987) Construction of plasmid vectors for gene transfer experiments in Clostridium acetobutylicum. Abstract CEC-BAP Meeting, Ioannina, p.51.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)	Yes	
Joint meeting(s)	Yes	

Descriptive information for the above data.

1. Exchange of materials

The pMTL series of E. coli cloning vectors have been distributed not only to the contractual partners of this project but to additional BAP contractors. These include, Dr M Gasson (AFRC, Reading, UK), Dr Casey (John Innes, Norwich, UK) and Dr S Bron (University of Groningen, Haren, The Netherlands). Aberystwyth University (Dr M Young) provided the Leu^- Clostridium acetobutylicum auxotrophic mutants for the studies described in the above report.

2. Joint experiments

Functional analysis of the replication region of the clostridial plasmid pCB101 was undertaken jointly at Aberystwyth and Porton Down. Studies on the use of pAMB1 cointegrates to test the ability of commonly used Gram $^+$ve plasmids to replicate in Clostridium spp. are ongoing.

A synthetic oligonucleotide is currently being synthesised to allow the cloning of clostridial enoate reductase at Munich.

Functional analysis of the pAMB1 replicon is currently being undertaken in collaboration with Dr Ehrlich's laboratory in Paris.

3. Joint Meetings

An informal meeting of research staff at Aberystwyth and Porton Down working within the framework of the BAP contract was held in Aberystwyth on 10-11 March 1987. As Dr Staudenbauer's contract did not begin until April 1987, a meeting between all three contractors took place for the first time in Ioannina at the BAP sectorial meeting.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Univ. College of Wales, Aberystwyth
Contract no.: BAP - 0044 - UK
Project leader: M. YOUNG
Scientific staff: D.R. Williams, A. Davies

Address:
University College of Wales
Department of Botany and Microbiology
UK - ABERYSTWYTH, DYFED SY23 3DA

Telephone no.: +44.970.3111 EXT.3058

Telex no.: 35181 ABYUCW G

Other contractual partners in the joint project:

N.P. Minton, Microbial Technology Laboratory (Salisbury)
W.L. Staudenbauer, Technische Universität München

Title of the research activity:

Development of host/vector systems in clostridia of industrial and agricultural importance.

Key words:

Clostridia, Conjugal plasmids, Plasmid mobilisation, Conjugal transposons, Vector construction

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Biotechnological exploitation of the saccharolytic clostridia is hampered by the lack of suitable procedures for promoting genetic exchange in these organisms. Our objectives are to develop methods based, initially at least, on conjugal streptococcal plasmids, for obtaining gene transfer in both mesophilic and thermophilic organisms. In parallel with this, plasmid vectors will be developed that will enable the regulated high level expression of cloned genes to be obtained in clostridia. The development and exploitation of cloning strategies based on conjugal transposons is also envisaged. With these new procedures it will be possible to adopt rational approaches to the optimisation of industrial fermentations employing saccharolytic clostridia.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

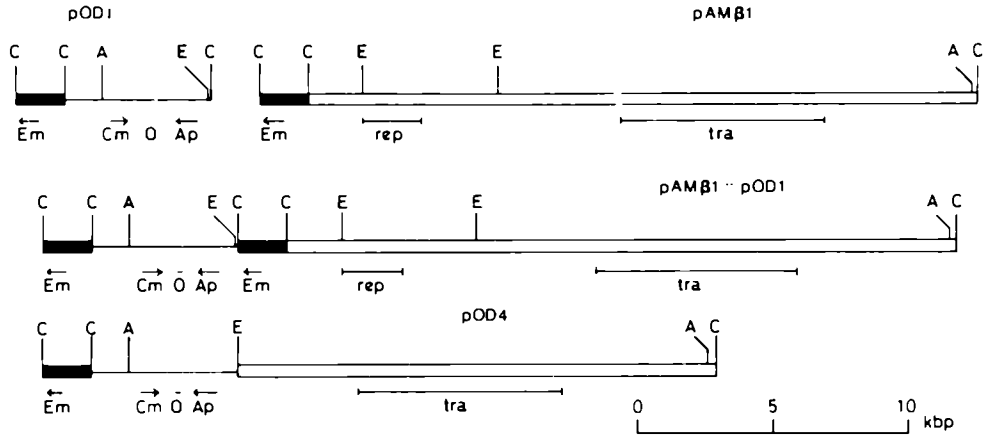
These were to exploit our recent finding that the conjugative streptococcal plasmid pAMB1 can be transferred to Clostridium acetobutylicum by using it to mobilise small non conjugative plasmids. This will permit the identification of replicons and antibiotic resistance markers that can be expressed in clostridia. It was also envisaged that derivatives of the mobilisable plasmids carrying streptococcal transposons would be constructed and transferred to selected clostridia so that procedures for obtaining transposon mutagenesis could be explored.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Methodology. The 26.5kbp MLS^R plasmid pAMB1, from Streptococcus faecalis (1) can be transferred to Clostridium acetobutylicum and a number of other clostridia from a variety of donor organisms including Bacillus subtilis (2, 3). This is accomplished by a filter mating procedure, full details of which have been published (2, 4). Standard methods were used for the manipulation of DNA samples in vitro and their introduction into and extraction from Escherichia coli (5).

Results and Discussion. pAMB1 is known to mobilise small non-conjugative plasmids during filter matings between different strains of streptococci (6). The mechanism of this "natural mobilisation" is not known. Repeated attempts were made to demonstrate "natural mobilisation" of several B. subtilis plasmids, viz pHV33, pBC16, pUB110, pTV1 using donor strains containing these plasmids as well as pAMB1. However, if mobilisation does indeed occur, the frequency was too

Figure 1 Construction of plasmid pOD4



Key : ——— , pJH101 DNA; ▭ , pAMB1 DNA; ▬ , 1.8 kbp ClaI fragment of pAMB1. Restriction sites are denoted: A, AvaI; C, ClaI (selected sites shown); E, EcoRI. The replication origins derived from pBR322 and pAMB1 are denoted "O" and "rep", respectively.

low to be detectable in these experiments. Given a transfer frequency for pAMB1 alone of 10^{-6} (transcipients per recipient), a mobilisation frequency of 10^{-3} would have been detected. Rather than pursue "natural mobilisation" using donor organisms such as Streptococcus lactis that transfer pAMB1 more efficiently than B. subtilis (2), we decided to retain B. subtilis as donor, because genetic analysis in this species is well advanced, and to follow instead an alternative approach; i.e. to force mobilisation by cointegrate formation.

A plasmid, pOD1 (7.2 kbp; Cm^R, Em^R, Ap^R), was constructed by inserting a 1.8 kbp ClaI fragment of pAMB1 that encodes the MLS^R gene into the vector pJH101 (5.4 kbp; Cm^R, Ap^R, Tc^R). The vector, pJH101 is unable to transform B. subtilis, because it lacks a Gram-positive replication origin (Rep^-), but pOD1 does transform strains that harbour pAMB1. Southern hybridisation was used to prove that transformants contain a pAMB1::pOD1 cointegrate plasmid which is transferred between B. subtilis and C. acetobutylicum at a frequency similar to that of pAMB1. Resolution of the pAMB1::pOD1 cointegrate has been demonstrated in C. acetobutylicum but only one of the component plasmids, pAMB1, survives and is able to replicate autonomously.

In order to transfer small Rep^+ plasmids from B. subtilis, it was necessary to construct a Rep^- derivative of pAMB1 that (a) would form cointegrates with them and (b) retained the functions needed for conjugal transfer. This was accomplished by isolating the pAMB1::pOD1 cointegrate, deleting from it two adjacent EcoRI fragments, and re-establishing the resultant plasmid, pOD4 (24 kbp; Em^R,

Cm^R), in E. coli (Fig.1). This plasmid will not transform strains of B. subtilis unless they harbour pBR322-derived sequences with which recombination can occur. Thus, pOD4 forms cointegrates with plasmids such as pHV33(7), pMK3(8), pUBR1(9) and pAP26 (10) which are chimaerae between pBR322 and various Gram-positive plasmids. Transfer of these cointegrates from B. subtilis into C. acetobutylicum is currently in progress. Plasmid pOD4 should be generally useful for mobilising small plasmids in other non-transformable Gram-positive species of medical and biotechnological interest.

Experiments to introduce transposons into C. acetobutylicum are still at an early stage. A derivative of pOD1 containing Tn916 (15 kbp; Tc^R) has been constructed and is being tested for use as a suicide plasmid for delivering this transposon. Highly thermosensitive derivatives of the pTV series of plasmids constructed by Youngman (11) which may be suitable for delivering Tn917 (Em^R) into C. acetobutylicum via cointegrate formation with pAMB1, are also being explored. An unexpectedly simple method for delivering conjugative transposons such as Tn916 was very recently discovered when it was found that transfer occurs at a frequency of about 10^{-6} in filter matings between S. faecalis or B. subtilis and C. acetobutylicum. Another similar element Tn1545 (25kbp; Em^R, Tc^R, Km^R) is transferred at frequencies as high as 10^{-3} in matings between Streptococcus faecalis and C. acetobutylicum (Table1). Experiments are currently in progress to determine whether these transposons insert at many different sites into the C. acetobutylicum chromosome, and to recover Tn-induced mutants.

Table 1 Transfer of conjugal transposons into C. acetobutylicum by filter mating

Donor	Recipient	Selection	Frequency
S. faecalis FAO14 (Tn916)[a]	C. aceto. AAOO4 (pAMB1)[b]	Em^R Tc^R	2.3×10^{-6}
B. subilis QB666 (Tn1545)	C. aceto. ATCC 8052	Em^R	1.0×10^{-6}[c]
S. faecalis JH2-Sm (Tn1545)	C. aceto. ATCC 8052	Em^R Tc^R Em^R	1.5×10^{-6}[d] 1.0×10^{-3}[e]

a This strain is S. faecalis DS16 cured of pAD2
b This strain is C. acetobutylicum ATCC 8052 carrying pAMB1
c These colonies were also Tc^R; Km^R was not tested
d These colonies were also Km^R
e These colonies were also Km^R and Tc^R

References

(1) *LeBlanc, D. J. and Lee, L. N. (1984). Journal of Bacteriology, **157**, 445.*

(2) *Oultram, J. D. and Young, M. (1985). FEMS Microbiology Letters, **27**, 129.*

(3) *Oultram, J. D., unpublished results.*

(4) *Oultram, J. D., Davies, A. and Young, M. (1987). FEMS Microbiology Letters, (In Press, July).*

(5) *Maniatis, T., Fritsch, E. F. and Sambrook, J. (1982). Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory, New York.*

(6) *Smith, M. D. (1985). Journal of Bacteriology, **162**, 92.*

(7) *Primrose, S. B. and Ehrlich, S. D. (1981). Plasmid, **6**, 193.*

(8) *Sullivan, M. A., Yasbin, R. E. and Young, F. E. (1984). Gene, **29**, 21.*

(9) *Moir, A., unpublished results.*

(10) *Pennock, A. P. and Young, M., unpublished results.*

(11) *Youngman, P. J. (1987). In "Plasmids, a practical approach", Ed. Hardy, K. G., p79. IRL Press Oxford.*

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

1. *Young, M., Oultram, J. D., Pennock, A. and Richards, D. F. (1987). Gene transfer in clostridia. In, Proceedings of the Fifth International Symposium on the Genetics of Industrial Microorganisms, pp403-413. Ed. Alacevic, M., Hranueli, D. and Toman, Z., Split, Yugoslavia.*

2. *Oultram, J. D., Davies, A. and Young, M. (1987). Conjugal transfer of a small plasmid from Bacillus subtilis to Clostridium acetobutylicum by cointegrate formation with plasmid pAMB1. FEMS Microbiology Letters (July 1987).*

3. *Barstow, D. A., Brehm, J. K., Minton, N. P., Pennock, A. and Young, M. (1987). Analysis of the replication origin of plasmid pCB101 from Clostridium butyricum. Abstract of CEC-BAP Meeting, Ioannina, Greece, p54.*

4. *Brehm, J. K., Barstow, D. A., Pennock, A., Young, M. and Minton, N. P. (1986). Characterisation of a replication origin from a cryptic plasmid of Clostridium butyricum. Abstract of 14th International Congress of Microbiology, Manchester, UK, p132.*

5. *Oultram, J. D. and Young, M. Conjugal gene transfer in clostridia. Abstract of 14th International Congress of Microbiology, Manchester, UK, p102.*

6. *Oultram, J. D., Williams, D. R. and Young, M. Conjugal plasmid transfer in Clostridium acetobutylicum. Abstract CEC-BAP Meeting, Ioannina, Greece, p53.*

7. *Young, M. and Minton, N. P. Development of host-vector systems for saccharolytic clostridia. Abstract of IV International Conference on Genetics and Biotechnology of Bacilli, p47.*

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Exchange of materials.

A student, Ms D. F. Richards, who is not directly funded by the EEC, but who is undertaking cognate work, has benefitted greatly from plasmids and strains sent to us by Dr Staudenbauer in München. Dr Minton and I have regular contact and freely exchange plasmids and strains. He has also generously provided us with unpublished sequence information.

Exchange of staff.

My former colleague, Dr J. D. Oultram is now working in the laboratory of my collaborator, Dr N.P. Minton. The student mentioned above, Ms D. F. Richards is currently attempting to transfer pAMB1 to Clostridium thermohydrosulfuricum and if, as her preliminary results suggest, she is successful, Dr. Staudenbauer and I have agreed that it would be provident for her to spend a short period (1-2 weeks) in his laboratory this autumn.

Joint experiments.

The experiments concerning the use of plasmid pOD4 to test several Gram-positive replicons for the ability to replicate in Clostridia and the analysis of the replication origin of plasmid pCB101 are being jointly undertaken at Aberystwyth and Porton.

Joint meetings.

We had a joint one day meeting with the Porton group which was held in Aberystwyth in March, 1987. We also attended the large BAP sectoral meeting in Ioannina in April, when we had extended trilateral discussions involving all participating laboratories.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

BAP - 0038 - IRL

Contractor: Trinity College, Dublin
Contract no.:

Project leader: D. McCONNELL / K.M. DEVINE
Scientific staff:

Address: Trinity College
Department of Genetics
IRL - DUBLIN 2

Telephone no.: +353.1.772941 EXT.1872

Telex no.: 25442 TCD EI

Other contractual partners in the joint project:

S.D. Ehrlich, Université Paris VII

Title of the research activity:

Studies of segregational and structural plasmid stability in Bacillus subtilis.

Key words:

Plasmid instability, Segregational and structural stability

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Two types of plasmid instability have been identified in B. subtilis; structural instability where plasmids undergo rearrangements especially deletions, and segregational instability where plasmids are lost from the cell population. Such instability is particularly prevalent in recombinant plasmids. The objective of this project is to determine (a) the mechanism(s) by which structural rearrangements occur and (b) to determine the plasmid and host functions involved in plasmid maintenance. This information can then be utilised to construct stable cloning vectors for B. subtilis.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The minimal replicon of a segregationally stable Bacillus plasmid, pBAA1, had previously been identified. Our objectives are (a) to sequence the minimal replicon; (b) to identify and analyse elements of the minimal replicon essential for replication and (c) to identify sequences involved in the stable maintenance of this plasmid.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Replication of pBAA1.

The minimal replicon of pBAA1 was localised to a 1400bp fragment. Deletion of 350bp from one end or 250bp fron the other end of this fragment abolished the ability to replicate in B. subtilis. Thus functions essential for replication are localised to these regions. The 350bp deletion overlaps an open reading frame with the potential to code for a protein of 287 amino acids. Comparison of this protein with proteins of other plasmids from B. subtilis and S. aureus known to be involved in replication revealed homology with the α-peptide of pUB110 and with the Rep A protein of pC194. The degree of homology with pUB110 is greater than that with pC194.

The 250bp fragment whose removal also abolishes the ability to replicate contains six sets of inverted repeats ranging from 13bp to 4bp in size . Comparison of this region with regions of other B. subtilis and S. aureus plasmids known to be involved in replication, revealed an 15bp

sequence perfectly conserved in pUB110 and pC194 (Fig. 1).

```
                    ___________
(1) G T C T T T C T T A T C T T G A T A C T A T A T A G A A A C A A C A T

       * *                   *                 *     *
(2) G T T C T T T C T T A T C T T G A T A C ^ A T A T A G A A A T A A C G T

    *  * *                   *   * * * * *   * *  * * * *
(3) T T T C T T T C T T A T C T T G A T A A T A A G G G T A A C T A T T G C
```

Figure 1 Conserved 15bp sequence in pBAA1(1), pUB110(2), and pC194(3). The nick site of ØX174 is overlined in the pBAA1 sequence.
*=mismatch and ^=gap.

There is greater homology between pBAA1 and pUB110 in the DNA flanking the conserved region than between pBAA1 and pC194. It is likely that this conserved region is located at the origin of the plasmid since it has been shown that the origin of pC194 is located in a 55bp sequence which spans this conserved region (Ehrlich, personal communication). It is also interesting that the sequence C T T G A T A contained within the 15 bp conserved region is the nick-site sequence for the phage ØX174.

Segregational stability of pBAA1.

Segregational stability of plasmids was estimated by growing cells in the absence of selection for 100 generations and then testing for the presence of the plasmid. Results indicated that the plasmid pDE22 which contains a 2200bp *Hin* D III fragment of pBAA1 is segregationally stable (100% cells contain plasmid after 100 generations), whereas plasmids containing the minimal replicon (1400bp contained within the 2200bp fragment) are segregationally unstable (79% cells contain plasmid after 100 generations). It is suspected that this instability is caused by a replication defect since this instability correlates with the production within the cell of single stranded plasmid DNA (see next section).

Production of single stranded plasmid DNA

It was found that the plasmid pDE22 was segregationally stable and no detectable single stranded plasmid DNA was present within cells. However the plasmids pDE14 and pRP17 were both segregationally

unstable and single stranded plasmid DNA was found in cells containing either of these plasmids. The plasmid DNA was deemed single stranded by the following criteria (i) it migrates faster than CCC monomeric plasmid in agarose gels containing ethidium bromide; (ii) it binds to nitrocellulose without denaturation and (iii) it is preferentially degraded following treatment with S1 nuclease. The presence of single stranded plasmid DNA in cells containing unstable plasmids suggests that this instability may be caused by a replication defect.

Discussion.

The minimal replicon of pBAA1 has been localised and sequenced. Two regions essential for replication have been identified; an open reading frame coding for a protein of 287 amino acids and a region containing a series of inverted repeats which contains a 15bp sequence conserved in pBAA1, pC194 and pUB110. In addition to the 15bp sequence there exists homology in regions involved in replication between pBAA1 and pC194 and pUB110 suggesting that they may replicate by the same mechanism. The presence of the nick site sequence of ØX174 and the production of single stranded DNA upon deletion of certain plasmid sequences suggests that the mode of replication of these plasmids may be similar to the rolling circle model of ØX174. The nature of the sequences which when deleted cause instability of the plasmids and production of plasmid single stranded DNA is currently being investigated.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	No
Exchange of staff	No
Joint experiment(s)	No
Joint meeting(s)	Yes

Descriptive information for the above data.

A meeting of both groups was held in Paris in March of this year. There were three presentations from this group and four presentations from Dr. Ehrlich's group. Discussion focussed on aspects of segregational and structural instability of plasmids. It was decided that people from this laboratory would work in Dr. Ehrlich's laboratory later this year.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Assoc. Dévelop. de la Recherche et de la Biologie Moléculaire, Paris

Contract no.: BAP - 0141 - F

Project leader: S.D. EHRLICH

Scientific staff:

Address: Université Paris VII
Institut Jacques Monod
Tour 43, ler étage
2 place Jussieu
F - 75251 PARIS CEDEX 05

Telephone no.: +33.1.43.29.53.70

Telex no.: 200094

Other contractual partners in the joint project:

K.M. Devine, Trinity College (Dublin)

Title of the research activity:

Studies of segregational and structural plasmid stability in Bacillus subtilis.

Key words:

Plasmid segregation, DNA rearrangements, Recombination

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

(1) Understanding of the mechanisms whereby Bacillus plasmids are partitioned

(2) Use of partition functions of stably inherited plasmids to stabilize homologous and heterologous replicons

(3) Understanding of the role of single-stranded DNA in plasmid rearrangements, and construction of structurally stable cloning vectors

(4) Understanding of the role of nicks in plasmid rearrangements.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

(1) Studying the stimulation of illegitimate recombination by single-stranded DNA

(2) Construction of structurally stable B. subtilis cloning vectors.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

METHODOLOGY

- Isolation and characterization of plasmids from E. coli and B. subtilis

- Construction of plasmids by cloning techniques

- Isolation and characterization of single-stranded DNA.

RESULTS AND DISCUSSION

(1) Single-stranded DNA stimulates illegitimate recombination

Plasmid rearrangements are often a consequence of recombination between short (< 20 bp) homologous sequences.

To study the effect of production of single-stranded (ss) DNA on such recombination (often named illegitimate recombination) we used the following approach.

First, we constructed a plasmid composed of pBR322 and the replication origin of phage M13. This plasmid replicates as a double-stranded (ds) molecule unless the phage replication protein (the product of the M13 gene II) is present in the host cell. In that case the plasmid generates large amounts of ssDNA.

Next, we isolated insertions of a Tn10-derived transposon in the Ap^R or the Tc^R gene of the plasmid. Such insertions have two consequences - they inactivate a gene and duplicate a 9 bp sequence of the gene such that the duplication flanks the inserted transposon. Recombination between these short duplications can easily be followed, since it excises the transposon, restores the intact gene and leads to the appearance of the resistance to Ap or Tc.

Recombination frequencies were low (close to 10^{-5}) when the plasmid replicated as a ds molecule. They were much higher (up to 10^{-1}) when ssDNA synthesis was induced. This shows that the production of ssDNA stimulates recombination between short duplicated sequences.

(2) Structurally stable B. subtilis cloning vectors

We used the test described above to compare recombination frequencies in B. subtilis plasmids which generate ssDNA, which do not generate ssDNA (these were identified in our

laboratory) and in the bacterial chromosome. The frequencies were 100-1000 times higher in the first type of plasmids than in the second or in the chromosome. These results confirm that ssDNA stimulates illegitimate recombination.

One of the plasmids which replicates as dsDNA is pAMβ1, a broad host-range plasmid isolated from Streptococcus faecalis. We joined a 3 kb segment which carries its replication region to a hybrid composed of pBR322 and a Cm^R gene well expressed in B. subtilis (the gene derives from pC194). In that way we obtained a shuttle E. coli/B. subtilis cloning vector. We compared this vector (labeled pHV1432) to a similar shuttle vector (pHV33) composed of pBR322 and pC194, a plasmid which generates ssDNA. In a shot-gun experiment we efficiently cloned large (up to 30 kb) DNA segments in the first but not in the second vector (the largest segment cloned in pHV33 was about 2 kb).

Structural stability of segments between 5 kb and 30 kb carried on pHV1432 was tested. All segments were stable for 150 generations. These results indicate that plasmids which do not generate ssDNA are structurally stable. The failure to clone large DNA segments in plasmids which generate ss DNA is likely to be due to their structural instability. That instability is a consequence of the stimulation of illegitimate recombination by ssDNA.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

L. Jannière and S.D. Ehrlich (1987)

Recombination between short repeated sequences is more frequent in plasmids than in the chromosome of *Bacillus subtilis*.

Mol. Gen. Genet., in press.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

(1) Sequence data were exchanged

(2) A joint meeting with the Dublin laboratory was held from March 7th to March 10th 1987 in Paris. The next one, subject to solving the problems of financing it, is planned to take place in Ireland next October.

BIOTECHNOLOGY OF PLANTS AND ASSOCIATED MICROORGANISMS

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Rothamsted Experiment. Station, Harpenden

Contract no.: BAP - 0101 - UK

Project leader: M.G.K. JONES

Scientific staff: H. Jones, N. Fish, S. Cooper-Bland, A. Karp, C. Symonds

Address: Rothamsted Experimental Station
Biochemistry Dept.
UK - HARPENDEN, HERTS AL5 2JQ

Telephone no.: +44.5827.63133 EXT.479

Telex no.: 825726

Other contractual partners in the joint project:

M.J. Tempelaar, University of Groningen
M. Jacobs, Vrije Universiteit Brussel
L. Van Vloten-Doting, Research Institute ITAL (Wageningen)
F. Sala, University of Pavia
M. Caboche, I. N. R. A. (Versailles)

Title of the research activity:
Genetic manipulation and regeneration in model and crop plants in vitro.

Key words:
Protoplasts, Potato, Electroporation, Electrofusion, gene expression

Reporting period: August 1986 - July 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The aim of the work is to develop and apply systems for the genetic manipulation of important European crop plants by exploiting cell biological techniques. In an integrated approach, model species (e.g. *N. plumbaginifolia*) and crop species (potato, sugar beet, tomato, brassiccas, cereals) are being examined. The transfer of genetic information ranging in size from complete genomes to partial genomes to single genes is being studied, with analysis of the fate of introduced DNA and characterization of plants regenerated following such manipulations.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

1. Development of methods of direct gene transfer of DNA constructs into protoplasts of potato and related wild species by electroporation.

2. Characterisation of somatic hybrids obtained by protoplast fusion (*S. tuberosum* + *S. brevidens*).

3. Evaluation of partial genome transfer in potato after irradiation of donor protoplasts and fusion with acceptor protoplasts.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY

Electroporation and electrofusion

Mesophyll protoplasts have been isolated from a range of commercial potato cultivars and the wild species *S. brevidens*. Tuber protoplasts have also been isolated from minitubers produced on shoot cultures *in vitro*. Commercial rectangular pulse and capacitor discharge electroporation systems have been assessed for electroporation. From this experience a workshop built capacitor discharge system was designed and is now used routinely for electroporation of a range of crop plant protoplasts (0-450V pulse, 50 - 2000 µF capacitance). Conditions for electroporation are: medium 20 mM KCl (mannitol osmoticum), pH 7.2, electrode chamber resistance 1.7 KΩ; pulse duration, τ, was calculated from equation $\tau=RC$ to be 85 ms for the applied field to decay to 1/e. After electroporation protoplasts were cultured for 48 h, percentage survival estimated and activity of marker genes assessed.

Comparison of electrofusion with chemical fusion using the model system *N. plumbaginifolia* has been carried out.

Partial genome transfer

Donor protoplasts of commercial potato cultivars have been irradiated (50 - 100 Krad) and fused with acceptor potato protoplasts by electrofusion.

2. RESULTS

Electroporation

Using 50 µF capacitor discharges (3 pulses) at a range of pulse voltages, the expression of the marker gene chloramphenicol acetyl transferase (35S CaMV promoter) was greatest at pulses of 225 Vcm^{-1} for mesophyll protoplasts of *S. brevidens* (Fig. 1A). This corresponded to protoplast viability of 50% (Fig. 1B). Similar results were obtained following electroporation of mesophyll protoplasts of commercial varieties (e.g. Maris Piper). With larger capacitors (80 to 150 µF), protoplast viability decreased but almost 100% conversion of chloramphenicol to acetylated forms was achieved.

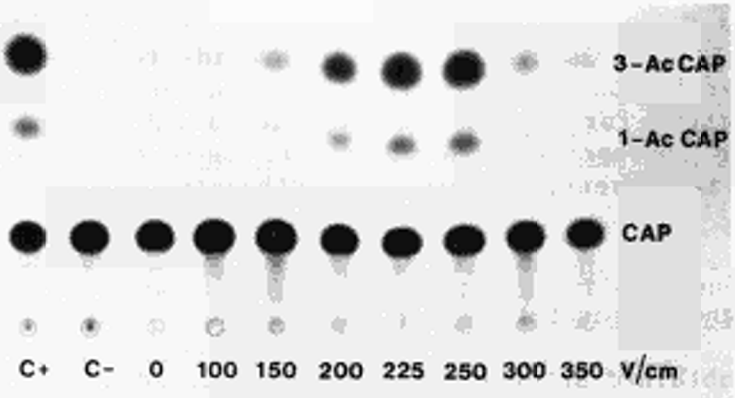


Fig. 1A. Transient expression assay for 35S CaMV-CAT gene electroporated into *S. brevidens* protoplasts with pulses of increasing field strength; C+ positive control, C- negative control, 1 and 3-AcCAP chloramphenicol acetylated at positions 1 or 3.

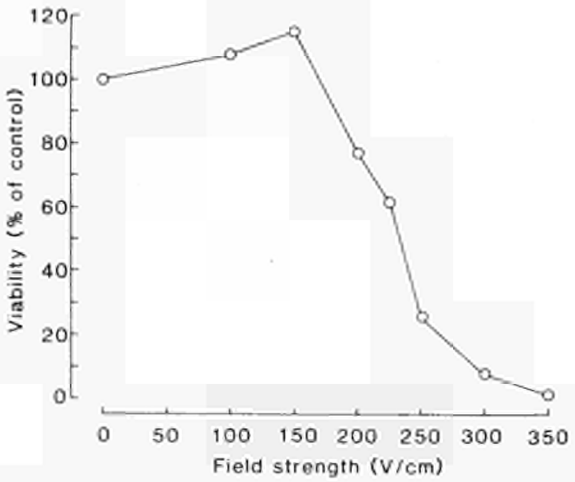


Fig. 1B Viability of mesophyll protoplasts of *S. brevidens* after electroporation with increasing field strengths.

Tuber protoplasts, isolated from cultivar Desiree, were also electroporated (as a system to study expression of tuber specific genes). Using the same marker gene, the optimum pulse voltage for transient expression was 150 Vcm^{-1}, and this is consistent with the larger size of tuber protoplasts compared to mesophyll protoplasts.

Experiments designed to obtain stable integration of genes introduced by electroporation are in progress.

Joint experiments on electrofusion and chemical fusion using complementary mutants of *N. plumbaginifolia* to assess relative efficiencies of the two techniques are in progress. Both approaches produce many hybrid colonies for this model species.

Field experiments of somatic hybrids

Somatic hybrid plants, produced both by chemical and electrical fusion, have been obtained between *S. tuberosum* (dihaploid) and *S. brevidens* (diploid). Tests for PLRV resistance have shown that this character, present in the *S. brevidens*, is expressed in somatic hybrids. In order to carry out field experiments on somatic hybrids to assess their agronomic potential, permission was required from a government committee, the Advisory Committee for Genetic Manipulation (ACGM). This was sought and obtained, with appropriate precautions to be taken during field growth. Field testing is currently underway at Rothamsted, and the plants are being monitored.

Partial genome transfer

Colonies obtained after electrofusion of irradiated donor and acceptor potato protoplasts have been cultured successfully, but plants have not yet been regenerated.

3. DISCUSSION

The results clearly show that electroporation is an efficient method of direct gene transfer into crop plant protoplasts. The production of tissue specific protoplasts (eg. tuber protoplasts) will allow rapid assessment of transient expression of suitable gene constructs without the need, in the first instance, for stable integration. However, some caution is required when this approach is used, since the tissue specific nature of isolated protoplasts is a temporary phenomenon.

Conditions of electroporation required for stable integration of selectable DNA for the production of transformed colonies are probably somewhat different (less harsh) than those required for transient expression. This is because it is necessary to have improved viability for colony formation. The use of this approach to study aspects of gene integration, and shotgun transformation, will be examined.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

1. PUBLICATIONS IN SCIENTIFIC JOURNALS

Tempelaar , M.J., Duyst, A., De Vlas, S.Y., Krol, G., Symonds, C. and Jones, M.G.K. (1987). Modulation and direction of the electrofusion response in plant protoplasts. Plant Science 48, 99-105.

De Vries, S.W., Jacobsen, E., Jones, M.G.K., Loonen, A.E.H.M., Tempelaar, M.J. Wijbrandi, J. and Feenstra W.J. (1987). Somatic hybridization of amino acid analogue-resistant cell lines of potato (*Solanum tuberosum*) by electrofusion. Theor. Appl. Genet. 73, 451-458.

Fish, N., Karp, A., and Jones, M.G.K. (1987). Improved isolation of dihaploid *S. tuberosum* protoplasts and the production of somatic hybrids between dihaploid *S. tuberosum* and *S. brevidens*. In Vitro, in press.

De Vries, S.E., Jacobsen, E., Jones, M.G.K., Loonen, A.E.H.M., Tempelaar, M.J., Wijbrandi, J. and Feenstra, W.J (1987). Electrofusion of biochemically well characterised nitrate reductase deficient *Nicotiana plumbaginifolia* mutants. Studies on optimisation and complementation. Plant Science, in press.

Jones, H., Tempelaar, M.J. and Jones, M.G.K. (1987) Recent advances in plant electroporation. In 'Oxford Surveys of Molecular and Cell Biology', Ed. Miflin, B.J., in press.

2. SHORT COMMUNICATIONS

Tempelaar, M.J. and Jones, M.G.K. (1986). Analytical and preparative aspects of electrofusion of plant protoplasts in a widely-spaced electrode system. Acta. Bot. Neerlandica, 35, 43-44.

de Vries, S.E., Jacobsen, E., Jones, M.G.K., Loonen, A.E.H.M., Tempelaar, M.J. and Wybrandi, J. (1986). Somatic hybridization of two amino acid analogue-resistant cell lines of *Solanum tuberosum* by means of electrofusion. Acta. Bot. Neerlandica, 35, 51.

Jones, M.G.K., de Both, M.T.J., Wu, Q.S., Sparks, C.A., Maddock, S.E. and Gleddie, S. (1986). Transient expression of CAT in wheat protoplasts. In: Proceedings of VI International Congress of Plant Tissue and Cell Culture, Minneapolis. Ed. D.A. Somers, B.G. Gengenbach, D.D. Biesboer, W.P. Hackett & C.E. Green, P.19.

Foulger, D., Fish, N., Karp, A., Bains, A., Cooper-Bland, S., Bright, S.W.J. and Jones, M.G.K. (1986). Protoplast fusion of *Solanum* species. In: Proceedings of the VI International Congress of Plant Tissue and Cell Culture, Minneapolis. Ed. D.A. Somers, B.G. Gengenbach, D.D. Biesboer, W.P. Hackett, & C.E. Green, p. 420.

Jones, M.G.K. (1986) Electrofusion of crop plant protoplasts. Proc. Congress Eur. Soc. Plant Physiol., Hamburg, p.49.

3. PATENTS - None

4. DOCTORATE THESIS. - Foulger D. (1987). Protoplast regeneration and somatic hybridization of potato (*Solanum tuberosum* L.).

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Exchange of materials

Reciprocal exchange of cultures between Rothamsted and Groningen; also of materials (cloned repetitive DNA sequences from Rothamsted and DNA selectable vectors from Groningen).

Exchange of staff

Staff from Groningen have worked at Rothamsted to carry out experiments on transient expression of DNA constructs in protoplasts.

Joint Experiments

Two series of joint experiments between Rothamsted, Groningen and Brussels, with participants from each laboratory, have been carried out at Brussels: to compare chemical and electrical fusion methods with model protoplasts (*N. plumbaginifolia*); and to compare methods of direct gene transfer by electroporation.

Joint meetings

Joint meetings to discuss progress and cooperation with all the participants in the joint project (Rothamsted, Groningen, Brussels, Versailles, Wageningen and Pavia) have been held routinely in rotation at different participating laboratories and at BAP meetings. These include at Ital, (Wageningen), Versailles and Louvain. Summaries of different techniques and cultures available in each participating laboratory have been circulated. The format of the joint meetings at participating laboratories is normally a two day, one night, workshop, with 2 representatives from each laboratory and more representatives from the host laboratory.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Rijksuniversiteit Groningen Contract no.: BAP - 0082 - NL

Project leader: M.J. TEMPELAAR
Scientific staff: G. van der Steege, R.A.L. Hartman, L.J. Drenth-Diephuis

Address: University of Groningen
Dept. of Genetics - Biological Centre
P.O. BOX 14
Kerklaan 30
NL - 9751 NN HAREN

Telephone no.: +31.50.632136 or +31.50.632092 or +31.50.632020

Telex no.:

Other contractual partners in the joint project:

M. Caboche, I. N. R. A. (Versailles)
F. Sala, University of Pavia
L. Van Vloten-Doting, Research Institute ITAL (Wageningen)
M. Jacobs, Vrije Universiteit Brussel
M.G.K. Jones, Rothamsted Experimental Station (Harpenden)

Title of the research activity:
Genetic manipulation and regeneration in model and crop plants in vitro.

Key words:
Electroporation, Electrofusion, Protoplasts, Transformation, DNA

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Genetic manipulation of plant cells employs an array of methods and techniques for the transfer of genetic elements and complexes varying from well defined DNA fragments, incorporated into plant vectors to complete nuclei by means of fusion. In between the two extremes, optimal transfer of e.g. naked DNA and cell organelles requires the further development of cellular engineering followed by investigations into integration and expression of the transferred material in the recipient cell. The development of this approach is the central part of this project.

In an integrated approach, a wide range of cell biological methods for genetic manipulation is examined.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

I Establishing DNA uptake by electroporation

II Generating hybrids with irradiated donor protoplasts

III Establishing principles underlying the various processes, ie

- -uptake properties of protoplasts in electric fields in relation to fusion properties and field/system parameters.
- -interactions in fusion hybrids between donor and recipient genomes, effects of irradiation on partial genome transfer.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1 METHODOLOGY

For transformation in electric fields, existing electrofusion generators have been used. In addition, a generator has been built to provide fields from combinations of different waveforms.

DNA constructs of several sizes were made containing various antibiotic-resistance genes, promotors and reporter genes.

Directed preparative electrofusion for partial genome transfer was done as descibed by Tempelaar and Jones (1985b), analytical electrofusion in small scale experiments to determine fusogenic properties according to Tempelaar and Jones (1985a).

Isolation and culture of protoplasts, screening for resistance or enzymatic reaction have been executed according to various procedures derived from several sources.

2 RESULTS

Fusogenic state and DNA uptake

1 The fusogenic state of various types of protoplasts has been chararacterized for a number of electric conditions (selected for probable importance in DNA uptake) by analytical fusion. The results fit a model based on pulse-duration dependant membrane breakdown voltage. This in turn is used to correlate electric-field induced membrane disturbances with uptake capabilities (Includes joint experiments with ITAL, Wageningen).

Some experiments involving transient expression after electroporation have been carried during visits in Brussels and Harpenden.

Transformants have been obtained, induced by electrical-field mediated DNA uptake, in Nicotiana plumbaginifolia using various constructs conferring antibiotic resistance. Constructs have been generated and plants have been exchanged for planned joint experiments (Harpenden) for transformation of potato.

Partial genome transfer by fusion

Initial conditions for fusion (directed electrofusion, see Tempelaar and Jones 1985b) and for irradiation (cytological verification of chromosome damage) have been established. Fusion products between irradiated material from a mutant Solanum tuberosum cell-line and Nicotiana plumbaginifolia have been characterized by selection and cytological analysis. Incompatibility problems have delaved progress however. Recently, these problems have been solved and data have begun to be collected from early stages which may predict the frequency of gene transfer in this particular cell-biological transformation system.

Hybrids and (to be confirmed) partial hybrids of (mainly) modelspecies have been generated in joint experiments (with the Brussels' and Harpenden labs) which also may permit a comparison between electric field induced fusion and chemical fusion in a model species.

References:

Tempelaar MJ and Jones MGK (1985a) Fusion characteristics of plant protoplasts in electric fields, Planta 165, 205-216

Tempelaar MJ and Jones MGK (1985b), Directed electrofusion between protoplasts with different responses in a mass fusion system, Plant Cell Reports 4, 92-95

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.2 INTERNAL REPORTS

Tempelaar MJ (1986), Electrical Field manipulations for partial genome transfer. Rept of Meeting of contractants of BAP project 165, Versailles

Steege G van der, Hartman RAL, Drenth-Diephuis LJ, Faber R, Feenstra WJ, Tempelaar MJ (1987), Genetic Manipulation of model and cropplants by fusion and transformation, in : Abstracts from BAP meeting on genetic and cellular engineering of plants and micro organisms important for agriculture, Louvain-la-Neuve, 114-115

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	No
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Material exchanged:

With Harpenden (UK): Plant material for transformation experiments
DNA vectors with repititive plant DNA

With Brussels: Hybrid plant material for chromosome analysis

Joint Experiments: (during visits, host location underlined)

Harpenden/Groningen: Transient expression induced by DNA uptake in electric fields

Brussels/Groningen/Harpenden: Fusion experiments to generate hybrids and to compare fusion methodology; electropration (two visits)

Wageningen/Groningen: Uptake of molecules by protoplasts in electric fields, flow cytophotometry.

Joint Meetings:

Participation in two half-yearly meetings of contract partners to present and exchange data, coordinate efforts, organize exchanges of material and joint experiments:

Versailles 23-24 Oct '86, Louvain-la-Neuve 23-26 March '87

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: V. U. B., St.Genesius Rode
Contract no.: BAP - 0111 - B

Project leader: M. JACOBS, I. NEGRUTIU
Scientific staff: M. Ghislain, Y. Famelaer

Address: Vrije Universiteit Brussel
Laboratorium voor Plantengenetica
Paardenstraat 65
B - 1640 SINT GENESIUS RODE

Telephone no.: +32.2.3584547

Telex no.: 61051 VUBCO B

Other contractual partners in the joint project:

M.G.K. Jones, Rothamsted Experimental Station (Harpenden)
M.J. Tempelaar, University of Groningen
L. Van Vloten-Doting, Research Institute ITAL (Wageningen)
F. Sala, University of Pavia
M. Caboche, I. N. R. A. (Versailles)

Title of the research activity:
Genetic manipulation and regeneration in model and crop plants in vitro.

Key words:
Protoplast, Gamma-fusion, Asymmetric hybrid plants, Direct gene transfer

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

It is of fundamental importance to develop an array of techniques in genetic manipulation of plants, covering the transfer of single genes, of blocks of genes and chromosomes, and establish extrachromosomal vectors.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Use the above mentioned techniques to study problems on developmental genetics and develop alternative approaches to plant breeding.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

A. <u>Cell-mediated gene/chromosome transfer</u>.

Classical plant breeding demonstrated that in the cultivated wheats cytogenetics and field selection can be successfully combined as means of making directed genetic changes by chromosome engineering (Law, 1983). Protoplast fusion between species belonging to distinct genera, tribes, or families produced sterile plants of non-morphogenic cell cultures, the elimination of chromosomes in such fusion products being most frequently random and progressive.

The production of fertile parasexual plant combinations is believed to lie in generating highly assymmetric "hybrids", by transferring single chromosomes or chromosome fragments. Gamma-fusion (fusion of protoplasts with an irradiated partner) opens up such ways of investigation. The donor-receptor technique, making use of various selectable and/or molecular markers, gives us the possibility to assess the role played in chromosome engineering by the following, mutually interacting factors: irradiation damage, somatic incompatibility reactions, specific selection pressure(s), *in vitro* culture passage

and plant regeneration. To what extent such complex methodology enables us to direct the unidirectional elimination of redundant donor genetic material and to generate recombination events, is shown below.

(1) The incompatibility barriers.

The recipient *N. plumbaginifolia* (2n=20) protoplasts were fused with non-irradiated and gamma-irradiated protoplasts of *N. tabaccum*, *N. sylvestris*, *Datura innoxia*, *Atropa belladonna*, *Petunia hybrida*, *Lycopersicon aesculentum*, *Solanum tuberosum* and *Hyoscyamus muticus*, *Brassica napus*, *Arabidopsis thaliana*, *Medicago sativa*, *Beta vulgaris*. Only the combinations with tobacco, *N. sylvestris*, *Atropa belladonna* and sugarbeet were successful.

The gamma-fusion did not allow us to extend the range of successful fusion combinations. Thus, the heavy irradiation dammage does not seem to be sufficient in breaking or by-passing the existing post-zygotic incompatibility barriers. However, access to wider cross combinations seems to be possible by protoplast fusion as compared to sexual crosses.

(2) Irradiation, fragmentation of donor chromosomes, and regeneration of asymmetric hybrid plants.

Irradiation favours a rapid and unidirectional elimination of most or part of the donor chromosomes. The donor genome undergoes important fragmentation which increases with the dose. Deleted or minichromosomes are maintained mitotically and/or meiotically. By analysing sufficient clones (20 to 100) one can identify individuals with very few donor chromosomes. In practical terms, the method holds promise at present for chromosome transfer in interspecific intrageneric combinations at least (for example between cultivars and wild species).

Fertile plants can be regenerated from asymmetric somatic hybrids. They usually have a recipient-type phenotype. Recipient-type asymmetric hybrids may represent cases were large portions of the maintained donor chromosomes became "silent" because of radiation dammage and/or "under-expression" in a highly asymmetric genomic context. However, gamma-fusion may facilitate the obtention of plants containing or expressing but few foreign genes.

Sub-clonal analysis in asymmetric hybrid plants showed that slight variation in genotype occurs. Further work is required to establish whether the passage through meiosis can exert a stabilizing effect on an asymmetric hybrid structure.

(3) Elimination-maintenance of the donor chromosome.

In the absence of specific selection pressures it is probably impossible at present to control the extent of chromosome elimination or to direct the maintenance or elimination of specific chromosomes.

Regeneration of recipient-type plants (developmental pressure) may operate to select specifically against or in favour of certain chromosomes of the donor species.

(4) Recombination of donor genetic material into the host genome.

A constant feature in fusion experiments is the physical separation within a common nucleus of the chromosomes of the fusion partners (Gleba *et al.*, 1987), which may explain the apparent lack of recombination between donor and recipient chromosomes in asymmetric hybrids (normal sets of the recipient genome are systematically observed). It represents so far a serious handicap in the transfer of chromosome segments by means of gamma-fusion.

B. Gene transfer experiments.

Direct gene transfer into plant protoplasts has been recently developed, and conditions for high frequency transformation of SR_1 tobacco protoplasts established (Shillito *et al.*, 1985). In a recent paper (Negrutiu *et al.*, 1987a) we analysed numerous transformation parameters in a comparative study on SR_1 *Nicotiana tabacum* and *N. plumbaginifolia*, and reported on a simple chemical technique for very efficient protoplast transformation. It is based on the synergistic interaction of $MgCl_2$ and PEG. The technique yielded up to 1400 transformants per $3x10^5$ treated *N. tabacum* protoplasts (up to 4.8 % of the survivors, late selected clones). Using *N. plumbaginifolia*, the frequencies were 10-fold lower, indicating that the "competence" for transformation has a species-specific component.

The technique also yielded high and reproducible values in transient expression experiments with protoplasts from both species.

Cloned sequences from total DNA of *N. plumbaginifolia* that exhibited ARS function in yeast (M. Horth, ULB Brussels) were assayed for autonomous replication in plant cells and showed to stably integrate within the plant genome (Negrutiu *et al.*, 1987 b).

Gleba *et al.* (1987) PNAS 84, 3709
Law (1983) Genetic Engineering: Principles and Methods (Setlow J.W., Hollaender A. eds.),Vol 5, Plenum Press, 157;
Negrutiu *et al.* (1987a) Plant Mol Biol 8, 363;
Negrutiu *et al.* (1987b) Plant Physiol Biochem (in press);
Shillito *et al.* (1985) Biotechnology 3, 1099

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1. I. Negrutiu, R. Shillito, I. Potrykus, G. Biasini and F. Sala : Hybrid genes in the analysis of transformation conditions. I. Setting up a simple method for direct gene transfer in plant protoplasts. Plant Molecular Biology 8 : 363-373 (1987).

Several other manuscripts are in preparation:

- *universal hybridizer*

- *comparison between PEG- and electrofusion.*

- *production of asymmetric hybrid plants (3 papers)*

- *genetic analysis and linkage in transgenic plants.*

IV.3. a patent was deposited through CIBA-GEIGY (Basel) on direct gene transfer.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Contractual partners meet once a year to discuss their projects, results and exchange technical information. A list of available techniques, plasmids, cloned genes, plant material has been established and made available within the group. Joint experiments on protoplast fusion and gene transfer have been performed in Rothamsted and Brussels.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: ITAL, Wageningen

Contract no.: BAP - 0083 - NL

Project leader: L. VAN VLOTEN-DOTING

Scientific staff: H.C.P.M. van der Valk, H.A. Verhoeven, D. Sree Ramulu, J. Blaas, P. Dijkhuis

Address: Research Institute ITAL
P.O. Box 48
NL - 6700 AA WAGENINGEN

Telephone no.: +31.8370.91263 or +31.8370.91911

Telex no.: 45856 ITAL NL

Other contractual partners in the joint project:

F. Sala, University of Pavia
M. Caboche, I. N. R. A. (Versailles)
M. Jacobs, Vrije Universiteit Brussel
M.J. Tempelaar, University of Groningen
M.G.K. Jones, Rothamsted Experimental Station (Harpenden)

Title of the research activity:

Genetic manipulation and regeneration in model and crop plants in vitro.

Key words:

Chromosome isolation, Synchronization, Chromosome transfer, Micronuclei, Microinjection, Flow cytometry

Reporting period: June 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Genetic manipulation of plant cells employs an array of methods and techniques for the transfer of genetic elements and complexes, varying from well defined DNA fragments incorporated into plant DNA vectors to complete nuclei by means of cell fusion. In between the two extremes, optimal transfer of e.g. naked DNA and cell organelles requires the further development of cellular engineering followed by investigations into integration and expression of the transferred material in the recipient cell. The development of this approach is the central part of this project.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

1) Improvement of the yield of isolated metaphase chromosomes by optimizing the culture, synchronization and isolation procedures.
2) Development of a system in plant cells for the induction and isolation of micronuclei.
3) Development of a microinjection system suitable for the injection of organelles into plant cells.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY

Flow cytometric analysis of cellular DNA content was applied to optimize the cell cycle synchronization. Cells were incubated with increasing concentrations (0-10 mM) of hydroxy urea (HU) for 24 h to block the cells in S-phase.

Subsequently, cells were washed free from HU and arrested in metaphase by the addition of 10 ppm amiprophosmethyl (APM), immediately or 6-8 h after washing (Verhoeven et al. 1987).

DNA histograms were determined and mitotic indices were calculated from lactopropionic orcein-stained squash slides of the cells. Also the occurrence of micronuclei was determined from these slides.

Chromosomes and micronuclei were isolated from synchronized cultures, and analysed and sorted by flow cytometry (De Laat et al. 1987).

2. RESULTS

Cells incubated with 10 mM HU for 24 h were arrested in S phase (fig. 1). Lower concentrations (1 and 2.5 mM) were not sufficient to inhibit DNA synthesis.

Higher concentrations resulted in slower recovery of the HU block after washing the cells free from HU.

After washing, and in the presence of 10 ppm APM, cells continued the cell cycle until they reached metaphase. At this point, passage to anaphase was completely prevented by the action of APM on the spindle, causing the scattering of metaphase chromosomes throughout the cell.

For chromosome isolation, protoplasts were isolated at this stage, and lysed in chromosome buffer for flow cytometric analysis and sorting. Large quantities of chromosomes (> 15.000) were sorted on the basis of their DNA content.

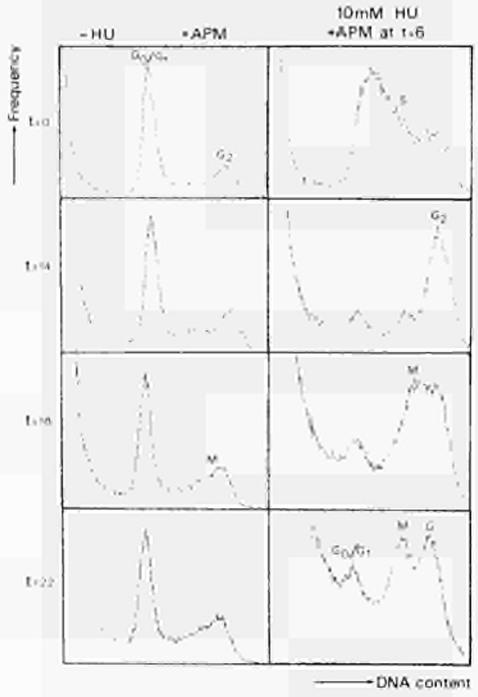


Fig. 1.

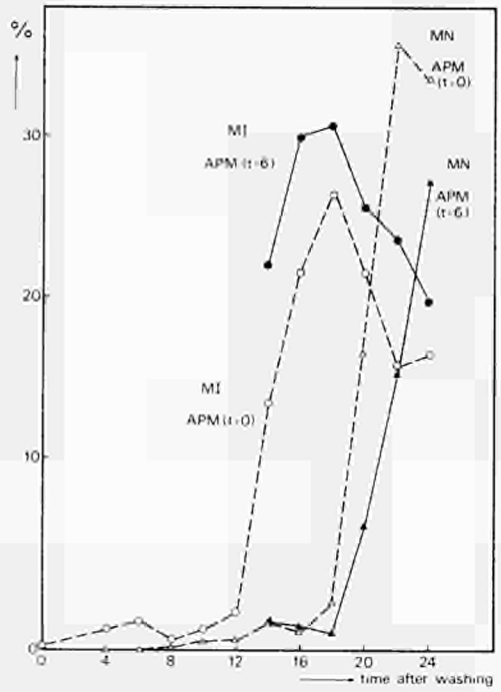


Fig. 2.

Fig. 3.

Fig. 1. Frequency distribution of DNA content of protoplasts from HU and APM treated suspension cells, at different times after washing out HU.

Fig. 2. Time course of mitotic index and occurrence of micronuclei in suspension cells during synchronization.

Fig. 3. Clump of fluorescently labelled mitochondria (arrow), injected into an unstained regenerating protoplast.

After prolonged exposure to APM, the chromosomes formed small groups, decondensed and formed micronuclei. Micronuclei were isolated employing the procedure for metaphase chromosomes, and sorted on their DNA content. In this way, subpopulations were obtained, containing 1-2 chromosomes, as was confirmed by microdensitometric analysis of Feulgen stained, sorted fractions.

Because micronuclei interfere with the isolation of metaphase chromosomes, the effect of delayed addition of APM after removal of HU was investigated. It appeared that APM addition could be delayed by 6-8 h, without affecting the mitotic index, whereas the amount of micronucleated cells was substantially reduced (fig. 2).

Microinjection has been improved: On average, 50% of the cells survive injection with needles of 5 μm diameter. We have obtained microcalli from cells, in which cytoplasm from a donor protoplast had been injected (fig. 3).

3. DISCUSSION

With the improved techniques, it is possible to isolate large numbers of metaphase chromosomes and micronuclei for subsequent transfer into recipient cells by microinjection. So far, no reports on the induction and mass isolation of micronuclei from plant cells have been published. Because micronucleation is a well established technique in animal cells, by which the human genome has been mapped (reviewed in Fournier 1982), it could also offer new possibilities in plant genetic manipulation. Both in plants and animals, the introduction of metaphase chromosomes by microinjection or poly-ethylene glycol induced uptake has so far never resulted in the construction of a mitotically stable addition line (Griesbach 1987, Szabados *et al.* 1981, De Jonge *et al.* 1985). By microcell mediated chromosome transfer, however, the transfer of intact chromosomes has been achieved with mammalian cells (Fournier 1982).
This opens prospects for similar applications of micronuclei in plants.

REFERENCES

De Jonge, A.J.R., De Smit, S., Kroos, M.A. and Reuser, A.J.J. (1985). Hum. Genet. 69: 32-38.

De Laat, A.M.M., Verhoeven, H.A., Sree Ramulu, K. and Dijkhuis, P. (1987). Planta, in press.

Fournier, R.E.K. (1982). In: Techniqes in Somatic Cell Genetics (Ed. J.W. Shay), Plenum Press, New York, London, pp 309-327.

Griesbach, R.J. (1987). Plant Sci. 50: 69-77.

Szabados, L., Hadalaszky, G., Dudits, D. (1981). Planta 151: 141-145.

Verhoeven, H.A., De Laat, A.M.M., Sree Ramulu, K., Dijkhuis, P., Van Vloten-Doting, L. (1987). In: Genetic and cellular engineering of plants and microorganisms important for agriculture, Commission of the European Communities meeting, Louvain-la-Neuve, March 23-26, pp. 119-120.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1 PUBLICATIONS IN SCIENTIFIC JOURNALS, MONOGRAPHS, ..

Verhoeven, H.A., Blaas, J. (1987) Direct cell to cell transfer of cytoplasm and organelles by microinjection. Plant Sci. (submitted).

De Laat, A.M.M. and Blaas, J. (1987). An improved method for protoplast microinjection suitable for transfer of entire plant chromosomes. Plant Sci. 50: 000-000 (in press).

De Laat, A.M.M., Verhoeven, H.A., Sree Ramulu, K. and Dijkhuis, P. (1987). Efficient induction by amiprophos-methyl and flow cytometric sorting of micronuclei in N. plumbaginifolia. Planta, in press.

Sree Ramulu, K., Verhoeven, H.A. and Dijkhuis, P. (1987). Mitotic dynamics of micronuclei induced by amiprophos-methyl and prospects for chromosome-mediated gene transfer in plants. Submitted to Theor. Appl. Genet.

IV.2 SHORT COMMUNICATIONS, INTERNAL REPORTS, ..

Verhoeven, H.A., De Laat, A.M.M., Sree Ramulu, K., Dijkhuis, P., Blaas, J. and Van Vloten-Doting, L. (1987). Induction, isolation and flow cytometric sorting of micronuclei from cell suspension cultures of Nicotiana plumbaginifolia. Acta Bot. Neerl. 36: 64.

Verhoeven, H.A. and Blaas, J. (1987).
Vital staining of mitochondria for protoplast manipulation. Acta Bot. Neerl. 36: 63.

Verhoeven, H.A. and Van der Valk, H.C.P.M. Flow cytometric analysis of cellular DNA content after hydroxy urea treatment of N. plumbaginifolia suspension cells. Acta Bot. Neerl. in press.

TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)	Yes	
Joint meeting(s)	Yes	

Descriptive information for the above data.

Exchange of plasmids, fluorescent dyes and protocols took place with Dr. Tempelaar, University of Groningen, in a series of joint experiments on the use of flow cytometry in combination with electroporation.
In these experiments, expertise from the Groningen team on electroporation, and their equipment was combined with the flow cytometer facility in Wageningen.

Several meetings (Wageningen 1986, Versailles 1986 and Louvain-La-Neuve 1987) with the contractual partners of project group 165 took place, on which occasions results, methodologies and future plans were exchanged and discussed.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: University of Pavia

Contract no.: BAP - 0084 - I

Project leader: F. SALA

Scientific staff: R. Cella, S. Castiglione, L. Marchesi, G. Gavazzi, C. Tonelli, M. Terzi, F. Lo Schiavo

Address: University of Pavia
Dept. of Genetics and Microbiology
via S. Epifanio 14
I - 27100 PAVIA

Telephone no.: +39.382.303852

Telex no.:

Other contractual partners in the joint project:

M.G.K. Jones, Rothamsted Experimental Station (Harpenden)
M.J. Tempelaar, University of Groningen
M. Jacobs, Vrije Universiteit Brussel
L. Van Vloten-Doting, Research Institute ITAL (Wageningen)
M. Caboche, I. N. R. A. (Versailles)

Title of the research activity:

Genetic manipulation and regeneration in model and crop plants in vitro.

Key words:

Cultured cells, Somaclonal variation, Lycopersicon esculentum, Repeatitive DNA, Gene Transfer

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

In an integrated approach with the other partners of the joint project, the following cell biological methods for genetic manipulation of crop plants are being examinated:
1 - regeneration systems from single cells to plants; 2 - transfer by protoplast fusion of defined genetic attributes between different plant species; 3 - isolation and transfer of single chromosomes; 4 - use of existing and construction of new selectable DNA vectors; 5 - entry and fate of DNA introduced by direct gene transfer; 6 - characterization of regenerated plants following these manipulations.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

These were: a) to induce, select and characterize mutants in agronomically important and model plants; b) to set up simple methods for direct gene transfer in plant protoplasts.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Induction, selection and characterization of marker genes

a) In tomato: plants carrying mutant traits have been produced both by exploiting somaclonal variation in plants regenerated from callus and by chemical mutagenesis (ethyl methane sulphonate) at the seed and pollen level. Scoring of nonparental phenotypes has been performed in the second generation in order to recover recessive mutations, to overcome problems related to chimerism in first generation plants, to restrict the study to fertile variants and to obtain data on genetic segregation.

Isolated mutants can be grouped into one of the following categories: seedling lethality, male sterility, tolerance to NaCl and to drought, resistance to Verticillium, short stature, change in number of later shoots or in leaf shape.
The results add further strengh to the proposed used of somaclonal variation to recover at high frequency new genetic variability from existing crop varieties. In fact, the two sources of variability (somaclonal variation and EMS mutagenesis) differ in their effects changing the spectrum and frequency of the mutants.

b) In model organisms (Daucus carota and Medicago coerulea). Carrot cell lines resistant to methotrexate (Mtx) have been selected by plating suspension cultured cells in the presence of the inhibitor. Among Mtx-resistant lines, those which showed normal Mtx uptake were analyzed for the level of the target enzyme: dihydrofolate reductase (DHFR). All tested lined were found to overproduce the enzyme (up to 7 times), while in no cases resistance could be ascribed to inactivation of the inhibitor or to the presence of on altered form of the enzyme. DHFR overproduction was stable even in cells grown for 6 months in the absence of the inhibitor. Measurement of DHFR activity in all lines grown in the presence of Mtx was made possible by the use of polyclonal rabbit antibodies against the carrot enzyme.

When these antibodies were used to probe semicrude extracts of different plants (by western blot analysis), low or no recognition was found in the case of Nicotiana plumbaginifolia and of N. tabacum. This suggested the possible use of DHFR as a specific marker for the charcterization of asymmetric hybrids between distantly related species. Experiments are now under way, in collaboration with dr. Dudits (Szeged, Hungary) to verify this possibility by analyzing fusion products between N. tabacum leaf protoplasts (X-ray irradiated) and Mtx-resistant carrot protoplasts.

Nineteen chlorate-resistant variants have been isolated after mutagenesis from cells of Medicago coerulea. The level of nitrate reductase activity is variable in these lines and ranges from 100 to less than 5% of the wild type level.

Methylammonium-resistant variants were also isolated from the same cells. They show a different regulation of nitrogen utilization. In particular, the enzymatic level of nitrate reductase, which in wild type cells is sensitive to ammonium repression, is much less affected in the variants. Differences are also seen in the regulation of other functions of the nitrogen-utilizing pathway: xanthine dehydrogenase and, possibly, adenine uptake.

Gene transfer

In collaboration with I. Negrutiu (a Vrije Universiteit partner in this joint project) and with R. Shillito and I. Potrykus (F.M.I., Basel) simple techniques have been worked out for the chemical (PEG-induced) transformation of N. plumbaginifolia protoplasts. Numerous transformation parameters have been analyzed in a comparative study on N. tabacum and N. plumbaginifolia and the importance of several major factors has been established in optimizing transformation schemes. The optimized technique is based on the synergic action of $MgCl_2$ and PEG. Specific requirements are different for the two species and the transformation frequency for N. plumbaginifolia is anyhow in the order of 10-fold lower than in the cases of N. tabacum.

Preliminary results are also showing that electroporation (without PEG treatment) can be affectively used to substitute PEG-induced transformation in the case of N. plumbaginifolia.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

1) Cella R., Albani D., Carbonera D., Etteri L., Maestri E. and Parisi B.: Selection of Methotrexate-resistant cell lines in Daucus carota: biochemical analysis and genetic characterization by protoplast fusion. J. Plant. Physiol. 127, 135-146, 1987.

2) Zheng K.L., Castiglione S., Biasini M.G., Biroli A., Morandi C. and Sala F.: Nuclear DNA amplification in cultured cells of Oryza sativa L. Theor. Appl. Genet. 74: 65-70, 1987.

3) Sala F.: Trasferimento genico in vegetali superiori: risultati e prospettive. Atti Soc. Agr. Lomb., 74: 17-32, 1986.

4) Negrutiu I., Shillito R., Potrykus I., Biasini M.G. and Sala F.: Hybrid genes in the analysis of transformation conditions. I. Setting up a simple method for direct gene transfer in plant protoplasts. Plant Mol. Biol. 8: 363-373, 1987.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)	Yes	
Joint meeting(s)	Yes	

Descriptive information for the above data.

1) Exchange of plasmids and of plants with N. Negrutiu (Vrije Universiteit Brussel).
2) Joint experiments with N. Negrutiu (in collaboration with R. Shillito and I. Potrykus, F.M.I., Basel) to develope simple methods for direct gene transfer in protoplasts of Nicotiana plumbaginifolia and Nicotiana tabacum. The results have recently been published in: Plant Mol. Biol. 8, 363, 1987.
3) Joint meeting with the other contractual partners have been held at:
   a) Wageningen (NL) ITAL. January 30, 1985. This meeting was set up in order to organize co-ordination of research and collaborations between the proposing laboratories in preparation of the start of B.A.P.
   b) Versailles (France). I.N.R.A. Oct. 23-24, 1986. A two-day meeting to present and discuss results coming out from the different laboratories of the joint project.
   c) Louvain-la-Neuve (Belgium). March 24, 1987. A meeting held during the General BAP Meeting to discuss several points related to the joint project.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: I. N. R. A., Versailles

Contract no.: BAP - 0085 - F

Project leader: M. CABOCHE

Scientific staff:

Address:
I.N.R.A.
Route de Saint Cyr
F - 78000 VERSAILLES

Telephone no.: +33.1.30217422 or +33.1.39507522

Telex no.: 695269 INRAVER F

Other contractual partners in the joint project:

F. Sala, University of Pavia
L. Van Vloten-Doting, Research Institute ITAL (Wageningen)
M. Jacobs, Vrije Universiteit Brussel
M.J. Tempelaar, University of Groningen
M.G.K. Jones, Rothamsted Experimental Station (Harpenden)

Title of the research activity:

Genetic manipulation and regeneration in model and crop plants in vitro.

Key words:

Protoplasts, Potato, Electroporation, Electrofusion

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The general objective of the joint project is to develop new techniques of manipulation of the genome of higher plants and to adapt these techniques to plants of agronomic importance. Methodologies of somatic hybridization will be improved when possible and adapted to new purposes such as the introgression of foreign genetic material by gamma-fusion or introduction of purified chromosomes into a model species. Direct gene transfer techniques will be optimized and adapted to plants of agronomic importance.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

A technique of direct gene trasfer into tobacco involving the use of liposomes has been developed in our laboratory. The objectives of our laboratory for the reporting period were to compare this direct gene transfer technique to other gene transfer techniques such as electroporation and to further characterize the obtained transformants. Another part of the project was to develop a new selectable marker for plant transformation: the structural gene for nitrate reductase.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Methodology

Liposome-mediated gene transfer involves the fusion with mesophyll protoplasts of negatively charged phosphatidylserine-cholesterol liposomes loaded with plasmid DNA. A step of sonication is required during the encapsulation procedure, leading to the random linearization of plasmids. With tobacco mesophyll protoplasts as recipient cells and using a plasmid carrying a kanamycin resistance selectable marker, transformation frequencies ranging from 10^{-5} to 10^{-4} were achieved. PEG and Ca^{++} were required for optimal transformation.

Electroporation was also used to introduce foreign DNA into tobacco mesophyll protoplasts. Electroporation conditions were optimized on the basis of transient expression signals obtained after transfer of a plasmid carrying a CAT reporter gene. Various factors were found to affect electroporation conditions including the size of capacitor, the lenght and

intensity of electric pulses, the ionic strenght of the medium. Optimal conditions of electroporation were observed with a 16 uF capacitor charged at 300 volts and discharged in a 1 cm cubic square electroporation chamber containing 2.10^6 protoplasts resuspended in a medium containing 5 mM KCl and 0.5 M mannitol. Using these electroporation conditions kanamycin resistant clones were obtained at a frequency of 0.2-1%. Electroporation was found to be approximately 20 times more efficient than the liposome procedure on the basis of transient expression signal measurements.

2. Results and Discussion

The comparison of optimal conditions of transformation of tobacco mesophyll protoplasts by liposome treatment or by electroporation has shown that electroporation is more suited to routine transformation experiments than liposome transfection. Electroporation is presently being adapted for the transformation of plants of agronomic importance such as potato, tomato, lettuce and rapeseed. Liposome-mediated transformation has a low reproducibility in terms of transformant frequencies. However this procedure was efficient enough to perform cotransfer experiments and allowed us to demonstrate that a 4.3 kb EcoR1 fragment of the T-DNA of *A. rhizogenes* alone confers the hairy root phenotype. The liposome procedure seems to be well adapted to experiments of viral RNA transfection (up to 80% of the protoplasts with liposomes) but seems inappropriate for direct gene transfer if large DNA fragments need to be transferred. Liposomes may be a good DNA carrier for organelle transformation since they interact with the outer mitochondrial membrane. A series of 100 independant transformants selected for kanamycin resistance were isolated at random and regenerated. A significant proportion of the regenerated plants showed morphology abnormalities. Whether these abnormalities were related to the insertion of foreign sequences inserted in the host genome was studied by progeny transmission analysis. Among twenty independant transformants expressing and/or segregating a new phenotype in their progeny none of them cosegregated this new genetic trait with kanamycin resistance. It appears therefore that these abnormalities result rather from somaclonal variations than from insertion mutagenesis of the transfected sequences. Work is under progress to further characterize two transformants Ka 2 and Ka 120 apparently unable to transmit the kanamycin resistance trait through pollen grains.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1.

GUERCHE P. BELLINI C. LE MOULLEC JM and CABOCHE M. Use of a transient expression assay for the optimization of direct gene transfer into tobacco mesophyll protoplasts by electroporation. Accepted for publication in Biochimie.

JOUANIN L, VILAINE F, TOURNEUR J, TOURNEUR C. PAUTOT V, MULLER JF, CABOCHE M. Transfer of a 4.3 kb fragment of the TL-DNA of *Agrobacterium rhizogenes* strain A4 confers the pRi transformed phenotype to regenerated tobacco plants. Accepted for publication in Plant Science.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	No
Exchange of staff	No
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

- Joint experiments are planned with the laboratory of Professor Jacobs. This final aim of the project would be to demonstrate the complementation of a nitrate reductase deficiency expressed in *N. plumbaginifolia* mutants by somatic hybridization with other plant species such as sugar beet. In gamma fusion experiments for instance the presence of foreign genetic material can sometimes no longer be detected by karyotype analysis. Evidence for true genetic complementation requires the use of specific probes for the structural gene (cDNA) of nitrate reductase or for the corresponding protein monoclonal antibody. These probes are being characterized in our laboratory.

- Joint meetings of the contractants to the program are being organized every six months. The first of these meetings was organized last Autumn in our Institute. Each contractant laboratory presented the latest developments of this project.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: M. P. I., Köln Contract no.: BAP - 0013 - D

Project leader: H. LÖRZ
Scientific staff: R. Töpfer (April-October 1986)
B. Junker (since January 1987)
R. Lührs (non EC-funding)

Address: Max-Planck-Institut für Züchtungsforschung
Egelspfad
D - 5000 KÖLN 30

Telephone no.: +49.221.5062316

Telex no.: 8881028 MPIZ D

Other contractual partners in the joint project:

Y. Demarly, Université Paris-Sud (Orsay)

Title of the research activity:

Research on genetic transformation and plant regeneration from protoplasts of wheat and barley.

Key words:

Gramineae, Cereals, Protoplasts, Gene transfer, Genetic manipulation

Reporting period: April 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

a) Development of reproducible and efficient culture conditions for plant regeneration from isolated protoplasts of wheat and barley.

b) Development of methods for the transfer of isolated genes into cereal cells.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Our efforts are concentrating on protoplast culture of barley and the possible use of Agrobacterium as a natural vector system for cereal transformation.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Methodology

Cell suspensions of barley (Hordeum vulgare L.) were established from embryogenic callus cultures cv. "Dissa" and cv. "Golden Promise". Protoplasts were isolated 2-3 days after subculture of the suspensions and then cultured in liquid or agarose- solidified medium. Modified Kao- and N6- media were used for culturing the protoplasts. In the beginning cultures are kept in the dark and only when embryogenic or organogenic structures have been detected, cultures are transferred to light. Agarose-solidified media with different combinations and concentrations of phytohormones are used to improve plating efficiency and the regeneration protocol.
For Agrobacterium-mediated gene transfer the procedure of Graves and Goldman (Plant Mol. Biol. 1986, 7: 43-50) was followed. Additionally in vitro cultures of maize and wheat were treated with agrobacteria according to the so-called "co-cultivation"- and "leaf-disc-method" originally developed for dicotyledonous species.

Results

Progress towards plant regeneration from barley protoplasts was achieved by selection of genotypes with good response in tissue culture. From more than 40 lines and varieties tested, cvs. "Golden Promise" and "Dissa" gave best results in terms of callus formation from immature embryos, induction of somatic embryogenesis, and efficiency of plant regeneration (Lührs and Lörz, Theor. Appl. Genet. 1987, in press). Suspension cultures have been established from cv. "Dissa" and cv. "Golden Promise" which provided suitable material for the isolation of protoplasts. The cultured protoplasts reform cell walls and 0.1-10% form colonies. The formation of embryogenic and morphogenic structures in the protoplast-derived cultures is less frequent, and only few plantlets have been regenerated. So far all regenerants are chlorophyll deficient (albinos) and have to be kept under in vitro conditions. The protoplasts of barley provide a suitable material for transformation studies applying direct gene transfer methods.

Initial work towards the construction of the plasmid pRT100 - pRT104, carrying the 35S promoter and the polyadenylation signal of CaMV strain Cabb B-D, was done within the frame of this EC-project (Töpfer et al. 1987). Additional recombinant DNA constructions were done in the department "Genetische Grundlagen der Pflanzenzüchtung" headed by Prof. Dr. J. Schell to provide new marker genes and efficient selection schemes.

Considering the difficulties of plant regeneration from protoplasts alternative ways are sought to transfer foreign genes into cereal cells. Agrobacterium-mediated gene transfer is studied as one of such alternative approaches. Different strains of A. tumefaciens carrying NPT as a marker gene have been used for infection of maize and wheat. In both species NPT-activity could be measured 7-14 days after infection of the seedlings, but in these experiments it could not be absolutely excluded, that the enzyme activity measured was caused by bacterial activity.

Discussion

The protoplast culture work is a continuation of previous work in the laboratory. Initially numerous genotypes of barley (spring- and winter-type) have been tested for their response in tissue culture. Subsequently cell suspensions have been established from so-called "good" tissue culture varieties. Work with other cereal species such as rice, maize or Panicum has shown, that embryogenic cell suspensions are the most promising source of material for the isolation of totipotent protoplasts. The regeneration of albino plantlets from barley protoplasts confirmed this strategy. A problem still remains in the fact that embryogenic and regeneration capacibility frequently is lost during prolonged subculture, and that regenerants often show chlorophyll deficiency and other growth abnormalities. New suspension cultures therefore have to be established repeatedly to provide suitable material for protoplast isolation.

In the first period of the project chimaeric genes and plasmids have been constructed which could be used in addition to the already existing constructions, but were also of special interest as the "strong" promoter from the 35S gene of Cauliflower Mosaic Virus can be combined with selectable genes such as NPT or marker genes such as GUS (ß-glucuronidase).

Direct gene transfer into protoplasts has been developed in the last few years as a reproducible, efficient, and generally applicable system. For barley and wheat regeneration of the protoplast remains the bottleneck. To develop transformation procedures which are independent of the use of cultured protoplasts, Agrobacterium tumefaciens is studied as a possible vector system for cereals. Enzyme activity of the marker gene NPT could be detected in maize seedlings several days after treatment with genetically modified A. tumefaciens strains carrying this gene, but molecular evidence for the integration of the foreign genes into the plant genome has still to be provided. Further studies therefore are necessary to analyse in detail the processes during and after treatment of cereal tissue with agrobacteria.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

B. Junker, J. Zimny, P. Lührs, H. Lörz (1987)

Transient expression of chimaeric genes in dividing and non-dividing cereal protoplasts after PEG-induced DNA uptake.
Plant Cell Reports, in press.

B. Junker, H. Lörz (1987)

Transformation studies in maize and other cereals.
In: Electroporation and Electrofusion in Cell Biology, eds. E. Neumann et al., Plenum Publ., in press.

R. Töpfer, V. Matzeit, B. Gronenborn, J. Schell, H.H. Steinbiß (1987)

A set of plant expression vectors for transcriptional and translational fusions.
Nucleic Acid Research, 15, in press.

B. Junker (1987)
Studien zur Transformation und in vitro Kultur von Mais (Zea mays L.)
Thesis, Universität Köln, 1987

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff	Yes	
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

Cell suspension cultures of Triticum monococcum have been given to the laboratory at Rothamsted (Dr. M.G.K. Jones) and barley cultures to Brussels (Prof. M. Jacobs).

Visits (2 days up to 1 week) to the laboratory in Orsay and vice versa in Köln have been done during 1986 and 1987 to learn about the experimental details of the procedures for protoplast isolation and protoplast culture of barley, wheat and rice.

B. Junker participated the group meeting (Genetic manipulation and regeneration in model and crop plants in vitro) during October 1986 in Versailles and as a consequence of this and further discussions during the BAP-meeting at Louvain-la-Neuve in March 1987 we joint the ELWW in the field of "crop improvement through cell biotechnology".

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Laboratoire d'Amélioration des Plantes, Orsay

Contract no.: BAP - 0014 - F

Project leader: Y. DEMARLY, Y. DATTEE

Scientific staff: N. Boissot, M. Branchard, J. de Buyser, E. Guiderdoni (6 months), Y. Henry, D. Negri, M. Valdez

Address: Université Paris-Sud
Lab. d'Amélioration des Plantes
Bât. 360
F - 91405 ORSAY CEDEX

Telephone no.: +33.1.69417491 or +33.1.69416334

Telex no.: 692166 FACORS F

Other contractual partners in the joint project:

H. Lörz, M. P. I. (Köln)

Title of the research activity:

Research on genetic transformation and plant regeneration from protoplasts of wheat and barley.

Key words:

Cereals, Regeneration, Somatic embryogenesis, Protoplast culture, mt DNA variations

Reporting period: August 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Plant cellular and molecular biology is presently in a state of rapid development and it is to be expected that this will lead in a relatively short time to major developments in the fundamental understanding of the regulation of plant morphogenesis-physiology and biochemistry-and to applications of great value to plant breeding.In this development,howewer,the cereal plants are lagging behind mostly because the regeneration of whole plants from individual somatic cells or protoplasts is very difficult and the few reports to date are only partially confirmed.The main objective of this program is in particular to develop methods to regenerate wheat,barley and rice plants from single cells and/or protoplasts.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Research of a morphogenetic rice, wheat and barley cell culture for isolation of totipotent protoplasts. In fact, the basic idea of this work is that the success of the different techniques on plant regeneration depends on the embryogenic capacity of the cultured cells, and on the subsequent morphogenic ability of isolated protoplasts.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY

Investigations on rice and barley callogenetic response and regenerative ability have been carried out with several kinds of genotypes and explants (immature inflorescences, mature embryos, immature embryos, root segments and leaf bases of three and seven days old aseptic seedlings).

In order to understand the origin of the reduced amount of protoplast division, we investigated the regeneration ability, chromosome number and mitochondrial DNA from our wheat somatic tissue cultures. The part of this work dealing with the study of callus culture mt DNA organization has been carried out in Professor F. QUETIER laboratory.

2. RESULTS

The rice explants which produce the highest rates of callogenesis are leaf bases of 7 days old, root segments of 3 days old and mature em-

bryos (scutellum). The genotypes of lower production of calli are Apura (O. sativa ssp indica) and O. longistaminata (wild species).
After eight weeks of culture of the explants on callus induction medium, calli were transfered to regeneration medium. After two months of culture, only the genotypes Apura (leaf bases of 7 days) and O. longistaminata (leaf bases of 7 days and mature embryos) were able to regenerate.
Using immature barley embryos as explants, totipotent cultures were established. We have noticed strong differences among genotypes on rate of callogenesis, type of induced calli and in vitro regeneration ability. The effects of media composition were also investigated like basal medium, hormonal composition. Our results show also effects on callus induction according to genotype. From callus strains, embryogenic sectors were used directly to isolate protoplasts. No good rate of division was obtained. Difficulties remain on each step : isolation, purification and culture of protoplasts.
The embryogenic wheat somatic tissue cultures retain embryogenic competence over a long period of culture (3 years). Root tip analysis indicate a high degree of cytological changes, since 42 % of the regenerated plants exhibit an abnormal chromosome complement : 40, 41, 41 + t, 43,... Changes in chromosomes are commonly associated with altered phenotype and reduced fertility.
The comparison of Sal I restriction patterns and the probing of the corresponding filter blots with Sal I fragments from a wheat mt DNA library have allowed us to show extensive variations in non embryogenic callus mt DNA (cv Aquila) and, to a lesser extent in embryogenic callus mt DNA (cv Chinese Spring). These variations are characterized by either the appearance, the disappearance, the decrease or the increase of several Sal I restriction bands in their relative stoichiometries.

3. DISCUSSION

According to the experimental protocol of Coulibaly (1986) further experiments on isolation of rice protoplasts were carried out directly from coleoptile and scutellum calli of the Cigalon genotype. Nevertheless, we are facing several problems concerning these techniques : a difficulty in the repeatability of the experiments and no cellular division after cell wall formation. Consequently, we are

starting further investigations on :

a - callogenetic response and embryogenic ability of seven others genotypes of O. sativa

b - initiation and establishment of cell suspension cultures for the isolation of totipotent protoplasts on Apura and O. longistaminata genotypes.

The embryogenic barley material does not seem to be a convenient source for protoplast isolation. However it could be used to establish embryogenic suspensions cultures. The transfer in liquid medium is an important step because difficulties like dissociation of embryogenic cells clusters remained. The best material should be friable embryogenic callus. Another problem is the decrease of the regeneration ability in long term cultures. At present, our purpose is to examine the effects of age and type of calli on initiation of liquid cells cultures.

Our results suggest the occurrence of particular recombination events during cell culture and agree with the hypothesis of different replication rates among the various wheat mt DNA subgenomic molecules. The mechanism by which mt DNA variation arises in wheat tissue culture is currently under investigation. The possible significance of these mt DNA variations in affecting plant fertility and regeneration ability is currently studied.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1 **PUBLICATIONS IN SCIENTIFIC JOURNALS, MONOGRAPHS,..**

DE BUYSER J., HENRY Y., HARTMANN C. and RODE A. Variations in long term wheat somatic tissue cultures : chromosome number, plant morphology and mitochondrial DNA. Plant Cell Reports (submitted for publication).

RODE A., HARTMANN C., FALCONET D., LEJEUNE B., QUETIER F., BENSLIMANE A., HENRY Y., DE BUYSER J. Extensive mitochondrial DNA variation in somatic tissue cultures initiated from wheat immature embryos. Current Genetics (in press).

IV.2 **SHORT COMMUNICATIONS, INTERNAL REPORTS,...**

DE BUYSER J., HENRY Y., HARTMANN C., RODE A., ORY C. and YOLLE N., 1987. Morphology, chromosome and mt DNA variations in wheat somatic tissue culture. BAP Meeting, Louvain la Neuve, March 23-26, 1987.

GUIDERDONI E., BOISSOT N., VALDEZ M., YOLLE N. and DATTEE Y., 1987. Research of morphogenetic rice cell culture for isolation of totipotent protoplasts. BAP Meeting, Louvain la Neuve, March 23-26, 1987.

NEGRI D. and BRANCHARD M., 1987. Obtention of protoplasts from embryo derived cultures of Hordeum vulgare L. BAP Meeting, Louvain la Neuve, March 23-26, 1987.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)		No
Exchange of staff	Yes	- 1
Joint experiment(s)		No
Joint meeting(s)	Yes	- 2

Descriptive information for the above data.

1 - Visit and discussion at the Max Planck Institut with Dr Lörz. E. GUIDERDONI, Jan. 1987.

2 - Visit and discussion at Nottingham University. E. GUIDERDONI, Dec. 1986.

Participation at BAP Meeting, Louvain la Neuve, March 23-26, 1987. Y. HENRY, J. DE BUYSER, M. VALDEZ, M. BRANCHARD, D. NEGRI, N. BOISSOT, Y. DATTEE, S. AGACHE.

Participation at the French-German workshop on plant biotechnology Grünbach, R.F.A., May 5-6-7, 1987. Y. HENRY, Y. DATTEE.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: CNR, Pisa Contract no.: BAP - 0092 - I

Project leader: M. TERZI
Scientific staff:

Address: Istituto di Mutagenesi e Differenziamento,
CNR
Via Svezia 10
I - 56100 PISA

Telephone no.: +39.50.574161

Telex no.: 500241 IMD I

Other contractual partners in the joint project:

S.C. de Vries, Agricultural University (Wageningen)

Title of the research activity:

Molecular analysis of carrot somatic embryogenesis.

Key words:

Daucus carota, Extracellular glycoproteins, Phosphorylation, Developmental mutants, Somatic embryogenesis

Reporting period: January 1987 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Regeneration of plant cells after genetic manipulation in to fertile adult plants presents a major problem in many crop species. Since Daucus carota regenerates through massive production of somatic embryos from suspension cultures we want to isolate in this species temperature-sensitive mutants impaired in somatic embryogenesis and characterize them in molecular terms at mRNA and protein level.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Definition and preliminary characterization of the lesion present in the temperature-sensitive mutants impaired in embryogenesis that were isolated.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Methodology:

1D and 2D-PAGE of proteins of cell extracts of, and medium conditioned by, wild-type and ts59 and ts11c

mRNA extraction and in vitro translation

Western blot and biotinylated lectin binding of glycoproteins separated by SDS /PAGE , detected by avidin-peroxidase, for the analysis of sugar modification.

Results:

Carrot cell line ts59 was characterized in physiological, genetical and biochemical terms. It shows two phenocritical times at preglobular stage and heart transition. Its defect lies in the lack of phosphorylation of a subset of heat-shock proteins. Interestingly enough the mutation is dominant in somatic hybrids.

tsllc mutation is recessive, its phenocritical time is at globular stage and it shows a defect in glycosylation. Both high-mannose chains and fucose-modified chains are affected.
The physiological meaning of glycosylation is being investigated.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

F.LoSchiavo, G.Giuliano and Z.R.Sung: Charcterization of a carrot cell mutant impaired in somatic embryogenesis. Plant Science

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff	Yes	
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

The mutant cell lines isolated in this laboratory have been made available to the contractual partner.
M.Terzi, F.LoSchiavo and M.R.Vergara visited the Wageningen laboratory for various periods of time.
Dr.S.C.deVries has visited Pisa

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Agricult. University, Wageningen

Contract no.: BAP - 0093 - NL

Project leader: S.C. DE VRIES

Scientific staff: P. Sterk (per 010487)

Address:
Agricultural University
Dept. of Molecular Biology
De Dreijen 11
NL - 6703 BC WAGENINGEN

Telephone no.: +31.8370.84325 or +31.8370.84706

Telex no.: 45015 BLHWG NL

Other contractual partners in the joint project:

M. Terzi, C. N. R. (Pisa)

Title of the research activity:

Molecular analysis of carrot somatic embryogenesis.

Key words:

Carrot, Extracellular glycoproteins, Somatic embryogenesis, Antibodies, Protein microsequencing, Developmental mutants

Reporting period: January 1987 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Regeneration of plant cells after genetic manipulation into fertile adult plants still present a major problem in many crop plants. Since fundamental knowledge about the molecular processes that take place during regeneration is limited our approach is to study regeneration through somatic embryogenesis in Daucus carota, a species that regenerates through massive production of somatic embryos from suspension cultured cells. To find genes and gene products responsible for somatic embryogenesis, mutants impaired in somatic embryogenesis are being studied at both mRNA and protein level. Identification of these genes could then lead to a better understanding of the molecular processes necessary for early plant development.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The isolation of antibodies against proteins that have shown to be altered in the emb$^-$ mutants impaired in a particular developmental state, and isolation of the corresponding cDNA clones.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Methodology

a). Both 1D and 2D PAAGF of cellular and extracellular proteins of wild-type and mutant cell lines under embryo conditions (low cell density without 2,4-D) and cell proliferation conditions (high and low cell density with 2,4-D). ts emb$^-$ lines were also compared at permissive and non-permissive temperatures

b). Preparation of mouse antisera against spots cut out from Western blotted 2D gels and subcutaneously implanted in mice.

c). Preparation of antiserum against total extracellular proteins produced by somatic embryos and proliferating cells.
d). Screening of λgt11 expression library made on embryo poly A^+ RNA with crude extracellular protein antiserum.
e). Preparation of monospecific antisera against λgt11 fusion proteins purified by preparative PAAGE.
f). Micro protein sequencing of extracellular proteins after blotting on activated glass fiber filters.
g). Complementation assay. To test the activity of extracellular proteins on somatic embryogenesis, both somatic embryo and suspension produced extracellular proteins were added to mutant and tunicamycin blocked cell cultures.

2. Results

a). The procedures to obtain specific antisera raised against proteins that were 2D separated, blotted on n.c. and subcutaneously implanted in mice were not succesful, partly due to excessive death rates of the mice, partly due to very low antibody titers of the sera obtained. The proteins involved here are heat shock induced embryoproteins, present in the normal phosphorylated form in line HA80, and present in unphosphorylated form in line ts $emb^{-}59$. It is probably more worthwhile to use direct protein micro sequencing procedures for these proteins. (see section 2.e)
b.) Employing both 1D and 2D PAAGE it was found that extracellular proteins with apparent molecular weights of 13, 17, 29, 38 and 46 kDa were being excreted by developing somatic embryos in the absence of 2,4-D. When proembryogenic masses were inoculated in media containing increasing amounts of 2,4-D, NAA and 6-BAP respectively, embryogenesis was blocked depending on the concentration and type of phytohormone used. Concomittant with the inhibition of somatic embryogenesis, the amount of the 13, 17, 29, 38 and 46 kDa extracellular proteins decreased to undetectable levels. Cytokinin treatment did not block embryogenesis but resulted in many abberantly formed somatic embryos. In these cultures, only the 38kDa extracellular protein disappeared. A doublet of two extracellular proteins of 52/54 kDa remained present, regardless of the differentiation state of the cultures.
c). Mutant or variant cell lines impaired in their ability to produce somatic embryos as ts11c, α_5^{II} and C15 all exhibited alterations in their extracellular protein profile under embryo-promoting conditions. In some cases a protein was found to be lacking altogether e.g. 38 kDa in ts11C, 46 kDa in C15. In other cases, e.g. $\alpha 5^{II}$, the amounts present of 13, 17, 29, 38 and 46 kDa proteins were greatly reduced compared to embryogenic counterparts as $\alpha 5^{IIIR}$ and A^+. Prolonged culturing over 2 years reduced embryogenic capacity to zero in once regenerative cultures. The loss of embryogenic capability was reflected in the absence of proembryogenic masses and the synthesis of higher molecular weight forms of 38 and 52/54 kDa extracellular proteins in cell line FG10, when compared to embryogenic cultures of cultivar 10 (cv. Trophy). In some cases, eg. ts11C, α_5^{II} and C15 somatic embryogenesis could be partly restored by addition of extracellular proteins obtained

from embryogenic cultures. In the case of FG10, this was not possible, indicating that the lack of one or several extracellular proteins could be complemented, but that the presence of altered forms of the extracellular proteins irreversibly blocked somatic embryogenesis.

d). Most of the extracellular proteins appear to be heavily glycosylated as detected by concanavalin A staining. Inhibition of somatic embryogenesis but not unorganized proliferation of cells by tunicamycin was fully reversible by addition of extracellular proteins from developing somatic embryos and also with extracellular proteins from cultures proliferating in the presence of 2,4-D. Complementation was also possible with two protease inhibitors, leupeptin and aprotinin indicating that stability of some extracellular proteins was reduced after removal of the sugar moiety by tunicamycin treatment, and with extracellular proteins from α_5^{II} and C15 indicating that these lines still produced the complementing proteins. Complementation was not possible with extracellular proteins from FG10 cultures producing altered forms of the 38 and 52/54 kDa extracellular proteins. Analysis of extracellular proteins on 1D PAAGE showed that glycosylation of the 46 and 52/54 kDa proteins was affected resulting in the presence of unglycosylated forms of these proteins in the media. Since all other proteins were unaffected, the tunicamycin- complementing effect is probably due to the addition of either the 46 or the 46 and 52/54 kDa extracellular proteins. Morphological observations on tunicamycin blocked cultures indicated that the proembryogenic masses dissociated into smaller clusters and single vacuolated cells. Somatic embryogenesis but not unorganized proliferation could also blocked by deoxynojirimycin, a substance interfering with the high mannose chain trimming reactions. This treatment resulted in higher molecular weight forms of 52/54 kDa protein, similar to that observed in FG10. Likewise, deoxynojirimycin treatment could not be complemented, again indicating that the correct sugar modifications of the 52/54 kDa proteins are essential for its function during embryogenesis. From these experiments we conclude that the correct, phytohormone controlled expression of several extracellular glycoproteins, possibly organized into an extracellular matrix together with oligo-(?) and polysaccharides is essential for the expression of embryogenic potential in carrot suspension cultures.

e). Direct microsequencing of the gel-purified 29kD extracellular protein has yielded the N-terminal 24 amino acids of this protein. These data are now being used to prepare synthetic DNA probes to isolate the corresponding genes. Antibody screening has yielded a clone encoding the 38KD extracellular protein, which is the extracellular protein missing in cell line ts11c. Further characterization of these clones is in progress.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

De Vries, S.C. and van Kammen, A (1987) Extracellular proteins control both embryogenic potential and somatic embryogenesis in carrot. EMBO Workshop Vallombrosa, Italy, July 19-23, Book of Abstracts.

De Vries, S.C. et al. (1987) Extracellular proteins and developmental mutants in carrot somatic embryogenesis. CEC Meeting Louvain-La-Neuve, March 23-26, Book of Abstracts

Everink, A. (1987) Extracellulaire eiwitten en somatische embryogenese bij Daucus carota. Student report.

Sterk, P. (1987) Screening of a lambda gt11 library representing the messenger RNA population in somatic embryos of carrot. Student report.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

- Cell lines deficient in embryogenesis were obtained from Dr. M. Terzi, Univ. of Naples, Italy.
- Dr. M. Terzi has visited our laboratory for 6 months in 1986, short visits were from Dr. F. LoSchiavo and Dr. R. Vergara from Dr. Terzi's laboratory. Dr. de Vries has visited the CNR lab in Pisa in Oktober 1986.
- Frequent discussion of experiments to be planned and carried out occurs on a routine basis by letter and telephone.
- Joint meeting with other BAP groups working in the area of regeneration and early plant development will be scheduled in Wageningen in the fall of 1987 or spring of 1988.
- Dr. de Vries has visited the laboratories of Prof. Dr. P. Albersheim, Univ. of Georgia, Prof. Dr. N.-H. Chua, Rockefeller Univ. New York and Dr. T. Thomas, Texas A&M University, Texas to discuss joint experiments.
- Ir. P. Sterk is in Dr. Thomas' laboratory to carry out protein-micro sequencing and probe synthesis experiments (010487 - 011187)

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Univ. degli Studi di Roma

Contract no.: BAP - 0209 - I

Project leader: A. BALLIO

Scientific staff: P. Aducci, M. Marra, M.R. Fullone, P. Di Domenico

Address: Università "La Sapienza"
Dipartimento di Scienze Biochimiche
P.le Aldo Moro 5
I - 00185 ROMA

Telephone no.: +39.6.494.08.04 or 494.06.06

Telex no.: 620564 UNISAP I

Other contractual partners in the joint project:

M.A. Hall, University College of Wales (Aberystwyth)
D. Klämbt, University of Bonn
K.R. Libbenga, University of Leiden
M.A. Venis, East Malling Research Station (Maidstone)

Title of the research activity:
Plant hormone receptors.

Key words:
Chromatography, Endogenous ligands, Fusicoccin, Proteoliposomes, Receptors

Reporting period: March 1987 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Understanding of the primary mechanisms responsible for plant hormone action, with particular attention directed to membrane-bound and "soluble" auxin-receptor systems, ethylene-receptor systems and fusicoccin-receptor system and with careful consideration of yet unsolved specific technical problems, such as application of immunological methods, solubilisation and purification of hydrophobic receptor proteins, inactivation of receptors during isolation and purification, reconstruction of functional in vitro perception and transduction systems.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

1. Fusicoccin binding sites.
   1.1. Isolation, solubilisation and characterization of sites from corn roots and shoots.
   1.2. Entrapment of the above solubilised sites into unilamellar liposomes, a step towards the reconstitution of a H^+-excreting system sensitive to fusicoccin (FC).
2. Endogenous ligands.
   Extraction from corn roots and purification of endogenous ligand(s) for FC binding sites.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY.

1.1. Preparation of soluble sites. Microsomal fractions of both corn roots and shoots were solubilised by the same procedure used to solubilise spinach leaf binding sites (1), except for the grinding medium which contained 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1 mM p-hydroxymercuribenzoate (PMB), to avoid proteolytic degradation.

1.2. Entrapment of soluble sites into liposomes. A mixture of soybean L- α -phosphatidylcholine (25 mg) and L- α -phosphatidylethanolamine (25 mg) was dissolved in chloroform (0.5 ml), evaporated under nitrogen, suspended in 50 mM Tris-MES buffer pH 6.5 (1 ml) and stirred for 1 hour at room temperature. The suspension was gassed with nitrogen for 1 min, sonicated for 1 min (six times 10 sec on followed by 60 sec off) at 4°C and centrifuged at 10,000 x g for 5 min. Liposomes (150 µl) were added to soluble sites (100 µl containing 0.4 mg protein in 50 mM Tris-MES buffer pH 6.5) at 4°C, freezed in liquid nitrogen for 1 min and thawed at 27°C for 2-3 min.

1.3. Binding assays. The binding assays of the soluble fractions and of the proteoliposomes with ^{3}H-labelled FC (^{3}H-FC) were performed according to Aducci et al. (2).

1.4. Extraction and purification of endogenous ligand for FC binding sites. Hydroponically grown corn roots (7 days) were squeezed in a Braun multipress to get a liquid which was heated 15 min at 100°C, cooled and then centrifuged 30 min at 40,000 x g. The clear supernatant was repeatedly fractionated by HPLC on RP columns according to conventional methods. The activity of the endogenous ligand was detected in chromatographic fractions by competition with ^{3}H-FC in a specific binding test with acetone-dried preparations of spinach leaves (1).

2. RESULTS.

2.1. Solubilisation of binding sites from corn roots and shoots. FC binding sites were solubilised from corn roots and shoots by the same procedure previously used for spinach leaves (1). The only exception was the further addition of PMSF and PMB in the grinding buffer in order to inhibit the endogenous proteinase activities. Shoot preparations were investigated more carefully with the following results. The pH-dependence of ^{3}H-FC binding shows a sharp optimum at pH 6.5, a value similar to that observed in microsomal preparations of shoots. The time-course of the ^{3}H-FC binding reaction at 27°C shows that saturation is reached after 60 min incubation, as with microsomal preparations. The addition of cold 10^{-5} M FC displaces about 15% of the bound radioactivity after 30 min at 27°C, a figure close to that observed for microsomal preparations.

2.2. Proteoliposomes of solubilised binding sites from corn. Incorporation of the binding sites from corn roots or shoots required some modifications of the procedure previously used for spinach leaves (2). In fact, the phospholipid vesicles giving best entrapment were composed of L- α -phosphatidylcholine and L- α -phosphatidylethanolamine in a 1:1 ratio. Using otherwise the same techniques reported in a previous paper (2) the following results were obtained. The amount of FC binding proteins specifically entrapped corresponds to 60% of the total used for the preparation of proteoliposomes both for shoots and roots. The time-course of ^{3}H-FC binding reaction is very close to that of the soluble preparations and still shows a very poor reversibility of the binding by a chase of cold 10^{-5} M FC.

2.3. Endogenous ligands. A careful fractionation by reverse-phase HPLC of crude corn root sap afforded small amounts of an apparently pure, but as yet uncharacterized substance which shows a very high affinity for FC binding sites. At variance with the crude starting material (3), this purified substance is unable to inhibit the FC-stimulated H^+ extrusion. Instead, this activity is found in the most polar fractions and is devoid of affinity for FC binding sites.

3. DISCUSSION.

Corn tissues were the first material to be investigated for the occurrence of FC binding sites in higher plants (3,4,5,6), but the best characterization of these binding proteins was achieved with microsomal

fractions and solubilised preparations of spinach leaves (1,7). The present results on the solubilisation and entrapment into liposomes of FC binding proteins from corn allow a preliminary evaluation of similarities and differences between preparations from monocots and dicots. The solubilisation of binding sites from monocots with chaotropic agents or detergents has been reported for corn coleoptiles (8) and for oat roots (9). We have obtained very satisfactory results by processing corn tissues with the same detergent-free procedure previously used for spinach leaves. The influence of lipids on the binding preparations from corn tissues is different from that of spinach leaf preparations. In particular, it is confirmed that the release of bound radioactive FC by a chase of cold FC is very poor for both microsomal and solubilised preparations of corn shoots (5), while that from spinach is quite prompt (2). Furthermore, the optimal composition of phospholipids required to get proteoliposomes capable to bind FC is different with the two tissues.

Work concerning the characterization of endogenous ligands to FC binding sites is of importance for the elucidation of their function. It has been postulated that these substances may modulate the physiological system acted upon by FC. The chromatographic procedures worked out for the purification of these substances has yielded an apparently pure compound. It is present in minute amounts (ppb) in corn roots, thus making structural characterization a very difficult problem. Its affinity for FC binding proteins is very high, while it is unable to influence FC-stimulated H^+ extrusion. We have found that the latter process is strongly inhibited by a different substance present in corn root sap.

1. Aducci P., Ballio A., Fiorucci L., Simonetti E. (1984). Planta 160, 422.
2. Aducci P., Ballio A., Fullone M.R., Persichetti F. (1986). Plant Sci. 45, 83.
3. Aducci P., Crosetti G., Federico R., Ballio A. (1980). Planta 148, 208.
4. Dohrmann U., Hertel R., Pesci P., Cocucci S., Marré E., Randazzo G., Ballio A. (1977). Plant Sci. Lett. 9, 291.
5. Pesci P., Cocucci M.S., Randazzo G. (1979). Plant Cell and Environm. 2, 205.
6. Radice M., Scacchi A., Pesci P., Beffagna N., Marré M.T. (1981). Physiol. Plant. 51, 215.
7. Ballio A., Federico R., Pessi A., Scalorbi D. (1980). Plant Sci. Lett. 18, 39.
8. Pesci P., Tognoli L., Beffagna N., Marré E. (1979). Plant Sci. Lett. 15, 313.
9. Stout R.G., Cleland R.E. (1980). Plant Physiol. 66, 353.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	No
Joint experiment(s)	No
Joint meeting(s)	No

Descriptive information for the above data.

A reference sample of 5-azidonaphtylphtalamic acid, synthesized in our laboratory from phtalic anhydride and 5-azido-1-aminonaphtalene, has been sent to Dr. M.A. Venis. The compound was necessary for experiments related to the research on receptors of phytotropins and their endogenous ligands, planned in collaboration with the group of Dr. Venis.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Univ. College of Wales, Aberystwyth
Contract no.: BAP - 0208 - UK

Project leader: M.A. HALL
Scientific staff: I.O. Sanders, C. Connern

Address: Univ. College of Wales
Dept. of Botany & Microbiology
King Street
UK - ABERYSTWYTH, DYFED SY23 3DA

Telephone no.: +44.970.3111 EXT.3063

Telex no.: 35181 ABYUCW G

Other contractual partners in the joint project:

D. Klämbt, University of Bonn
K.R. Libbenga, University of Leiden
M.A. Venis, East Malling Research Station (Maidstone)
A. Ballio, Università "La Sapienza" (Roma)

Title of the research activity:
Plant hormone receptors.

Key words:
Ethylene, Receptor, Proteins, Antibodies, Immunoassay

Reporting period: March 1987 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The general objectives of the project are to promote collaboration between five European laboratories working in the area of plant hormone receptors. The Aberystwyth group is specifically concerned with receptors for ethylene, the production of polyclonal and monoclonal antibodies to such proteins and the development of immunoassays therefrom for use in studies on the transduction of the ethylene response.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

In the current first reporting period the objectives of the laboratory were to begin to raise polyclonal antibodies to the ethylene receptor protein.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

METHODOLOGY

Extraction and Purification of Ethylene Receptor Protein

i. Extraction of tissue and pelleting of membranes

60g of Phaseolus vulgaris L. cotyledons were peeled and their embryos removed. They were chopped finely with razor blades in 0.02M (N-Tris [hydroxymethyl] methylglycine) Tricine, 8% sucrose buffer at pH 9.0. A 3.4cm diameter Polytron head (Kinematica Gmbh) was used to homogenise the tissue pieces before straining through 20µm Miracloth (Henry Simon). The brei was centrifuged in a Sorvall (OTD65) centrifuge using a swinging bucket rotor (AH629) at 96Kxg_{av} for 0.75h at 4°C. The pellet was discarded and the supernatan centrifuged again at 96K x g_{av} at 4°C for 3.75h. The

resulting 96K x g membrane pellet was resuspended in 0.1M KCl at pH 12 using a 0.7cm diameter Polytron head and then centrifuged at 96K x g_{av} for 1 h at 4°C. This procedure has been shown to remove peripheral membrane proteins (Weidenmann et al. 1985).

ii. Solubilisation of the pH 12 pellet

20% (w/w) Triton X-100 in 0.02M (Tris [hydroxymethyl] aminomethane) Tris-HCl at pH 8.9 was added to the pH 12 pellet in a ratio of about 0.5g detergent to 1g equivalent fresh weight and resuspended with the small Polytron head. The final concentration of detergent was 6.7% made by diluting with 0.02M Tris-HCl, pH 8.9 buffer. The resuspended pellet was centrifuged with 96K x g_{av} for 1h at 4°C. The supernatant was then frozen at -17°C or used directly on a DEAE-Sepharose fast flow column.

iii. Anion exchange chromatography

A DEAE-Sepharose fast flow anion exchange column, 2.6cm in diameter, 20cm high and of 106cm^3 volume was used. A linear salt gradient of 0-100% 0.35M NaCl in 0.02M Tris-HCl, pH 8.9, 0.5% Triton X-100 was generated using a Pharmacia FPLC system in a volume of four column volumes. The gradient was started after loading 10cm^3 of sample and washing the column with one volume of start buffer. A linear flow rate of 0.47cm^3 cm^{-2} min^{-1} for the gradient and wash, 0.19cm^3 cm^{-2} min^{-1} for sample loading. The sample eluting at 0.091M salt was concentrated to 0.5cm^3 by ultrafiltration in an ultrasart 10 cell with a 20KD cut off filter.

Preparation, Detection and Characterisation of Antibodies to Ethylene Receptor Protein

Rabbits were immunised with 60µg of protein, purified as described above, in an equal volume of Freund's complete adjuvant. The rabbits were injected intravenously and subsequently boosted twice subcutaneously at various sites on the back.

Serum was analysed by dot-blotting and Western blotting using

goat anti-rabbit antibodies conjugated to colloidal gold with silver enhancement.

RESULTS & DISCUSSION

The concentrated sample of receptor protein ran as a single band on denaturing and non-denaturing gels. The protein had a specific activity of 4.56×10^4 Bq mg^{-1} protein (60% of theoretical assuming 1:1 stoichiometry and a MW of 60KD) representing a 2667 fold purification over crude membrane preparations and a recovery of 18.6%.

Dot blotting gave a visible signal to serum from immunised rabbits but not to non-immune serum. The antibodies did not recognise Triton X-100.

Western blotting of pH 12 solubilised preparations and purified receptor protein run on 3-22% gradient polyacrylamide gels showed after visualisation that the antibodies recognised only one protein species. The serum was able to detect down to 3.75ng of the protein. We are now proceeding to further characterise the antibodies and to develop immunoassay systems.

Reference

Weidenmann, B. *et al*. (1985) Solubilisation of proteins from bovine brain coated vesicles by protein perturbance and Triton X-100. Journal of Cell Biology *101*, 12-18.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)		No
Exchange of staff	Yes	
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

Meeting between all members on Plant Hormone Receptor contract at Louvain-la-Neuve (23-26 March 1987).

Meeting between M A Venis and M A Hall, May 1987 to discuss matters of common interest and joint experiments.

Meeting arranged between M A Hall and A Ballio (14.10.87-17.10.87). Hall to give seminar and discuss matters of common interest in program.

Two members from Leiden laboratory to visit Aberystwyth for 1-2 weeks in Autumn 1987 to learn separation techniques for hydrophobic proteins.

One member of Aberystwyth laboratory to visit Leiden for 1-2 weeks in Spring 1988 to explore preparation of monoclonal antibodies to ethylene receptor protein.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: University of Bonn Contract no.: BAP - 0207 - D

Project leader: D. KLÄMBT
Scientific staff: M. Löbler, T. Hesse, G. Viola, B. Dehe

Address: University of Bonn
Botanisches Institut
Meckenheimer Allee 170
D - 5300 BONN 1

Telephone no.: +49.228.732680

Telex no.: 886657

Other contractual partners in the joint project:

A. Ballio, Università "La Sapienza" (Roma)
M.A. Hall, University College of Wales (Aberystwyth)
K.R. Libbenga, University of Leiden
M. A. Venis, East Malling Research Station (Maidstone)

Title of the research activity:
Plant hormone receptors.

Key words:
Auxin, Receptor, Corn coleoptile, Monoclonal antibodies

Reporting period: March 1987 - July 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Plant normones act tnrough hormone specific receptors.. There are encouraging results for characterizing and analysing membrane associated (Löbler, Klämot and Venis) and cytoplasmic (Mennes, Libbenga) auxin receptors. For fusicoccin (Ballio, Adducci) and ethylene (Hall, Smith) normone binding proteins are described, which nave to be further characterized. Searching for hormone receptors immunological methods are extremely nelpful. - The program of the joint project is to cooperate in separation, purification and characterization of tne different plant hormone receptors.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The main interest was directed to the production of monoclonal antibodies against tne membrane associated auxin receptor of corn coleoptiles. A monoclonal antibody should be the highest specific probe to confirm the receptor function of the membrane associated auxin binding protein. These monoclonals will give the opportunity to check the process and function of this receptor in other organs of corn stems, styles, root-tips and -hairs.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Methology

For the different purposes in searching for auxin receptors monoclonal antibodies are of great value. One crucial point in getting monoclonals is the preparation of purified antigen samples for immunization and final screening. A very quick and sufficient method to prepare the antigen is found in the application of a modified separation procedure according to Shimomura et al. (1986) (Klämbt, Löbler 1987). The final preparation contains tne auxin receptor besides some other proteins of higher molecular mass. Trials for further purification failed, therefore this sample was used as antigen.

Mice were employed in tne immunization program. Due to convential methods spleenocytes were isolated and fused with myelome cells (AG63) to produce hybridoma cell lines. Screening is done by immunostaining by the use of a dot blot technique. For this purpose antigen samples are separated by a very short electrophoresis in polyacrylamide gels and transferred onto nitrocellulose sheats. Small stripes containing the separated proteins are finally used for screening.

For immunofluorescence microscopical studies the techniques of cryotom sectioning of corn coleoptiles and other organs are learned and trained.

2. Results

After three months under contract there are no real results to report. But the mentioned techniques are well trained and applicable. The first hybridoma cell clones are selected for the auxin receptor as well as for the other proteins, which are included in the antigen samples. They are now multiplied. The next step will be the immunofluorescence microscopical examination in confirming the eralier results with monospecific polyclonal antibodies on sections of corn coleoptiles (Löbler, Klämbt 1985)

3. Discussion

Since the project just started I may direct the reader to the next year report.

4. References

Klämbt,D., Löbler,M.: Affinity chromatography is a powerful tool to prepare auxin receptors. in Plant Hormone Receptors Ed. D. Klämbt, Springer, Berlin 1987, pp 261-263

Löbler,M., Klämbt,D.: Auxin-binding protein from coleoptile e membranes of corn (Zea mays L.) II. Localization of a putative auxin receptor. J.Biol.Chem. 260, 9854-9859 (1985)

Shimomura,S., Sotobayashi,T., Futai,M., Fukui,T.: Purification and properties of an auxin-binding protein from maize shoot membranes. J.Biochem.99, 1513-1524 (1986).

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

No publication issued within the three month period of the BAs contract.

TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	No
Exchange of staff	No
Joint experiment(s)	No
Joint meeting(s)	No

Descriptive information for the above data.

Exchanges of materials and coworkers did not take place yet. Joint experiments are in preparation and joint meeting of all BAB contractors of Plant Hormone Receptors is planed and will be organized in April 1988.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Rijksuniversiteit Leiden

Contract no.: BAP - 0206 - NL

Project leader: K.R. LIBBENGA

Scientific staff: A.M. Mennes, H.J. van Telgen, E.J.J. van der Zaal

Address:
University of Leiden
Dept. of Plant Molecular Biology
Botanical Laboratory
Nonnensteeg 3
NL - 2311 VJ LEIDEN

Telephone no.: +31.71.272727 EXT. 5065

Telex no.: 39427

Other contractual partners in the joint project:

M. A. Venis, East Malling Research Station (Maidstone)
D. Klämbt, University of Bonn
M. A. Hall, University College of Wales (Aberystwyth)
A. Ballio, Università "La Sapienza" (Roma)

Title of the research activity:
Plant hormone receptors.

Key words:
Plant hormone, Auxin, Receptors, cDNA, In vitro transcription

Reporting period: January 1987 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

A joint effort to gain a better basic understanding of perception and transduction of plant hormones, plant growth regulators and plant-hormone transport regulators.
A major goal is to improve techniques for isolation, characterization and function analysis of receptors. The joint groups will exchange expertise (purification of low-abundant (membrane) proteins, immunochemistry, recombinant-DNA technology, etc) and they will exchange materials(antisera, cDNA clones, etc) to investigate possible similarities in structure and function between related plant-hormone receptors from different systems and species.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

a. Optimilization and scaling up of the isolation and purification procedures for soluble auxin receptors extracted from auxin-activated target cells (auxin-dependent batch-cultured cells from tobacco).
b. Construction of cDNA clones of early auxin-activated genes in the target cells.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Methodology

Auxin-target system

As auxin-target system a batch-cultured cell line from tobacco (Nicotiana tabacum L. c.v. White Burley) was used. This well-established cell line requires a nutrient medium containing the auxin-analogue 2,4-dichlorophenoxyacetic acid (2,4-D) as a sole growth factor. For experimental purposes the cells were depleted from auxin by subculturing inocula from early stationary-phase cells in auxin-lacking medium. Addition of auxin (final concentration 2.2 x 10^{-6}M) to the auxin-starved cells reinitiated cell division activity after a lag of 10-15 h. (Van der Zaal et al (1987) Planta, accepted).

Construction of cDNA Library from auxin-induced mRNAs

RNA preparations isolated from cells treated for 4 h with 2,4-D (final concentration 2.2 x 10^{-6}M) were enriched for poly $(A)^+$ RNA. The thus obtained mRNA preparations were used to construct a cDNA library. By differential screening of this library with (ss) cDNA to mRNAs derived from auxin-starved cells treated with 2,4-D or water for 4 h, seven non-cross hybridizing cDNA clones to 2,4-D-induced mRNAs were obtained (Van der Zaal et al (1987) Plant Mol. Biol. submitted).

In vitro transcription

For in vitro transcription, nuclei equivalent to 20 µg of DNA were incubated at 26°C for 30 min as described by Mennes et al (1978) Plant Sci Lett. 13, 329-339. ^{32}P-labelled transcripts were isolated and hybridized for 72-96 h to Southern blots of Eco RI-digested and denatured plasmid DNA.

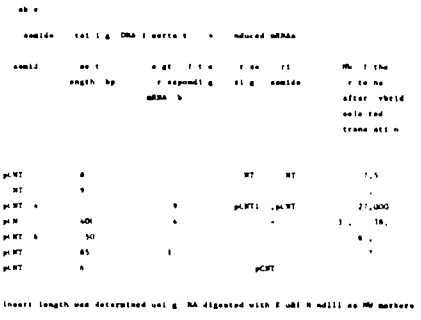
Purification of the soluble auxin receptor

For receptor isolation we used the same auxin-target system since we had found that addition of 2,4-D enhanced the level of auxin-receptor proteins in crude salt lysates of isolated nuclei (Libbenga et al 1987, UCLA Symp. 44, 229-243).

FPLC, using various columns (Superose 6 and 12, Mono Q) was introduced as a prepurification step, prior to ligand-affinity chromatography. The 5-hydroxy-IAA coupled to epoxy-activated Sepharose D was synthesized by the department of organic chemistry (University of Leiden).

2. Results

Auxin-induced mRNAs



An overview of plasmids containing cDNA inserts to 2,4-D-induced mRNAs is given in Table 1. Hybrid-selected in vitro translation demonstrated that several of the auxin-induced mRNAs encoded proteins with MWs in the range of those obtained by in vitro translation of total mRNA from auxin-treated cells. Induction kinetics, auxin-concentration dependence, hormone specificity and dependence on protein synthesis of the induction of the 7mRNA species were studied by Northern-blot analysis.

One of the most interesting auxin-induced mRNA species which is independent of protein synthesis, is the one encoded by cDNA clone pCNT103. Already 15 min after addition of 2,4-D, enhanced levels of this mRNA could be detected, and the level steadily increased up to ca. 4 h, but then apparently decreased as is shown by the intensity of the hybridization signal at 24 h (Fig. 1a). The dose-response curve of pCNT103 mRNA accumulation apparently exhibited simple saturation kinetics, the response being saturated over ca two decades of hormone concentration, with half-maximum response at ca 2.2 x 10^{-7}M. If we express cell-division activity as increase in cell density over the water control at 72 h after addition of 2,4-D, then the dose-response curve of pCNT103 mRNA accumulation roughly correlates with the dose-response of cell division activity, except for 2.2.10^{-5}M 2,4-D, which seems to be supraoptimal for cell division activity (Fig. 1b). Hormone specificity of pCNT103 mRNA accumulation, as well, correlated with that of cell division activity (Fig. 1c). Note that in both cases the natural auxin IAA is less active that the analogues 2,4-D and NAA. This is not surprising since IAA can easily be metabolized or conjugated by the cells.

In vitro transcription

Nuclei were isolated at 15 min after the addition of 2,4-D or water to the auxin-starved cells. When ^{32}P-labelled RNA made by in vitro transcription using nuclei from water-treated cells was hybridized to membranes containing plasmid DNA of the cDNA clones, no or only very weak signals were obtained. Nuclei isolated from 2,4-D-treated cells, however,

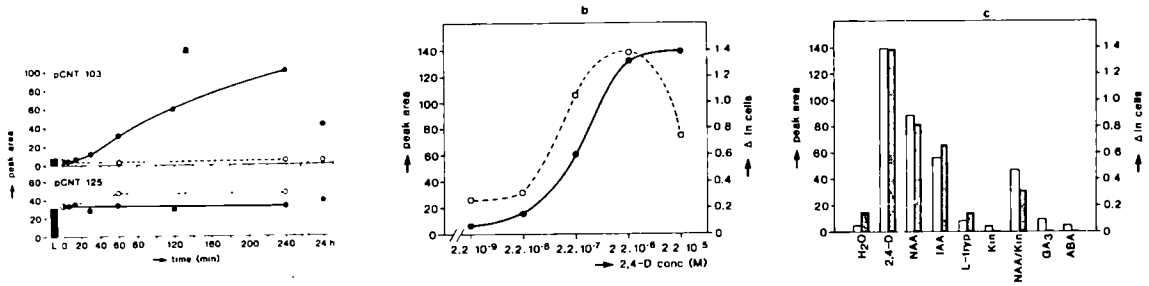


Fig.1.pCNT103 mRNA accumulation. a:Induction kinetics of 2,4-D-induced pCNT103 mRNA. Auxin-starved cells were treated for the indicated times with $2.2.10^{-6}$M 2,4-D(•-•) or water(o-o) as control.RNA blots were hybridized with ^{32}P-labelled plasmids.The autoradiographs were scanned in a densitometer and peak areas were integrated.A non-2,4-D-induced cDNA control clone(pCNT125) is shown in the bottom panel. b:Correlation between pCNT103 mRNA accumulation(•-•) and cell division activity(o-o) after treatment of auxin-starved cells with various 2,4-D concentrations. c:Correlation between pCNT103 mRNA accumulation(open bars) and cell division activity(hatched bars) after treatment of auxin-starved cells with different (non-)hormonal compounds(final conc. $2.2.10^{-6}$M).

synthesized 6 of the 7 mRNA species, including pCNT103 mRNA, at highly enhanced levels.

Purification of the soluble auxin receptor

As was suggested by some of the partners in the joint programme (Hall, Venis) FPLC was recently introduced as a fast prepurification step. Although excellent elution patterns were obtained in ca 5% of the time required for separation techniques used thus far, binding activity got lost as was revealed by analysis of the various pooled fractions. Direct ligand affinity chromatography of crude salt lysates of nuclei from auxin-activated cells, gave active preparations, but with low yield and still being contaminated with various other proteins. Although affinity chromatography can be used as a final step, proper prepurification techniques remain necessary. Since FPLC is in principle an attractive technique, the problem we have in loosing binding activity will be studied by us in the laboratories of Hall en Venis.

Discussion

The results presented indicate that the gene encoding pCNT103 mRNA is a good candidate for a primary auxin-target gene involved in cell division. The cDNA has now been sequenced and cloning of the gene is in progress. We think that it is likely that transcriptional responses of the hormone-regulated genes are eventually mediated by sequence-specific DNA binding proteins, which thus act a key factors in the transduction chain of the hormone. The cloning and analysis of the auxin-regulated gene encoding pCNT103 mRNA should reveal whether or not the soluble auxin receptor directly serves as a transacting factor. Therefore, the problems encountered in large-scale purification of this very-low abundant protein have to be solved in collaboration with other members of the joint programme.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	No
Exchange of staff	No
Joint experiment(s)	No
Joint meeting(s)	Yes

Descriptive information for the above data.

The first half year of the BAP contract was used to organize the cooperation for the next 2 years. During the recent "BAP 1987" meeting in Louvain-la-Neuve we discussed the necessity of a follow up of the NATO-workshop on "Plant Hormone Receptor" of August 18-22 1986 in Bad Honnef (Germany). This has now resulted in the decision to organize a working seminar in the week between 11-15 of April 1988. The seminar will take place in Bad Honnef which is near the site of Bonn, and the local organizer Prof. Klämbt has already announced the meeting to the Commission. Participants of this meeting of ELWW are Prof. Ballio (Rome), Prof. Hall (Aberystwyht), Prof. Klämbt (Bonn), Prof. Libbenga (Leiden), and Dr. Venis (East Malling). All groups are working on hormone receptors and we decided to bring together the so-called "workers in the field", like Ph.D students and technicians. This seems to us the best way to discuss common problems, to exchange techniques and to evaluate experiments that can be done jointly. Some other groups which are interested in our work, like those of Dr. Jacobsen in Bonn, working on auxin binding in Pisum sativum, and of Dr. Zbell from Heibelberg, working on auxin-mediated phosphoinositide response in carrot suspension cells, will be invited if additional money from other sources can be obtained.
On the 20th of March 1987 the group of Prof. Klämbt from Bonn visited our Lab. in Leiden and we have presented and discussed our last results. On the 8th of October our group will visit Bonn in order to discuss recent results in Klämbt's and Jacobsen's Labs. We also will discuss the possibility to get some of the antibody against the solubilized maize receptor. In May of this year we got the facility of FPLC in our lab, so we could start with some experiments on the purification of the receptor, using this technique. Regularly contact by phone with Dr. Venis and Prof. Hall has resulted in the decision that 2 scientists of our group will visit both labs in East Malling and Aberystwyth in order to be trained in the FPLC technique. These visits will take place in November of this year. In our last phone call to Prof. Hall we have proposed that one scientist of his group will visit our lab a few weeks before the working seminar in April 1988 in order to exchange expertise in auxin immunochemistry and gene technology (cDNA clones).

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Kent Incorporated Society

Contract no.: BAP - 0205 - UK

Project leader: M.A. VENIS

Scientific staff:

Address: East Malling Research Station
East Malling
UK - MAIDSTONE, KENT ME19 6BJ

Telephone no.: +44.732.843833

Telex no.: 957251

Other contractual partners in the joint project:

A. Ballio, Universtià "La Sapienza" (Roma)
M. A. Hall, University College of Wales (Aberystwyth)
D. Klämbt, University of Bonn
K.R. Libbenga, University of Leiden

Title of the research activity:
Plant hormone receptors.

Key words:
Auxin, Receptor, Phytotropin, Antibodies, Plant membrane

Reporting period: March - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

To improve techniques of identification, isolation, characterisation, localisation and functional analysis of plant hormone receptors. Particular emphasis to be placed on:

1. Membrane-bound auxin receptors from maize.

2. Isolation of putative endogenous ligand(s) for high affinity receptors binding synthetic inhibitors of auxin transport (phytotropins).

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

1. Purification of maize membrane auxin receptor sufficient for immunisation purposes to generate monoclonal and polyclonal antibodies.

2. Synthesis of phytotropin-protein conjugates for immunisation to produce anti-phytotropin antibodies for isolation of endogenous phytotropin.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Methods

Receptor purification. Membranes from etiolated shoots of maize were prepared by differential centrifugation. Auxin binding proteins were solubilised, after acetone treatment of the resuspended membranes (1), using 0.25 M sucrose-5 mM Mg SO_4-10 mM MES, pH 5.5. After centrifugation, this extract was applied to a column of DEAE Bio-Gel equilibrated in the same buffer and auxin binding activity was eluted with 0.1 M NaCl in this buffer. These proteins were precipitated with ammonium sulphate (75% saturation) and fractionated on a gel filtration column (Sephacryl S200) equilibrated in 250 mM sucrose-100 mM NaCl-20 mM Tris-HCl pH 7.3. Fractions with auxin binding activity (ca. 40-50,000 daltons) were pooled, diluted with an equal volume of the same buffer minus NaCl and fractionated on a Pharmacia FPLC anion exchange (Mono Q) column using a gradient of 50-350 mM NaCl in this buffer. Active fractions (1-2 ml) were either:

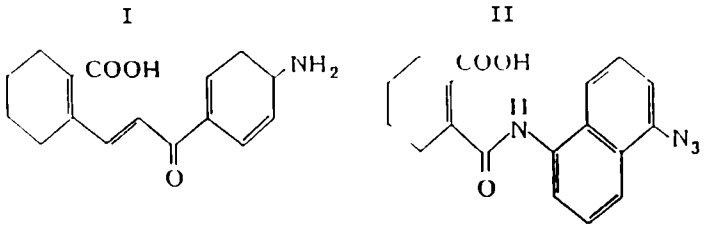
a) lyophilized and used to immunise rats, for subsequent production of monoclonal antibodies;

or, b) desalted to 0.25 mM PMSF, lyophilized, reconstituted in 5% w/v sucrose and electrophoresed on a preparative non-dissociating 10% polyacrylamide gel (2). At the end of the run, a small fraction of the separated proteins was transferred to nitrocellulose by brief

electroblotting (10 mA, 3 min). The blot was rapidly stained (3) and used to locate the protein bands remaining in the gel. Initially, the gel slices containing these bands were crushed and the protein eluted and assayed for auxin binding. Thereafter, the relevant gel slice was simply excised on the basis of its electrophoretic position and used for rabbit immunisation to produce polyclonal antibodies.

Binding assays. Auxin binding activity was monitored using 1-naphthyl[1-^{14}C] acetic acid, either by equilibrium dialysis (1) or by centrifugation to completion through YMT ultrafilter membranes (Amicon).

Synthesis of phytotropin-protein conjugates



A) CCA-KLH. 4-(2-carboxycinnamoyl)-aniline (CCA, I, a gift from Dr. G.F. Katekar) was diazotised and azo-coupled to keyhole limpet haemocyanin (KLH), yielding a conjugate of 0.45 μ mol CCA/mg protein.
b) 5-azido-NPA-KLH. 5-azido-N-1-naphthylphthalamic acid (5-azido-NPA, II) was prepared by reaction of equimolar quantities of 5-azido-1-naphthylamine and phthalic anhydride in toluene at room temperature. The product was coupled photolytically to KLH (365 nm peak emission, 1.8 mw cm^{-2}, 20 min) to give a conjugate of 0.13 μ mol ligand/mg protein.

2. Results

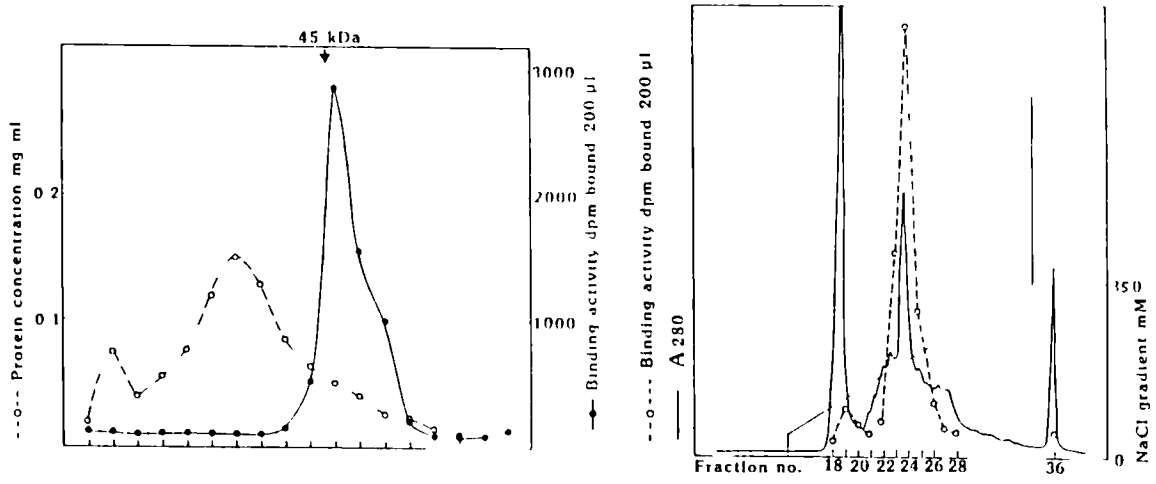


Fig. 1 Purification of post-DEAE receptor on Sephacryl S200 (left) followed by Mono Q (right)

Auxins: We are now able to purify the auxin receptor by a routine, straightforward procedure (see Fig 1 and Methods) that yields preparations of about 50% purity (range 30-80%) in three steps. These preparations have

been used to immunise rats for monoclonal antibody production. On SDS-PAGE the major polypeptide is seen at 22 kDa, usually associated with a minor 21 kDa species that elutes fractionally earlier on Mono Q.

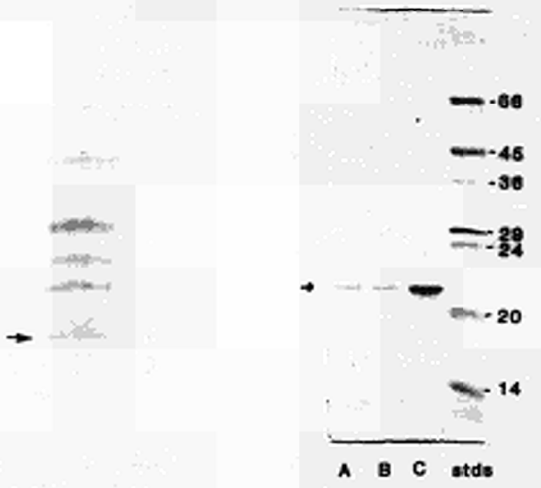


Fig 2. Left: Native PAGE of post-Mono Q receptor
Right: SDS-PAGE of three separate receptor preparations excised and eluted from native gels

To produce pure antigen the post-Mono Q fraction is separated by native PAGE. The highest mobility band, which has auxin binding activity and represents a dimer of the major 22 kDa polypeptide (Fig 2) is eluted and used for polyclonal antibody production.

Phytotropins. Two phytotropin-protein conjugates have been prepared and will be used to generate monoclonal antibodies (mAbs). It is intended to select a suitable mAb to use for immunoaffinity isolation of the putative endogenous ligand(s) for the phytotropin receptor, in conjunction with the Rome Laboratory (Ballio).

3. Discussion

Antigens suitable for production of both monoclonal and polyclonal antibodies to the maize membrane auxin receptor have been successfully produced, as well as phytotropin conjugates to use as the starting point for endogenous phytotropin isolation. Immunisation schedules are in progress.

References

1. Venis, M.A. (1977). Nature 66, 268-269.
2. Blackshaw, P.J. (1984). Methods in Enzymology 104, p 242.
3. Kumar, B.V. et al., (1985). Biochem. Biophys. Res. Commun. 131, 883-891.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

None

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

1. Reference sample of 5-azido-NPA supplied by Ballio (Rome).

2. Meeting of all research leaders at Louvain, March 1987, when all aspects of the research programme and arrangements for future meetings to include all research participants were discussed.

3. Meeting with Hall (Aberystwyth) in May 1987 to exchange information on progress with ethylene and auxin receptors.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: A/S De Danske Sukkerfabrikker, Copenhagen
Contract no.: BAP - 0078 - DK

Project leader: P. OLESEN
Scientific staff: M.G. Pedersen, J.M. Andersen, J.E. Nielsen

Address:
De Danske Sukkerfabrikker
Biotechnology Section
P.O. Box 17
Langebrogade 1
DK - 1001 COPENHAGEN K

Telephone no.: +45.1.954100

Telex no.: 31436 DDSENG DK

Other contractual partners in the joint project:

K. Roberts, John Innes Institute (Norwich)
R. Rajagopal, Royal Veterinary & Agricult. University (Copenhagen)

Title of the research activity:

Control of the differentiation of plant cells and of their regeneration into entire plants with special emphasis on cell membrane.

Key words:

Regeneration, Sugar beet, Hormones, Histology, Habituation

Reporting period: September 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

1: The three laboratories in Copenhagen and Norwich aim at understanding the mechanisms that govern cell differentiation and regeneration through embryogenesis in an important crop species, sugarbeet, as well as in a model species, carrot. Three different approaches are combined:
* Tissue culture in combination with biochemical and histological analysis
* Immunology and cell surface
* Immunology and hormone metabolism

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

2: We aim to select and characterize sugar beet callus populations with enhanced embryogenic/morphogenic potentials. For the present period cultures have been carefully characterized by morphological description and extensive anatomical analyses, thus establishing visual and histological selection criteria. Special emphasis is given to the elucidation of habituation processes which constitute a main obstacle to reproducible in vitro regeneration in sugar beets.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Methodo logy:
The general strategy for the tissue culture work has been described (1). Starting material comprised a number of varieties relevant to breeding (DDS-Maribo accessions) including a homozygotic variety (a dihaploid line resulting from ovule culture)in order to estimate the genotypic variation expressed under in vitro conditions.
At each subcultivation during callus induction, proliferation, induction of regeneration and shoot/root formation samples were fixed and sectioned for histological examination. Care was taken to correlate visual appearance of the samples (nodulation etc.)with orientation during sectioning. The desired sectioning plane was marked on a photograph of each sample.The samples were fixed in aldehydes, infiltrated with detergent (Tween 80) and embedding medium (Tissue Tek O.C.T.)before freezing. 5 µm. frozen and unstained sections were examined for autofluorscences by UV-microscopy.

Results:
Tissue culture: The initial calli were compact and dark green with a relatively high degree of nodulation. When transferred to substrate without hormones either general growth stopped and the nodules slowly started to develop - or a loose callus consisting of large cells started to grow very fast. When calli were transferred to the regeneration substrate, organogenesis took place originating from the nodules - or the prime callus was overgrown by the loose callus type. So far, root regeneration has been obtained several genotypes in all of the sugar beet varieties used, but virtually no shoots have been obtained. Calli from the same genotype performed very homogeneously. This was demonstrated very clearly in the cultures from the self-pollinated double haploid variety. Out of more than 600 callus lines (representing 300 different genotypes) 150 lines were subjected to regeneration attempts and about 100 lines were examined histologically.
Histology: In the cotyledon explants the primary callus originated mainly from areas close to the vascular tissue and in small amount from mesophyl and epidermis. Callus on hypocotyls originated both from the cortical tissue and from outer parts of the central vascular tissue. For both explant, the primary callus was dominated by a nearly spherical meristem type, where the meristematic cells partly look like a crescent surrounding a xylem bundle. These structures seemed to develop into other meristem types during further cultivation. Typically the meristems gave rise to a fan-shaped callus type with dark green flourescense which differentiated into xylem, a very loose callus and a so-called net-shaped callus. The net-shaped callus was loosely organized of enlarged cells with thin and wawy walls and contained scattered xylem cells. Interestingly, the net-shaped cells became totally dominant in one third of the calli after 12 to 28 weeks and was the most frequent type in all but two of the examined callus clones. Only two samples have showed indication of structures involved in shoot regeneration. In one case two small leaf-like structures without any shoot structures and, in a another case, two very small cotyledon-like leafy primordia.

Discussion: The lack of shoots is a very crucial factor in the experiments. For the time being work is done to evaluate the possibilities to control the development of roots at a very early stage to turn it into shoots, since it has proven impossible to form shoots on the allready formed roots. This is tried by the use of antiauxins, cytokinins (1) and through control of the ethylene synthesis or the effects of the ethylene (2). At the moment no results are avaible from these investigations.
In in the long term the lack of shoot regeneration clearly necessitates some change in strategy in the choice of substrates and subcultivation schedules. Both initial subcultivation and induction of regeneration will be attempted significantly earlier when more interesting meristem structures appear internally in the calli - and before the apparently

nonregenerating callus type takes over. Interestingly, the observations on this callus type indicate a significant correlation with habituation processes and will therefore be evaluated together with the coming biochemical data on habituation (3) in the near future.

References:

1; Andersen JM, Okkels FT, Ulvskov P, Marcussen J (1986): Endogenous cytokinins during embryogenesis in a carrot cell suspension. In: Genetic Manipulation in Plant Breeding (eds. Horn W et al.) Berlin-N.Y., pp. 449-451.

2; Okkels FT, Olesen P, Andersen JM, Jensen B (1985): Niacinamide as an inhibitor of ethylene production. In: Proc. Biotech. Plant Sci.: Relevance to Agriculture in the Eighties, Cornell University, USA.

3; Pedersen MG, Andersen JM, Okkels FT, Olesen P (1987): Characterization of embryogenic/morphogenic callus from sugarbeet seedlings: Identification and selection of competent cell and tissue types. - BAP Meeting, Louvain-la-Neuve, CEC, pp:179-180.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

NONE.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

1. Dr. S. C. De Vries from Agricultural University of Wageningen, The Netherlands, has been invited to Copenhagen this autum to discuss possibilities for project cooperation on somatic embryogenesis in carrot.

2. Dr. R. Pennell from the cooperating John Innes Institute, UK, and cooperating staff from Agricultural University of Copenhagen participated in a joint meeting at DDS-Copenhagen ,6th - 7th April together with DDS-AGC research collaborators from 3 UK institutions.

3. Dr. R. Pennell and mr. G. Scofield from the cooperating John Innes Institute, UK, visited DDS-Copenhagen and DDS-Maribo Breeding Station 1th - 4th June to be familiar with DDS material and tecknology. Sugar beet cell cultures were transferred from Maribo to John Innes, Where they have been successfully established and will be worked upon in the future. During this visit good contacts were made to research staff at Risø National Laboratory, Roskilde, DK, who also work on cell surface determinants specific to regeneration/embryogenesis.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: John Innes Institute

Contract no.: BAP - 0098 - UK

Project leader: K. ROBERTS

Scientific staff: R. Pennell, G. Scofield

Address: John Innes Institute
Dept. of Cell Biology
Colney Lane
UK - NORWICH NR4 7UH

Telephone no.: +44.603.52571

Telex no.: 975122 JIINOR G

Other contractual partners in the joint project:

P. Olesen, De Danske Sukkerfabrikker (Copenhagen)
R. Rajagopal, Royal Veterinary & Agricult. University (Copenhagen)

Title of the research activity:

Control of the differentiation of plant cells and of their regeneration into entire plants with special emphasis on cell membrane.

Key words:

Monoclonal antibody, Plasma membrane, Regeneration

Reporting period: October 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The long term aims of the combined labs in Norwich and Copenhagen are to understand fully the mechanisms of somatic embryogenesis through cell suspension and tissue culture technology. Specifically, it would be desirable to understand regeneration competance, and then to develop a mechanism for the identification and isolation of competant cells in a mixed suspension culture. We believe that cell surface phenomena may be associated with the capacity to regenerate, and therefore much of our emphasis is upon the plasma membrane.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

We aim to identify a number of macromolecules unique to the plasma membranes of plant cells by immunological methods. By raising monoclonal antibodies (McAb) against plasma membrane of both carrot and regenerable and habituated sugar beet, we intend then to describe cell surface macromolecules which are both species-specific and which may serve as markers during an in vitro selection system for competant cells.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Methodology

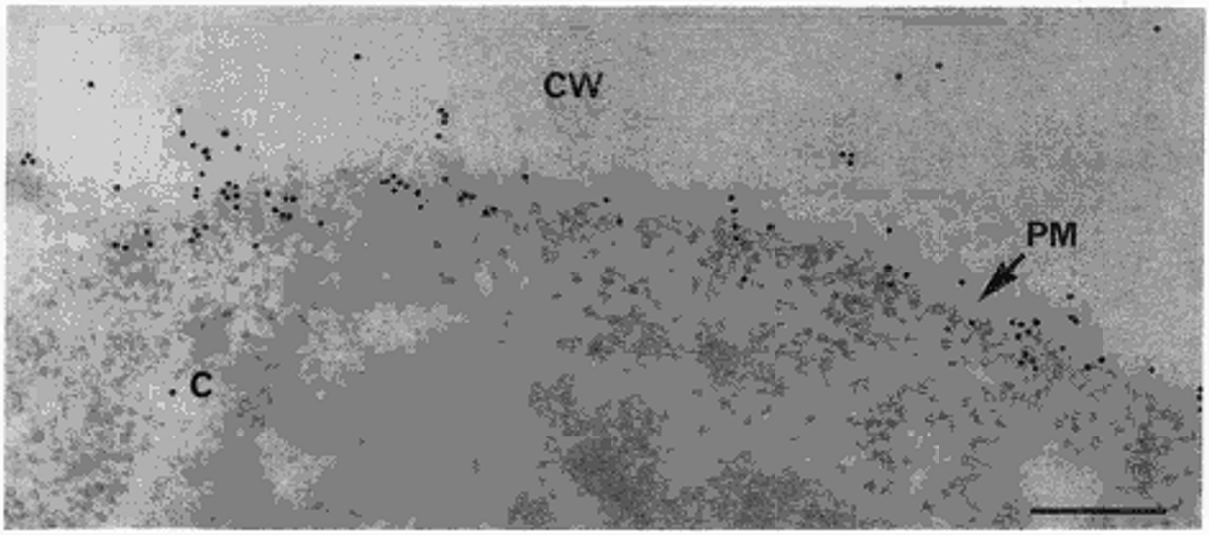
We have pioneered two novel approaches to the immunological characterisation of the plasma membrane of plant cells. First, we have immunised rats with living carrot protoplasts and prepared immune sera. These sera have for the first time been used in immunogold electron microscopy to observe the binding pattern of the mixture of antibodies. Second, a number of antibodies raised against a peribacteriod membrane fraction from pea infected with Rhizobium (Bradley et al, 1987) were screened against carrot protoplasts by immunofluorescence and against embedded cells of a number of crop plants and representatives of the major divisions of vascular plants in search of cross-reacting species. One antibody of interest (MAC 207) was then used to probe for plasma membrane vesicles isolated during 2-phase partitioning in a mixture of polyethylene glycol and dextran, and developed for specific and sensitive immunosassay of plasma membrane.

The antigen recognized by MAC 207 has been examined by protease digestion and periodate oxidation and during cross-competition experiments with a number of simple sugars and derivatives in an ELISA system.

2. Results

Immunisation of rats with viable carrot protoplasts gave rise in each instance to similar results. Immunogold localisation of the binding of the sera indicated that the principal localisation took place upon the plasma membrane. Auxillary binding occurred at the middle lamella of carrot cells and in some cases upon membranous inclusions in the cell walls that appear to be formed from evaginations from the plasma membrane. Some sera also located dictyosomes.

The monoclonal antibody MAC 207 located the plasma membrane in ultrathin sections of all plant cells so far examined. It also bound in abundance to membranous inclusions in the cell walls of some species. Immunoblotting of polyacrylamide gels indicated that the MAC 207 epitope is present within many proteins of widely divergent molecular weights. Digestion with Pronase and oxidation with periodate suggested that the antigen is a glycoprotein, and competition experiments with simple sugars are beginning to provide information about the structure and composition of the oligosaccharide moiety in the molecules.



Binding of monoclonal antibody MAC 207 to the plasma membrane (PM) of carrot suspension culture cells. C = cytoplasm, CW = cell wall. Scale bar = 200nm.

3. Discussion

We have shown that it is possible to make a large number of antibodies against the plasma membranes of plant cells by immunisation with living protoplasts. This is probably due to the presence within the plasma membranes of plant cells of macromolecules which do not occur in animals and which are therefore very immunoactive in mammalian systems. In contrast, many of the molecules of the cytoplasm and other organelles are concerned with metabolic processes common to both plants and animals, and consequently scarcely appear 'foreign' when encountered by the immunocompetant cells of rats. Once the cells responsible for secretion of the anti-carrot

antibodies have been cloned it should be possible to propagate a large number of monoclonal antibodies directed against the plasma membrane.

The isolation of plasma membrane vesicles by 2-phase partitioning has been developed by using marker enzymes for plasma membrane. These markers may give misleading results, however, and only a more specific plasma membrane probe can improve the quality of plasma membrane isolations. The monoclonal antibody MAC 207 locates a number of plasma membrane molecules which occur in many plant cells. Preliminary chemical tests indicate that the molecules involved are glycoproteins, and that at least two simple sugars are present within the oligosaccharide moiety of each. Moreover, the antibody binds to plasma membrane during ELISA and dot-blot analysis, and hence can be used as alternative to enzyme markers to probe fractionated or phase partitioned membranes. The specificity offered by this approach is greater than afforded by enzyme assay. Highly purified plasma membrane is now being used to immunise animals.

Reference: Bradley, D.J., Wood, E.A., Larkins, A.P., Galfre, G., Butcher, G.W. and Brewin, N.J. (1987) Isolation of monoclonal antibodies reacting with peribacteroid membranes and other components of pea root nodules containing Rhizobium leguminosarum. Planta, submitted.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

None

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

One contractor from the John Innes Institute, Dr. Roger Pennell, visited the research laboratories of De Danske Sukkerfabrikker in Copenhagen on April 6th and 7th, 1987 to meet others from EEC-funded projects in England and continental Europe. In June (1st-4th) Roger Pennell returned to Denmark with Graham Scofield, a technician appointed on the same contract, and viewed at first hand a number of techniques of value in both Copenhagen and at the DDS Breeding Station in Maribo. Three sugar beet suspension cultures were collected from Maribo, and these have been successfully established in Norwich. These cultures include regenerable and non-regenerable lines and will form the basis of future research at the John Innes Institute. Since the aims in the two laboratories are similar, we anticipate future exchanges of both staff and of materials.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Royal Veterinary & Agric. University, Copenhagen

Contract no.: BAP - 0077 - DK

Project leader: R. RAJAGOPAL

Scientific staff: J. Marcussen, P. Ulvskov

Address:
Royal Veterinary and Agricultural University
Dept. of Physiological Botany
Thorvaldsensvej 40
DK - 1871 COPENHAGEN V

Telephone no.: +45.1.351788 EXT.2642

Telex no.: 15061

Other contractual partners in the joint project:

P. Olesen, De Danske Sukkerfabrikker (Copenhagen)
K. Roberts, John Innes Institute (Norwich)

Title of the research activity:

Control of the differentiation of plant cells and of their regeneration into entire plants with special emphasis on cell membrane.

Key words:
Plant Hormones, Immunogen synthesis, Immunoabsorption, Immunoassays

Reporting period: August 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The aim of this joint project is to understand the mechanisms governing cell differentiation and regeneration through embryogenesis, using carrot and sugarbeet as model systems. Unravelling the hormonal control of differentiation through immunological methods and developing methods to isolate embryogenic cells based on their cell surface characteristics are the two prime objects of this project. Studies concerning embryogenesis-inducing and/or -inhibiting substances released from tissue cultures will also be undertaken.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

We are engaged in standardizing analytical methods based on poly- and monoclonal antibodies to the following plant hormones : indoleacetic acid (IAA), abscisic acid (ABA) and the cytokinins zeatin and zeatin riboside. This involves synthesizing antigen conjugates through novel methods; 2) study hormone correlations and metabolism in plant organs where hormone responses are well characterized, and 3) we are also interested in the intracellular localization of IAA through the use of fluorescent antibodies.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

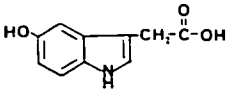
Methodology :

Immunological methods are increasingly preferred for the determination of trace amounts of diverse compounds. We are preparing poly- and monoclonal antibodies against plant hormones for immunoadsorption and immunoassay purposes. Since the small molecular weight plant hormones are only haptens, the specificity of the antibodies raised against them will depend strongly on the design of the antigen.

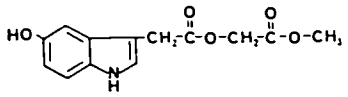
Two kinds of antigensare known, one in which the carrier protein is coupled to the -COOH group of the indole side chain, and the other in which the indole >NH group is the coupling site. We have synthesized a novel ring-coupled (at the 5 position) IAA antigen, as outlined in Fig. 1. The identity of all the intermediates involved in this synthesis has been confirmed by mass- and n.m.r spectra. Details of this synthesis, including the conditions needed for coupling 5-(N-succinimidoxy-carbonyl-ethyl)carbamoyl)-oxy-indolyl-3-acetoxyacetate methyl ester, to the protein carrier and the

subsequent enzyme-catalyzed deprotection reaction, form the basis of a patent application authored by Marcussen and Ulvskov (1986).

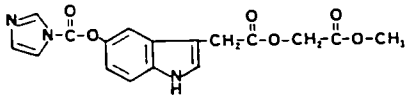
5-hydroxy-indolyl-3-acetic acid



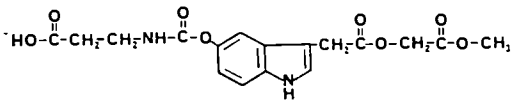
5-hydroxy-indolyl-3-acetoxy-acetate methyl ester



5-(N-(succinimidoxy-carbonyl)-oxy-indolyl-3-acetoxyacetate-methyl ester



5-(N-(carboxyethyl)-carbamoyl)-oxy-indolyl-3-acetoxyacetate-methyl ester



5-(N-(succinimidoxy-carbonyl-ethyl)-carbamoyl)-oxy-indolyl-3-acetoxyacetate methyl ester

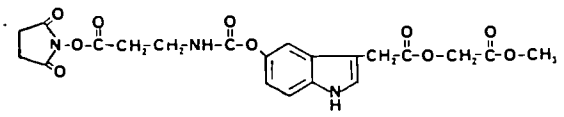


Fig. 1. Synthesis of ring-coupled (5-position) IAA hapten molecule.

2. Indole-3-acetamide (IAM), a metabolic precursor of IAA, presumably occurs only in plant-pathogen interactions. Agrobacterium tumefascians, commonly used as a vector for gene transfer into higher plants, carries genes on its plasmid for the synthesis of IAA via the indoleacetamide pathway. The formation of the neoplastic growth syndrome on the host plant following infection with Agrobacterium is dependent on the functioning of the introduced plasmid genes for plant hormone biosynthesis. We have exploited the clonal variation among monoclonal antibodies to IAA and selected antibodies suitable for the immunoaffinity purification of IAM. This work was carried out in collaboration with the Belgian group headed by Professor H. van Onckelen (Antwerp University) and the results have recently been published (see article 3 under item IV.1).

3. The enzyme-linked-immunosorbent-assay (ELISA) for cytokinins developed by others is satisfactory when combined with a prior HPLC purification step. We find that a single-step immunoaffinity purification prior to HPLC gives even better results, and this method has been routinely applied to many samples from Laboratory A during the past year.

Results :

At the BAP meeting in Brussels in March 1987, we presented our preliminary results supporting the assumption that antibodies raised against the new ring-coupled IAA-conjugate would be more specific. Data presented below further support this assumption.

Table 1: Antibody specificity towards the indole nucleus. Cross reactivity relative to IAA (=100%)

	IAA-N1-BSA	IAA-C5-BSA
N-methyl-IAA	14	0.9
4-Cl-IAA	100	27
5-Cl-IAA	100	4.5
7-Cl-IAA	100	5.3
5-OH-IAA	< 0.5	16

Table 2: Antibody specificity towards the C-3 substituent. Cross reactivity relative to IAA (=100%)

	IAA-N1-BSA	IAA-C5-BSA
Indole-3-carboxylic acid	< 0.5	< 0.5
Indole-3-propionic acid	3	< 0.5
Indole-3-acetamide	1.3	< 0.5
Indole-3-acetonitrile	1	< 0.5
IAA-L-Glu	< 0.5	< 0.5
Indole-3-aldehyde	1.3	< 0.1
IAA-L-Gln	< 0.5	< 0.1
IAA-L-Phe	< 0.5	< 0.1
IAA-L-Tyr	0.9	< 0.1
IAA-L-Ala	nd	0.12

nd = not determined

We plan to synthesize a stable IAA antigen coupled through its indole ring N and raise antibodies against it. We wish to devise an analytical procedure through the use of a single-step immunoaffinity purification based on one type of antibody and ELISA quantification based on the other type of antibody, and apply this tool to study relevant physiological problems.

Marcussen, J. and Ulvskov, P. 1986. - A method of preparing indoles or indole derivatives coupled via position 4,5,6 or 7, the indoles or indole derivatives concerned, as well as the use thereof. - Patent application nrs: US 898.608, JP 196.515/86, EP 86111670.5

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1 Publications in Journals, monographs etc.

1. R. Rajagopal, P. Ulvskov, & J. Marcussen - Effects of light on endogenous hormone levels in plants. In: Hormonal Regulation of Plant Growth and Development. (ed.) S.S.Purohit, 3: 29-90, Agro-Botanical Publishers, India, 1986.

2. R. Rajagopal, P. Ulvskov, J. Marcussen, J.M.Andersen & S. Allerup - Hormonal and phenolic changes accompanying and following UV-C induced stress in Spathiphyllum leaves. - J. Plant Physiol. In press, 1987.

3. P. Ulvskov, J. Marcussen, R. Rajagopal, E. Prinsen, P. Rüdelsheim & H. van Onckelen - Immunoaffinity purification of indole-3-acetamide using monoclonal antibodies. - Plant Cell Physiol. 28(5), 1987.

IV.2 Internal reports

Two internal reports on the preparation of immunoassay kits for cytokinin analysis were submitted to De Danske Sukkerfabrikker, Copenhagen.

IV.4 Doctorates awarded

Jan Marcussen and Peter Ulvskov obtained their Ph.D degrees from the Royal Vet. & Agricultural University, Copenhagen, in March 1987, based on their joint thesis "Immunological Methods in Plant Hormone Analysis".

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Dr. Jan Marcussen, Research Scientist supported by the contract, visited the John Innes Institute, Norwich during the summer of 1986 to familiarize himself with the research conducted at that Institute.

Dr. S. C. De Vries, of the Agricultural University, Wageningen, Netherlands has been invited to Copenhagen to discuss possible scientific cooperation on problems related to somatic embryogenesis in carrot cell suspensions.

Joint experiments were conducted with the team of Professor H. van Onckelen, Dept. of Biology, University of Antwerpen, Belgium, on the immunoaffinity purification of indoleacetamide (see article 3 under item IV.1).

One joint meeting was held at De Danske Sukkerfabrikker Copenhagen, during 6th and 7th, April 1987, with research groups from DDS, Copenhagen, Dept. of Plant Physiology, Agricultural University, Copenhagen and the John Innes Institute, Norwich, U.K. taking part. Progress to date was reviewed along with review lectures on relevant topics.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: C. N. R. S., Orsay

Contract no.: BAP - 0015 - F

Project leader: J. TEMPE

Scientific staff:

Address:
Université de Paris-Sud
Institut de Microbiologie
Bât. 409
F - 91405 ORSAY CEDEX

Telephone no.: TEL.+33.1.69417706

Telex no.: 692166 FACORS F

Other contractual partners in the joint project:

P. Costantino, Università di Roma

Title of the research activity:

Study of hairy root transformation : new strategy for plant genetic engineering.

Key words:

A. Rhizogenes, Transgenic plants, Rhizogenesis, T-DNA

Reporting period:

January 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

(i) - To study *Agrobacterium* T-DNA transfer and develop hairy root-based strategies for introducing foreign genes into plants.

(ii) - To understand the mechanism of hairy root morphogenesis.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Same as above.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY

Transformants obtained after inoculation of plant material *with Agrobacterium rhizogenes* and other *Agrobacterium* strains containing Ri or Ti plasmids derivatives were selected as hairy roots. Aseptic cultures were derived from primary hairy roots and plants were regenerated from these. Opine, T-DNA content and transcription were analyzed by high voltage paper electrophoresis and hybridization of Southern or Northern transfers. The legume *Lotus corniculatus* was used as a recipient for foreign DNA because it is easy to transform and readily regenerates whole plants from hairy roots.

The T-DNA region from *Agrobacterium rhizogenes* 2659 was identified by homology with that of pRi8196. Restriction maps were established by standard methods. T-DNA content of transformed roots was studied by hybridization of Southern transfers.

Cytological techniques were used to examine the response of plant tissues to inoculation with *Agrobacterium rhizogenes*. Different plant species and three different strains of *A. rhizogenes* were used.

2. RESULTS

(i) - Hairy root-based transformation strategies.

Because hairy root cultures derived from one single root tip as clonal and because it is easy to regenerate from hairy roots without loss of T-DNA we have proposed to use hairy root-derived strategies for plant transformation as an alternative to crown gall-derived strategies. Introduction of genes to be introduced into plants in hairy root T-DNA was achieved by homologous recombination. Using the legume *Lotus corniculatus* a transformation system was thus developed.

Multiple T-DNA inserts are often present in plant cells transformed with *Agrobacterium*. These result from one of two possible processes, namely multiple transformation of one plant cell by two different bacteria or transfer of several T-DNA copies from one single bacterium to one plant cell. Experiments showed that the first process, termed pluribacterial transformation, takes place with a frequency which is generally lower than 20 % whereas insertion of several T-DNA copies from one bacteria can be as high as 90 %. Both systems can be used to introduce unselectable makers in plant cells. This strategy by which the gene(s) to be introduced is (are) inserted independently from the hairy root T-DNA used as selectable marker allows for segregation of the unselectable marker in the progeny of transformed plants. It applies to a number of plants, including those for which biochemical markers such as resistance markers are not applicable.

(ii) - Studies on hairy root T-DNA genes.

A physical map of the T-DNA region of *Agrobacterium rhizogenes* 2659 has been established it will be used for mapping of the functions encoded by this region.

Studies on the hairy root phenomenon have been undertaken, in collaboration with the co-contractant and with other groups. These studies have allowed to understand the role of auxin in hairy root induction (see report by P. Costantino). Investigations at the cytological level on early events following inoculation of *Agrobacterium rhizogenes* to plants have shown that cells others than those giving normally rise to roots can undergo root meristem initiation after having been transformed with *A. rhizogenes*. Like with normal cells this initiation is dependent on auxin and occurs only after several cell divisions.

3. DISCUSSION

Hairy root-based strategies for transformation are now well established and in use in several laboratories. They have been applied for studying the expression of the soybean leghemoglobin gene in transgenic *Lotus corniculatus* which has demonstrated their general usefulness. They complete the array of existing plant transformation systems and when applicable provide one of the most expedient means to obtain cloned transformant tissues.

As investigations on the hairy root system progress it become more and more obvious that Ri plasmids are not just root-inducing Ti plasmid mutants. They have indeed evolved a completely independent way to induce plant cell proliferation which is not based on T-DNA-encoded synthesis of plant growth regulators. Thus instead of escaping the hormonal control of the organism like crown gall cells do by synthesizing their own hormones, hairy root cells have probably acquired the property to respond to endogenous levels of phytohormones by differentiating into root primordia. It is hoped that further investigations will result in the molecular description of this system and possibly to an understanding of physiological root morphogenesis.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1 PUBLICATIONS IN SCIENTIFIC JOURNALS

Petit, A., Berkaloff, A., Tempe, J. (1986) - Multiple transformation of plant cells by *Agrobacterium* may be responsible for the complex organization of T-DNA in crown gall and hairy root. Molec. gen. Genet. 202, 388-393.

Petit A., Stougaard J., Kühle A., Marcker, KA., Tempé, J. (1987) - Transformation and regeneration of the legume *Lotus corniculatus*: a system for molecular studies on symbiotic nitrogen fixation. Mol. Gen. Genet. 207, 245-250.

Dessaux Y., Tempé J., Farrand, Sk. (1987) - Genetic analysis of mannityl opine catabolism in octopine-type *Agrobacterium tumefaciens* strain 15955. Mol. Gen. Genet. 208, 301-308.

Combard A, Brevet, J., Borowski, D, Tempé, J. (1987) - Physical map of the T-DNA region of *Agrobacterium rhizogenes* strain NCPPB 2659. Plasmid (in press).

Cardarelli M., Spano L., Mariotti D., Mauro ML., Van Sluys MA., Costantino P. (1987) - The role of auxin in hairy root induction. Mol. Gen. Genet. 208, 457-463.

Tempé J., Schell J. (1987) - La manipulation des plantes. La Recherche, 188, 696-709.

Bercetche J., Chriqui D., Adam S., David C. (1987) - Morphogenetic and cellular reorientations induced by *Agrobacterium rhizogenes* (strains 1855, 2659 and 8196) on carrot, pea and tobacco. Plant Science (in press).

IV.4 DOCTORATE THESIS

Dessaux Y. (1987) - Contribution à l'étude des fonctions cataboliques des plasmides pathogènes d'*Agrobacterium tumefaciens*. Université de Paris Sud, Centre d'Orsay, France.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

We have a long standing collaboration with Pr. Costantino, our contractual partner in the frame work of the BAP programme. This involves exchange of information and material, as well as performing joint experiments. Short term visits help implement this collaboration.

The collaboration with Pr. Marcker on the use of *Agrobacterium rhizogenes* to develop a transformation system for studies on symbiotic nitrogen fixation has involved the exchange of material and information between the two laboratories.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Univ. degli Studi di Roma

Contract no.: BAP - 0018 - I

Project leader: P. COSTANTINO

Scientific staff: I. Capone, M. Cardarelli, P. Filetici, D. Mariotti, M.L. Mauro, L. Spano, M. Trovato, G. Vitali

Address:
Università di Roma
Dipartimento Genetica e Biologia Molecolare
c/o Ist. Fisiologia Generale
P.le A. Moro 5
I - 00185 ROMA

Telephone no.: +39.6.491721

Telex no.:

Other contractual partners in the joint project:

J. Tempé, Université Paris-Sud (Orsay)

Title of the research activity:

Study of hairy root transformation : new strategy for plant genetic engineering.

Key words:

A. rhizogenes, Transgenic plants, Rhizogenesis, T-DNA

Reporting period: January 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Characterization of the morphogenetic genes of the T-DNA of Agrobacterium rhizogenes for the exploitation of the extraordinary potentialities of the hairy root system in plant physiology and plant genetic engineering.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Definition of the relative role of TL and TR-DNA and auxin in the induction of hairy roots. Cloning, characterization and expression of individual TL-DNA genes of pRi1855 in transgenic plants and in heterologous systems.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

METHODOLOGY

Agrobacterium strains carrying different configurations of T-DNA have been constructed by cointegrating the whole of the TR-DNA of pRi 1855 with the RP4 derivative pNJ5000 and inserting the cointegrate in TR-less strains (mannopine strains) (1). Strains with only TR-DNA have been constructed trasferring the cointegrate in the T-DNA devoid strain LBA 4404 (1).
The above described strains have been utilized for carrot disc inoculations to elucidate the relative role of TL-DNA, TR-DNA and auxin in hairy root induction.

Comparison of T-DNA of different Ri plasmids has been carried out by extensive Southern cross-hybridizations (2).

Agrobacterium strains harbouring single or groups of TL-DNA genes (from pRI 1855) have been constructed by cloning several TL-DNA segments in the binary vector system Bin 19 and transferring the clones in LBA 4404. These strains have been utilized for infections on carrot discs and tobacco stems and to obtain transgenic tobacco plants from leaf disc infections.

Expression of individual pRi 1855 TL-DNA genes in E.coli has been achieved utilizing the cI λ fusion expression vector pEA305. Antibodies have been raised by immunizing mice with fusion proteins

Expression in yeast is currently underway through the secretion vector pEMBLyex2.

RESULTS AND DISCUSSION

The relative role of pRi1855 TL and TR-DNA (this latter harbouring auxin synthetic genes) has been elucidated by a complex series of infections and coinfections of carrot discs with Agrobacterium strains with or without TL or TR-DNA and/or auxin.
The conclusions of this analysis are that while the role of the TR-DNA aux genes is to allow hairy root induction

even in plant auxin limiting conditions, the TL-DNA is involved in conferring auxin responsiveness and morphogenetic potential to transformed plant cells (1).

The existence of two distinct regions conserved in the T-DNA of all three types of A.rhizogenes has been evidenced by means of an extensive series of Southern blot cross-hybridizations(2).

The key role of some TL-DNA genes in the induction of hairy roots and in controlling the morphology of transgenic plants has been pinpointed using bacterial strains harbouring single and groups of TL-DNA genes cloned in the binary vector system Bin 19. Thus, while ORF 11 is capable alone of inducing hairy roots in tobacco stem and controls most characteristic traits of hairy root plants (3), ORF 8 confers auxin sensitivity to transgenic plants; ORF 10,11 and 12 together are capable to induce hairy roots on carrot discs in the presence of adequate auxin supply and confer to transgenic plants a strong spontaneous rhizogenic potential (3).

Expression of ORF 11 in E.coli by means of the fusion vector pEA305 has been achieved and antibodies against the fusion protein (cIλ/ORF 11) have been raised.

These evidences constitute a major progress in the understanding of the hairy root system.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1

(1) M.Cardarelli, L.Spanò, D.Mariotti, M.L.Mauro, M.A.van Sluys and P.Costantino. 'The role of auxin in hairy root induction' Mol. Gen. Genet. in the press

(2) P.Filetici, L.Spanò and P.Costantino. 'Conserved regions in the T-DNA of different A.rhizogenes root-inducing plasmids' Plant Mol. Biol. in the press

(3) M.Cardarelli, D.Mariotti, M.Pomponi, L.Spanò, I.Capone and P. Costantino. 'Agrobacterium rhizogenes T-DNA genes capable of inducing hairy root phenotype'.
Mol. Gen. Genet in the press

IV.2

P.Costantino, M.Cardarelli, M.L.Mauro, M.Pomponi and L.Spanò 'The role of auxin and T-DNA genes in hairy root induction' Proc.EEC Meeting 'Genetic engineering of plants and microrganisms important for agriculture', Wageningen 1986

P.Costantino, M.Cardarelli, L.Spanò, D.Mariotti, P.Filetici, M.Pomponi and M.L.Mauro. 'Il ruolo dell'auxina nell'induzione di hairy roots' Atti del Convegno 'Embriogenesi e Differenziamento' Società Botanica Italiana, Perugia 1986

M.Cardarelli, L.Spanò, D.Mariotti, M.L.Mauro, G.Vitali and P. Costantino.'Ruolo dell'auxina e del T-DNA di A.rhizogenes nella induzione di hairy roots. Atti XXIII Convegno SIBBM, Pavia 1986

P.Filetici, L.Spanò, M.Cardarelli, M.Pomponi, S.Speranza, M.Trovato and P.Costantino.'Identificazione, clonazione ed espressione di regioni di T-DNA conservate in tutti i plasmidi Ri di A. rhizogenes'. Atti XXIII Convegno SIBBM, Pavia 1986

P.Costantino, M.Cardarelli, L.Spanò, D.Mariotti, P.Filetici, M.L.Mauro and M.Pomponi. 'The role of auxin and T-DNA genes

in hairy root induction'.Proc. Fallen Leaf lake Conference on Crown Gall, Lake Tahoe, California, 1986

L.Spanò, M.Cardarelli, M.L.Mauro, M.Pomponi, D.Mariotti, P.Filetici and P.Costantino. 'Role of A.rhizogenes T-DNA genes in the induction of root differentiation'. Atti XXX Convegno SIGA, Padova 1986

P.Costantino, M.Cardarelli, P.Filetici, D.Mariotti, M.L.Mauro,M.Pomponi, L.Spanò and M.Trovato. 'Molecular and physiological approaches to the dissection of the hairy root syndrome'.Proc. EEC-BAP Meeting 'Genetic and Cellular Engineering of Plants and Microrganisms important for Agriculture' Louvain-la-Neuve 1987

P.Costantino and J.Tempé. 'On the physiology, biochemistry and molecular biology of the hairy root syndrome'. Proc. EEC-BAP Meeting, Louvain-la-Neuve 1987

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	~~No~~
Exchange of staff	Yes	~~No~~
Joint experiment(s)	Yes	~~No~~
Joint meeting(s)	Yes	~~No~~

Descriptive information for the above data.

Exchange of material is customary in the form of bacterial clones tissue cultures, transgenic plants, protocols, preprints etc., with the laboratory of J.Tempé, Institut de Microbiologie, Orsay. Preprints, clones and others are also exchanged with other BAP contractors.

Exchange of staff consists mainly in frequent visit of P.Costantino to the laboratory of J.Tempé for joint experiment planning and meetings.

Joint experiments are frequently carried out exploiting the better molecular biology facilities and experience of Roma's group and the better tissue culture and plant set-up of Orsay.

P.Costantino and J.Tempè are currently promoting the establishment of an hairy root ELWW involving several BAP contracting laboratories.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Leiden University Contract no.: BAP - 0076 - NL

Project leader: R.A. SCHILPEROORT
Scientific staff: J.H.C. Hoge, P.J.J. Hooykaas, J. Memelink, R. Peerbolte, K. Roodenburg, M.J.J. van Haaren, G.J. Wullems

Address:
University of Leiden
Dept. of Plant Molecular Biology
Wassenaarseweg 64
NL - 2333 AL LEIDEN

Telephone no.: +31.71.27.47.65 or 27.47.67

Telex no.: 39427

Other contractual partners in the joint project:

M. Kreis, Rothamsted Experimental Station (Harpenden)

Title of the research activity:

Mechanisms controlling transfer and expression of developmentally regulated plant genes.

Key words:

Plant gene expression, Agrobacterium onc-genes, Cytokinin induced genes, Auxin induced genes, Plant development

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

a) Isolation and characterization of cis-acting regulatory sequences in plant genes
b) Development of techniques to transfer genes into monocots and dicots
c) Development of systems for the functional analysis of putative control regions
d) Identification and characterization of genes controlling plant growth and development

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

a) The characterization of the cis-acting regulatory sequences of the T-cyt gene
b) Further development of plant gene transfer systems
c) Isolation of transgenic tobacco plants with the hordein gene
d) Isolation of plant genes of which expression is regulated by the phytohormones auxin or cytokinin

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

a) Characterization of the cis-acting regulatory sequences of the T-cyt gene.

Deletion mutagenesis showed that 184 bp of the 5' non-coding region and 270 bp of the 3' non-coding region of the T-cyt gene, which is present in the T-DNA of octopine strains of Agrobacterium tumefaciens, are sufficient for expression of this gene in transformed plant cells at normal levels.
Within the 5' non-coding region two essential expression signals were identified: (i) an activator element located between positions -185 and -129 with respect to the translational start-codon, and (ii) either of two TATA-boxes, which are present between positions -79 and -29. The deletion of the activator element or both TATA-boxes at the same time was found to lead to an inactivation of the T-cyt gene.

Bisulphite mutagenesis (C → T and G → A transitions) revealed that a 13 bp sequence (CCACACTTTGTTG) located between -153 and -129 determines the function of the activator element. The T-cyt activator shares significant homology with activator elements of other plant genes. Based on this homology we propose the following

plant gene activator consensus sequence CCACANT/ATNNNT/AG. The deletion of both TATA-boxes inactivated the gene, but if only one of the two was deleted the gene remained active. Using primer extension assays it could be shown that each of the two TATA-boxes specifies a distinct (approximately 30 bp downstream) transctiption initiation site. Neither of the two putative CAAT-boxes, which are present in the 5' non-coding region, did significant affect the expression of the T-cyt gene in plant cells.

Within the 3' non-coding region of the T-cyt gene there are two poly-adenylation boxes (AATAAA), which flank a GT-rich sequence of which the significance for the expression of animal genes has been demonstrated. We could show that at least one of the poly-adenylation boxes as well as the GT-rich box are essential for the expression of the T-cyt gene.

b) Further development of plant gene transfer systems.

Binary vectors were constructed with synthetic 24 bp border repeats. These turned out to be inefficient at T-DNA transfer. Sequences, which in the wild-type Ti plasmid are present to the right of the right border repeat, proved to be necessary to obtain optimal T-DNA transfer frequencies. Said sequences comprise an enhancer for T-DNA transfer, which works equally well in both orientations in a position either to the left or to the right of the right border repeat. Vectors are being constructed now which contain not only the border repeats but also this enhancer sequence.

In order to learn more of the T-DNA transfer process, the vir-genes involved were cloned and (partially) sequenced. In agreement with data from other groups two vir-genes (virA and virG) were found to be involved in the regulation of expression of the other vir-genes. Sequence analysis of these genes revealed that virG probably codes for a transcriptional activator, while virA codes for a transmembrane protein. On the basis of these characteristics it was suggested by Ausubel et al that virA and virG belong to a particular class of two factor regulatory systems, namely one in which a (membrane) sensor protein (in this case VirA) becomes able to activate the positive regulatory protein (VirG) only under special conditions (in the case of VirA: the presence of plant phenolics). Recently we used antiserum against the VirA protein in order to obtain evidence showing that the VirA protein is indeed located in the inner membrane of Agrobacterium. We are now manipulating these regulatory genes in order to see whether we can isolate mutants which have a capacity to transfer T-DNA to plant cells at a higher frequency.

c) Isolation of transgenic tobacco plants with hordein genes.

Plasmids with hordein genes were received from the Rothamsted Experimental Station. These were transferred to tobacco via the Agrobacterium helper strain LBA4404. Seed-producing transgenic plants were obtained and these will be analyzed for hordein gene expression in collaboration with the Rothamsted group.

d) Identification and characterization of genes controlling plant growth and development.

Differential screening of cDNA libraries resulted in the isolation of five cDNA clones corresponding to mRNAs which are present at high levels in T-cyt gene transformed tobacco shoots, i.e. shoots lacking roots and apical dominance due to T-cyt activity, but at low levels in the shoot part of normal tobacco plants. Differential screening of cDNA libraries was also done to obtain seven cDNA clones corresponding to mRNAs of which the accumulation was shown to be auxin-dependent. The twelve mentioned cDNA clones as well as their corresponding genomic clones are being characterized in detail now.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

1. Govers, F., Moerman, M., Downie, J.A., Hooykaas, P., Franssen, H.J., Louwerse, J., Van Kammen, A. and Bisseling, T. (1986) Rhizobium nod genes are involved in inducing an early nodulin gene. Nature 323: 564-566.
2. Van Haaren, M.J.J., Pronk, J.T., Schilperoort, R.A. and Hooykaas, P.J.J. (1986) T-region transfer from Agrobacterium tumefaciens to plant cells: functional characterization of border repeats. In: Recognition in Microbe-Plant Symbiotic and Pathogenic Interactions, pp. 203-214. Edited by Lugtenberg, B.,Springer Verlag, Berlin.
3. Hooykaas, P.J.J. and Schilperoort, R.A. (1986) The molecular basis of the Agrobacterium-plant interaction-characteristics of Agrobacterium virulence genes and their possible occurrence in other plant-associated bacteria. In: Recognition in Microbe-Plant Symbiotic and Pathogenic Interactions, pp. 189-202. Edited by Lugtenberg, B, Springer Verlag, Berlin.
4. Melchers, L.S., Thompson, D.V., Idler, K.B., Schilperoort, R.A. and Hooykaas, P.J.J. (1986) Nucleotide sequence of the virulence gene virG of the Agrobacterium tumefaciens octopine Ti plasmid: significant homology between virG and the regulatory genes ompR, phoB and dye of E.coli. Nucl. Acids Res. 14: 9933-9942.
5. Mcneil, M., Darvill, J., Darvill, A.G., Albersheim, P., Van Veen, R., Hooykaas, P., Schilperoort, R. and Dell, A. (1986) The discernible structural features of the acidic polysaccharides secreted by different Rhizobium species are the same. Carbohydr. Res. 146: 307-326.
6. Offringa, I.A., Melchers, L.S., Regensburg-Tuink, A.J.G., Costantino, P., Schilperoort, R.A. and Hooykaas, P.J.J. (1986) Complementation of Agrobacterium tumefaciens Ti aux mutants by genes from the T_R-region of the Ri plasmid of Agrobacterium rhizogenes. Proc. Natl. Acad. Sci. USA 83: 6935-6939.
7. Ooms, G., Twell, D., Bosser, M.E., Hoge, J.H.C. and Burell, M.M. (1986) Developmental regulation of Ri T_L-DNA gene expression in roots, shoots and tubers of transformed potato (Solanum tuberosum cv. Desiree). Plant Mol. Biol. 6: 321-330.
8. Peerbolte, R. (1986) The fate of T-DNA during vegetative and generative propagation. Thesis, Leiden University.
9. Peerbolte, R., Leenhouts, K., Hooykaas-Van Slogteren, G.M.S., Hoge, J.H.C., Wullems, G.J. and Schilperoort, R.A. (1986) Clones from a shooty tobacco crown gall tumor I: deletions, rearrangements and amplifications resulting in irregular T-DNA structures and organizations. Plant Mol. Biol. 7: 265-284.
10. Peerbolte, R., Leenhouts, K., Hooykaas-Van Slogteren, G.M.S., Wullems, G.J. and Schilperoort, R.A. (1986) Clones from a shooty tobacco crown gall tumor II: irregular T-DNA structures and organization, T-DNA methylation and conditional expression of opine genes. Plant Mol. Biol. 7: 285-299.
11. Schilperoort, R.A. (1986) Integration, expression and stable transmission through seeds of foreign genes in plants. In: Genetic Manipulation in Plant Breeding. Eds. Horn, Jensen Odenbach, Schieder. Walter de Gruyter & C., Berlin. New York pp. 837-858.
12. Schilperoort, R.A. (1986) Plant-Agrobacterium interaction. In: Recognition in Microbe-Plant Symbiotic and Pathogenic Interactions. Edited by Lugtenberg, B., Springer Verlag, Berlin, pp. 431-432.

13. Squartini, A., Hooykaas, P.J.J. and Nuti, M. (1986) Genetic determinants of nodulation in pRle 1001a :nodD. In: Molecular Genetics of Plant-Microbe Interactions, pp. 162-164. Edited by Verma, D.P.S. & Brisson, N., Nijhoff, M. Publ., Dordrecht.
14. Van Haaren, M.J.J., Pronk, J.T., Schilperoort, R.A. and Hooykaas, P.J.J., (1987) Functional analysis of the Agrobacterium tumefaciens octopine Ti plasmid left and right T-region border fragments. Plant Mol. Biol. 8: 95-104.
15. Van Veen, R.J.M., Okker, R.J.H., Hooykaas, P.J.J. and Schilperoort, R.A. (1986) Plasmid molecular genetics of Rhizobium leguminosarum, Rhizobium trifolii and Rhizobium phaseoli. In: Nitrogen Fixation, Volume 4 - Molecular Biology. Edited by Broughton, W.J. & Puhler, A. Clarendon Press, Oxford, pp. 224-244.
16. Van Veen, R.J.M., Den Dulk-Ras, H., Schilperoort, R.A. and Hooykaas, P.J.J. (1987) Chromosomal nodulation genes: Sym plasmid containing Agrobacterium strains need chromosomal virulence genes (chvA and chvB) for nodulation. Plant Mol. Biol. 8: 105-108.
17. Wullems, G.J., Krens, F.A. and Schilperoort, R.A. (1986) In vitro transformation of Nicotiana tabacum protoplasts via co-cultivation with Agrobacterium tumefaciens and via DNA transformation with isolated Ti plasmid DNA. In: Handbook of plant cell culture, 4 (Ammirato, P., Evans, D., Sharp, W. and Yamada, Y., eds.) Macmillan Publ. Co., New York, pp. 197-220.

. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Exchange of materials.

Plasmid vectors, selectable marker genes, isolated plant genes and their mutated derivatives, and seeds of transgenic plants were exchanged between Leiden and Rothamsted. We provided Agrobacterium strains and vector systems to several other groups with a BAP-contract.

Exchange of staff.

Working as guests in our laboratory were dr.A.Squartini (University of Padova, Italy) and dr.E.Oxtoby (University of Newcastle, U.K.).

Joint experiments.

We received E.coli plasmids with hordein genes from Rothamsted and used these for the transformation of tobacco. Vice versa we sent out to Rothamsted a collection of T-cyt genes with promoter mutations for screening in potato. Our collaboration with Rothamsted resulted in a joint publication (Ooms et al., PMB 6 (1986) 321-330). A collaboration with dr.P.Costantino (University of Rome, Italy) also led to a joint paper (Offringa et al., PNAS 83 (1986) 6935-6939).

Joint meetings.

Dr.B.G.Forde, Dr.P.Gallois and Dr.G.Ooms visited our laboratory to discuss progress and joint experiments, in particular regarding the development of an Ri vector, the transfer of hordein genes into the genome of tobacco and the transfer of mutant T-cyt genes into potato.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Trustees of Lawes Agric. Trust Harpenden

Contract no.: BAP - 0099 - UK

Project leader: M. KREIS

Scientific staff: B.G. Forde, J. Forde, C. Marris, G. Ooms, J. Phelpstead

Address: Rothamsted Experimental Station
Molecular Sciences Division
Biochemistry Department
UK - HARPENDEN, HERTS AL5 2JQ

Telephone no.: +44.5827.63133 EXT. 2721

Telex no.: 825726

Other contractual partners in the joint project:

R.A. Schilperoort, University of Leiden

Title of the research activity:

Mechanisms controlling transfer and expression of developmentally regulated plant genes.

Key words:

Gene expression, Hordein, Glutamine synthetase, Patatin, Plant development

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

(a) Isolation and charcterization of cis-acting regulatory gene sequences.

(b) Development of techniques to transfer genes into monocot and dicots.

(c) Development of systems for functional analysis of putative control regions.

(d) Identification and characterization of genes controlling plant growth and development.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

1. Identification of putative control regions regulating gene expression in the developing cereal seed.

   (a) Isolation and structural analysis of barley β-amylase and chymotrypsin inhibitor-2 genes.

   (b) Functional analysis of the predicted control sequences in the barley B1 hordein gene by transformation into tobacco.

   (c) Development of techniques to be used for the identification of trans-acting regulatory factors (DNA-binding proteins).

2. Characterization of glutamine synthetase (GS) genes and molecular analysis of expression of individual gene members of the GS gene family in *Phaseolus vulgaris*.

3. Growth controlling genes in potato. Assessment of the feasibility of modifying potato biology in a desired way using *Agrobacterium* T-DNA genes under the control of endogenous plant promotor sequences, in particular from patatin genes.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Methodology. Most of the methods used are readily available in the literature and are described or referred to in our papers.

2. Results.

   (1) Identification of putative control regions regulating gene expression in the developing cereal seed.

   (a) Isolation of chymotrypsin inhibitor-2 and β-amylase genomic clones
   Two genomic clones of barley β-amylase and chymotrypsin inhibitor-2 have been isolated and purified. These are being characterized and sequenced.

   (b) Functional analysis of the predicted control sequences in the barley B1 hordein gene.

   The 549 bp of the immediate 5' flanking region of a B1 hordein gene containing a transcription start site, a "TATA" box, a "CAAT" like box and a putative regulatory sequence at -300 relative to the ATG, was

linked to the bacterial reporter gene CAT through insertion into the polylinker of the binary T_i vector pCATter (Bevan, PBI, Cambridge). The construct was mobilized by the helper plasmid pRK 2013 into *A. tumefaciens* LBA 4404. The chimaeric gene construct was stably transformed into the genome of tobacco (*N. tabacum* SR) using the leaf disc transformation system. Whereas no CAT activity was detected in transgenic tobacco leaves, enzyme activity was detected in the seeds from 11 days after pollination. Furthermore, CAT activity was found in the endosperm but not in the embryo (see Fig. 1). The consitutive 19S promoter from CaMV, however, directed expression in all the above tissues. It is concluded that the 549 bp of the 5' flanking region of the B1 hordein gene contain the necessary cis-acting regulatory sequences to direct tissue and developmental specific expression.

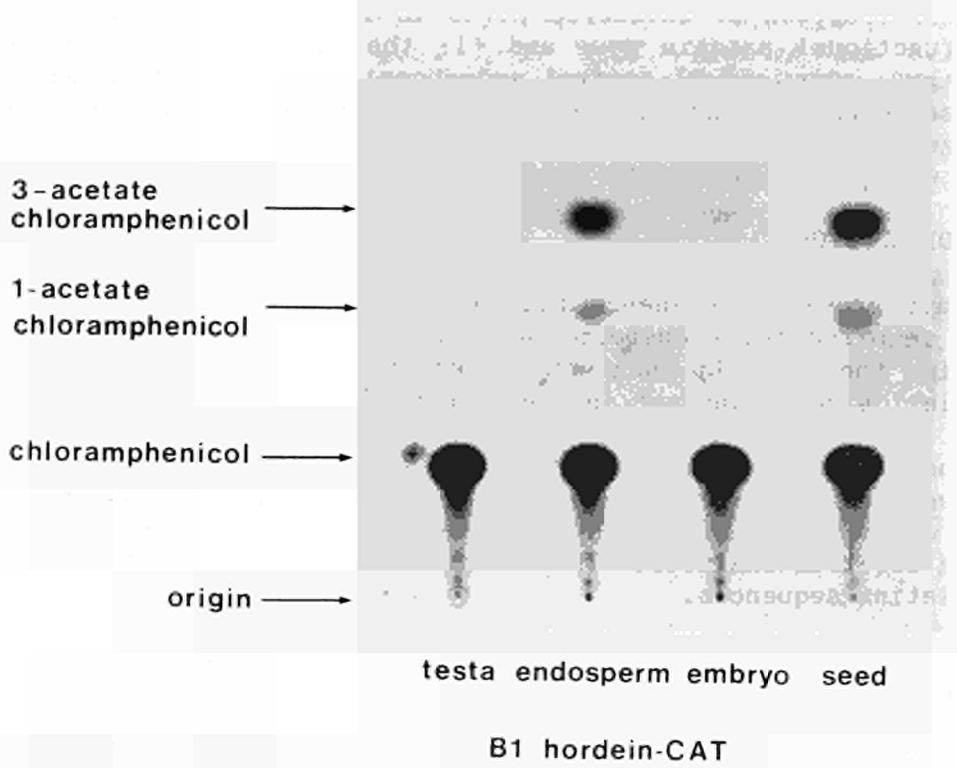


Fig. 1. Tissue specific expression of a B1 hordein-CAT gene in transgenic tobacco plants.

(c) Development of techniques for the identification of DNA-binding proteins.

Barley nuclei were isolated from endosperms and young leaves using a modified method of Willmitzer and Wagner (1984). A crude nuclear extract was prepared and probed for specific DNA-protein interactions using two techniques. First we used a procedure developed by Miskimins *et al.* (1985) for detecting high-affinity sequence-specific DNA binding proteins from crude nuclear extracts. A second method which is based on the altered mobility of a labelled DNA fragment on polyacrylamide gel electrophoresis when bound to protein (Galas and Schmitz, 1978). Preliminary results indicate that the barley endosperm contains several proteins which bind to putative regulatory sequences in the 5' flanking region of the B1 hordein gene.

2. Characterization of glutamine synthetase genes and molecular analysis of expression of individual members.

   Chimaeric genes consisting of 5' control sequences from the nodule specific GSN (for transcription and translation fusions) and the root/nodule enhanced GSR (for translation fusion) have been constructed using the reporter gene *gus*-Nos (with β-glucoronidase coding DNA and nopaline synthase terminator DNA). In parallel, an Ri shuttle vector for their transfer via *A. rhizogenes* into *Lotus corniculatus* has been isolated but its functionality remains to be proven.

3. Growth controlling genes in potato

   Construction of chimaeric genes is in progress using the 5' end of a functional patatin gene and (1) the coding DNA of the reporter gene (*gus*) plus *nos* 3' control sequences); (2) the coding and 3' regulator sequences of the Ti T-DNA cytokinin gene and (3) the Ti T-DNA aux-1 gene.

3. DISCUSSION

We have presented evidence that all the signals of the barley B1 hordein gene required for endosperm specific expression in transgenic tobacco are within the 549 bp segment immediately upstream of the translation initiation codon and not in the structural region or 3' end of the gene.

Having established a transformation system where we are able to observe tissue specific expression of the B1 hordein gene in transgenic tobacco, we have begun to delineate further the cis-acting regulatory region involved using deletion analysis. These experiments will identify the limits of the regulating sequences.

REFERENCES

1. Galas and Schmitz A. (1978) Nucleic Acid Res. 5, 3157-3170.

2. Miskimins, W. K., Roberts, M. P., McClelland A. and Ruddle, F. H. (1985) Proc. Natl. Acad. Sci. USA, 82, 6741-6744.

3. Willmitzer, L. and Wagner, K. G. (1981) Expl. Cell Res. 135, 69-77.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1.

1. M. S. Williamson, J. Forde, B. Buxton and M. Kreis (1987) Nucleotide sequence of barley chymotrypsin inhibitor-2 (CI-2) and its expression in normal and high-lysine barley. *Eur. J. Biochem.* 165, 99-106.

2. M. Kreis, M. S. Williamson, B. Buxton, J. Pywell, J. Hejgaard and I. Svendsen (1987) Primary structure and differential expression of β-amylase in normal and mutant barleys. *Eur. J. Biochem.* in press.

IV.2.

1. C. Marris, P. Gallois, J. Clark, J. Forde, B. G. Forde and M. Kreis (1987) Regulation of expression of barley seed protein genes. *BAP Meeting Louvain-La-Neuve, March 23-25 1987. Genetic and Cellular Enginering of Plants and Microorganisms important for Agriculture. Abstract p.154.*

2. P. Gallois, C. Marris, B. G. Forde and M. Kreis (1987) Seed specific expression of a B1 hordein gene in transgenic tobacco plants. *Plant Physiology Supplement* 83, No. 4, p.150.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Exchange of Material(s)

1. A gamma secalin cDNA clone was supplied to Dr. P. Joudrier (Montpellier, France)

2. A glyceraldehyde phosphatedehydrogenase cDNA clone was received from Prof. D. von Wettstein (Copenhagen, Denmark)

3. A barley genomic library was kindly given by Dr. W. Rohde (Cologne, Germany)

4. Dr. G. Ooms received T-DNA clones from Dr. P. Costantino (Rome, Italy)

Exchange of Staff

M. Kreis visited Dr. Rohde at the Max Planck Institute in Cologne in 1986 for one week. The aim was to gain experience in the construction of genomic libraries.

Joint Experiments

The collaboration with our partners in the laboratory of Prof. R. Schilperoort in Leiden involved the transfer of *E. coli* plasmids containing a complete B1 hordein gene and mutant genes. These genes have been transferred into the genome of tobacco by the Leiden group and transgenic kanamycin resistant plants have been regenerated.

Joint experiments have been going on with the Unviersity of Copenhagen and the Carlsberg Laboratory. These led to the identification of a β-amylase cDNA clone and the first nucleotide and protein sequence of a plant β-amylase.

Joint Meetings

Dr. B. G. Forde, Dr. P. Gallois (EEC, trainee) and Dr. G. Ooms visited the laboratory of Prof. Schilperoort at the University of Leiden to discuss progress and joint experiments, in particular the joint development of an R1 vector and the transfer of barley B1 hordein genes into the genome of tobacco.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: P.B.I., Cambridge Contract no.: BAP - 0106 - UK

Project leader: R.B. FLAVELL
Scientific staff: M.W. Bevan, V. Colot, L. Robert, A. Goldsbrough, D. Schnick, M. Thomas

Address: Plant Breeding Institute
Maris Lane, Trumpington
UK - CAMBRIDGE CB2 2LQ

Telephone no.: +44.223.840411

Telex no.: 818280 PBICAM G

Other contractual partners in the joint project:

P. Joudrier, I. N. R. A. (Montpellier)

Title of the research activity:
Analysis and manipulation of wheat protein genes related to grain quality.

Key words:
Glutenin, Gene, Wheat, Seed, Tobacco

Reporting period: June 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

(1) To determine the amino acid sequence differences between specific wheat seed proteins (high molecular weight (HMW) glutenin and gliadin subunits) that influence the contribution of the proteins to dough quality in the breadmaking process. This is to be achieved by isolating specific alleles and determining their nucleotide sequence.

(2) To determine the molecular mechanisms responsible for the expression of the wheat seed storage protein genes only in the endosperm of the seed.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

(1) To complete the characterisation of the amino acid sequence for high molecular weight (HMW) glutenin subunit number 10 and compare it with that of subunit number 12.

(2) To investigate the expression of a HMW glutenin gene in tobacco.

(3) To analyse tobacco plants into which various chimaeric genes of the wheat HMW and LMW glutenin promoters coupled to the bacterial coding sequence for chloramphenicol acetyltransferase (CAT) have been inserted to see if wheat glutenin gene promoters can be analysed using transgenic tobacco plants.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

METHODOLOGY

The gene encoding HMW glutenin subunit 10 from the wheat variety Hope was purified from a library of DNA fragments cloned in bacteriophage lambda and sequenced using the dideoxy method. A gene for a LMW glutenin was isolated from the variety Chinese Spring and sequenced by similar methods.

For inserting the previously isolated HMW glutenin subunit 12 into tobacco plants, an EcoRI fragment containing the gene was inserted into the polylinker site of the binary vector Bin 19. To study glutenin gene promoter activities, various fragments containing the promoter and upstream DNA were inserted into the polylinker of the binary vector plasmid polyCATter. This resulted in the coding sequence of the bacterial gene for chloramphenicol acetyl transferase being put under the control of the inserted wheat promoter and the terminator from the gene for nopaline synthase. The binary vector plasmids were mobilised into Agrobacterium

tumefaciens strain LBA 4404 using the Escherichia coli strain HB101 harbouring pRK2013 in a triparental mating. The integrity of the plasmids in Agrobacterium were verified. Leaf discs of Nicotiana tabacum (c.v. Samsun) were transformed by standard procedures, transformed cells selected on 100 µg/ml kanamycin and plants regenerated.

Extracts of tissues for CAT assays were made by grinding in 0.25 M Tris-HCl buffer pH 7.5 and heated to 60°C for 10 min. Enzyme activity was determined by the acetylation of radioactive (^{14}C) chloramphenicol and separation of the products of the reaction chromatographically on a TLC plate. The presence of HMW glutenin subunit 12 in tobacco seeds was determined by SDS-polyacrylamide gel electrophoresis of an extract prepared from seeds in the presence of β mercaptoethanol and also by Western blotting of the fractionated proteins onto nitrocellulose and incubation with radioactively labelled antibodies prepared against glutenin.

RESULTS

The gene for HMW glutenin subunit 10 has been sequenced. This gene is present at the 1D locus of the variety Hope together with another HMW glutenin gene. These two alleles are associated with better breadmaking quality than the equivalent alleles in the variety Chinese Spring one of which specifies the HMW glutenin subunit number 12. The amino acid sequences of subunits 10 and 12 are currently being compared by various methods, in order to assess which of the relatively few amino acid differences between them are likely to be responsible for the different dough qualities.

The complete gene encoding HMW glutenin subunit 12 and containing more than 1.5 kbp upstream from the coding sequence was inserted into tobacco plants using Agrobacterium tumefaciens transformation of leaf discs. Extracts of seeds from the tobacco plants were found to contain a protein of identical molecular weight to subunit 12 and which was precipitated by antibodies prepared against wheat glutenin.

When DNA fragments of the LMW glutenin gene promoter, of varying lengths but all containing the TATA and CAAT boxes, were fused to the CAT coding sequence and inserted into tobacco plants, CAT activity was detected only in seeds, not leaves, stems or roots providing at least 320 bp upstream of the transcription start site were present. If only 160 bp were present, no CAT activity was detected in seeds of the three plants tested. Thus there are DNA sequences residing between 160 and 320 bp upstream from the transcription start site which are essential for the seed-specific

expression of wheat LMW glutenin genes in tobacco. DNA sequences -140 to -930 bp from the start of transcription, when added upstream to a truncated CaMV 35S RNA promoter possessing a TATA and CAAT box but no enhancer, resulted in elevated levels of seed-specific CAT activity. Thus sequences within this region are capable of modifying the properties of other promoters.

Analyses on seeds dissected into endosperm and embryo fractions showed that the CAT activity was localised in the endosperm. This is the tissue in wheat seeds in which glutenin genes are expressed. Similar experiments with chimaeric genes containing HMW glutenin gene promoter fragments fused to the CAT coding sequence and inserted into tobacco have shown that 400 bp upstream of the transcription start site are sufficient to determine endosperm-specific expression in tobacco seeds.

DISCUSSION

During this period important progress has been made towards the long-term goal of manipulating HMW and LMW glutenin genes for the modification of breadmaking quality in wheat. Determination of the amino acid sequence of HMW glutenin subunit 10 and its comparison with subunit 12 is providing very valuable details on the amino acid differences that may determine variation in baking quality.

The HMW and LMW genes isolated from wheat chromosomes are likely to be complete, including all essential regulatory sequences, because the fragment containing HMW subunit 12 is sufficient to produce HMW subunit 12 in tobacco seeds and only a few hundred base pairs of DNA upstream from the transcription start site are essential for determining endosperm-specific expression in tobacco seeds. The finding that endosperm-specific signals of wheat (monocot) genes are recognised during tobacco (dicot) seed development is particularly interesting and implies that regulatory systems have been conserved during monocot and dicot divergence.

Now that the regulatory regions responsible for endosperm-specific expression have been localised approximately in the glutenin genes, more detailed experiments can be carried out to determine which particular DNA sequences are involved in, for example, recognition by the regulatory proteins that are likely to be responsible for the gene activation. Such experiments are in progress.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

None

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff	Yes	
Joint experiment(s)		No
Joint meeting(s)		No

Descriptive information for the above data.

As part of the cooperation, Therese Terce-Laforgue visited the PBI to construct a library of wheat DNA fragments in a bacteriophage lambda vector.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: I. N. R. A., Montpellier

Contract no.: BAP - 0106 - UK (sub-contract)

Project leader: P. JOUDRIER

Scientific staff:

Address:
I.N.R.A.
Lab. de Technologie des Céréales
9, place Viala
F - 34060 MONTPELLIER CEDEX

Telephone no.: +33.67612433

Telex no.: 490818 INRAMON F

Other contractual partners in the joint project:

R.B. Flavell, Plant Breeding Institute (Cambridge)

Title of the research activity:

Analysis and manipulation of wheat protein genes related to grain quality.

Key words:

Gene cloning, Wheat, Endosperm protein, Technological quality

Reporting period: July 1986 - July 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The aim of the project is to obtain in a first step :

- a better understanding of the storage regulation of gene expression during endosperm development

and in a second step :

- to manipulate the protein genes to improve nutritional and technological quality of wheat varieties.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

We would like to identify the genes involved in the synthesis of the gamma gliadin 45 or 42 components (electrophoretic mobilities according to the international nomenclature) because of the relationship observed between the presence or absence of these proteins and the Triticum durum gluten viscoelasticity.

Two hypothesis have been done : is it a functional relationship or a genetic linkage with a LMW glutenin subunit as suggested by Payne et al (1984) ?. This work should answer theses questions.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1) METHODOLOGY

- cDNA Libraries construction :

Total RNA was isolated by the guanidine hydrochloride extraction method (Chirgwin et al, 1979) from immature endosperms (20 Days After Flowering). Poly (A)+ mRNAs were purified by two cycles of oligo (dT)-cellulose chromatography and tested by in vitro translation in a wheat germ cell free system. Two Triticum durum cultivars were utilized for these study : Agathé (gamma gliadin 45 type) and Kidur (gamma gliadin 42 type).

cDNA cloning was done according to Gubler and Hoffman (1983) by dG-dC tailing in pUC 8. For the screening of the cDNA libraries, a Triticum aestivum gamma gliadin clone : pTag 64 (Bartels and Thompson, 1983) and a Secale cereale gamma secaline clone : pSC 503 (Kreis et al, 1985) were used.

- Genomic libraries construction :

The genomic libraries will be done by inserting genomic DNA fragments of 15-20 kb in Charon 35.

2) **RESULTS :** The *in vitro* synthesized proteins, labelled with 3H-Leucine, were analyzed by SDS-PAGE. For both cultivars, the molecular weight of *in vitro* traduction products ranged from 13,000 to 70,000 daltons.

According to 3H-Leucine incorporation, the *in vitro* protein synthesis capacity of poly (A)+ RNA isolated from Agathé is lower than for poly(A)+ RNA isolated from Kidur.

For each cultivar, about 500 cDNA recombinant clones were picked up to new plates and screened with two different probes. One filter was hybridized with the gamma gliadin probe (pTag 64) and the other with the gamma secalin probe (pSC 503). After the first screening, 99 Agathé clones and 102 Kidur clones were carried through a second screening. Among cDNAs clones giving positive signals, 19 Agathé clones and 30 Kidur clones were choosen for further analysis. cDNA insert sizes ranged from 250 to 1100 bp with an average size of 600 bp. Partial restriction maps and subcloning in phage M13 have been done. Nucleotide sequence analysis (Sanger procedure) are under investigations. From preliminary results (sequence homologies with published clones), it seems that we got partial clones corresponding to alpha/beta and gamma gliadins.

Concerning the genomic libraries construction, extraction of Charon 35 DNA and genomic DNA have been done. The genomic DNAs were extracted from nuclei isolated from embryos of both cultivars. Preparation of Charon 35 arms and size fractionated genomic DNA are undergoing.

REFERENCES

BARTELS D., THOMPSON R.D. (1983).
The characterization of cDNA clones coding for wheat storage proteins.Nucleic Acids Research, 11, 2961-2977.

CHIRGWIN J.M., PRZYBYLA A.E., McDONALD R.J., RUTTER W.J. (1979).Isolation of biologically active ribonucleic acid from sources enriched in ribonucleases.
Biochemistry, 18, 5294-5299.

GUBLER U., HOFFMAN B.J. (1983).A simple and very efficient method for generating cDNA libraries. Gene, 25, 263-269.

KREIS M., FORDE B.G., RAHMAN S., MIFLIN B.J., SHEWRY P.R. (1985).Molecular evolution of the seed storage proteins of barley, rye and wheat.J. Mol. Biol. 183, 499-502.

PAYNE P.I., HOLT L.M., THOMPSON R.D., BARTELS D., HARBERD N.P., HARRIS P.A., LAW C.N. (1984).The high molecular weight subunits of glutenin : classical genetics, molecular genetics and the relationship to breadmaking quality.Proceedings of the 6th International Wheat Genetics Symposium, Kyoto, Japan, 827-834.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

None.

. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	No
Exchange of staff	No
Joint experiment(s)	No
Joint meeting(s)	No

Descriptive information for the above data.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Carlsberg Lab., Copenhagen
Contract no.: BAP - 0091 - DK

Project leader: D. VON WETTSTEIN
Scientific staff: A. Brandt, V. Cameron-Mills, K. Brenner, J. Entwistle, G. Høyer-Hansen, M. Blom Sørensen, U. Rasmussen

Address: Carlsberg Laboratory
Dept. of Physiology
Gamle Carlsberg Vej 10
DK - 2500 COPENHAGEN VALBY

Telephone no.: +45.1.221022 EXT.5225

Telex no.: 15434 CARLS DK

Other contractual partners in the joint project:

H.G. Sarx, Friedrich Weissheimer Malzfabrik (Andernach)

Title of the research activity:
Improvement of protein quality in barley by means of genetic engineering.

Key words:
Hordein genes, Glucanase gene, Anther culture, Antibody screening

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

It is the aim of the present project to investigate at the molecular level the possibilities of improving the quality of the barley endosperm storage proteins. This requires detailed knowledge of individual genes in the mutigene families encoding endosperm storage polypeptides as well as the structure of the intercalated DNA segments. Also required is knowledge at the cellular level of the mechanisms directing the newly synthesized hordein polypeptides from the lumen of the endoplasmic reticulum to the vacuole in the endosperm cell. Ultimately, tailored genes are to be introduced into the barley genome by transformation and expressed in the developing endosperm.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

A total of three different hordein genes have been analysed at the nucleotide level. Regeneration of embryos from pollen grains of barley has been established and is expected to be an important tool for transformation of barley. Monoclonal antibodies against hordein polypeptides have been produced and a non-destructive screening method using fluorescing antibodies directed against endosperm proteins has been developed allowing rare recombinants and transformants to be detected. The expression of a barley (1-3,1-4) β-glucanase gene in yeast has been demonstrated and transfer of this gene to the genome of larger yeast has been attempted.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

We have isolated and determined the nucleotide sequences of one member of the Hor 1 locus and one gene encoding a gamma hordein polypeptide in order to gain information on a) the primary structure of the polypeptides these genes encode, b) to identify sequences which are important for the expression of the genes in the developing endosperm and c) to obtain information on the flanking regions of genes in order to facillitate the analysis of the sequences in between the individual genes in the multigene families.

The hor 1 gene encodes a 35 kd C-hordein polypeptide including a 19 amino acid long signal peptide. The major part of the coding region is made up of codons for glutamine and proline which appear in the deduced amino acid sequence as patterns of repeats, PQQPQQQP. The 5'-flanking region shows only a low homology to the 5'-region of the B-hordein gene hor2-4, indicating a differential regulation of these members of different multigene families. The gamma-hordein gene encodes a polypeptide of 30 kd and the deduced amino terminal sequence aligns with that known for a gamma-hordein polypeptide. Homology to the carboxyterminal 3/4 of a B-hordein polypeptide is recognizable as is extensive homology to a gamma gliadin polypeptide from wheat and a

40 kd secalin polypeptide from rye. Proline and glutamine repeats are found in the amino terminal part of the polypeptide. The signal peptide is 18 amino acids long and differs from those of B- and C-hordein polypeptides, respectively. The 5'-flanking region show homology to that of the B-hordein gene, hor2-4, suggesting that these members of two different multigene families are expressed under similar control. To determine the distances between the individual genes in a multigene family and the structure of the intercalated DNA segments, a number of different genomic clones in lambda vectors hybridizing to a hor2 probe have been isolated and analyzed by restriction site mapping. Since genomic clones containing two neighbouring genes have not been found, the nature of the intersitial segments has not yet been studied. The borders of the fragments in the analysed genomic clones are devoid of repetitive DNA. These border fragments can be used as probes to identify neighbouring fragments until a new hor2 gene is reached. A comparable structural analysis will be done in paralel to the hor1 and the gamma-hordein locus.

Site directed mutagenesis has been performed in the signal peptide coding region of a B-hordein gene changing the overall positive charge of the peptide as well as interupting the hydrofobic core. Five different mutated genes has been inserted into the expession plasmid pGem 3 and 4 and transcripts have been translated in vitro. Signal peptide cleavage and co-translational transport across the endoplasmic reticulum membrane are then to be studied in a homologous recontituted in vitro system, employing barley endosperm endoplasmic reticulum membranes, mRNA transcript from the mutated genes and wheat germ ribosomes in order to identify regions in the signal peptide sequence of importance for the transport across the ER membrane in the endosperm cell.

Monoclonal antibodies to hordein polypeptides have been produced and characterized. All antibodies reacted with more than one polypeptide, indicating that different hordein polypeptides have common epitopes. Of the seventeen isolated hybridoma lines, six secreted antibodies which reacted with both B- and C- hordein, five recognized only B-hordein and one secreted antibodies specific for C-hordein polypeptides. Two clones produced antibodies to an unidentified polypeptide doublet with an apparent molecular weight of 43 kd. These two polypeptides have at least one epitope in common with B-hordein polypeptides and are not extractable in water but in dilute alcohol. They are not stainable with Coomasie blue. The mutant hor2ca fails to synthesize B-hordein polypeptides due to a major deletion. It contains putative gamma hordein polypeptides in the B-hordein region of the gel, which have epitopes recognized by the B-hordein antibodies. The 43 kd hordein doublet was more abundant in the mutant than in the wild type endosperm, suggesting that these polypeptides like the C-hordein polypeptides are synthesized in larger amounts as a consequence of the absence of B-hordein polypeptides. Mutant lys3a which has an impaired synthesis of both B- and C-hordein polypetides was also found to be defective in the synthesis of the 43 kd polypeptide doublet.

The mutants, hor2ca and lys3a, were also used to test an immunological screening method utilizing fluorescein isothiocyanate labelled monoclonal antibodies recognizing only B-hordein polypeptides. The immunofluorescence was significantly greater from wild type than from the two mutant kernels in thin sections reacted with B-hordein antibodies.

A number of different strategies have been proposed to modify barley protein quantity and quality. The low lysine hordeins may be replaced with other storage proteins of higher lysine content e.g. with protein Z, β-amylase, chymotrypsin inhibitor 1 and 2 and an efficient selection technique for identification of these grains has been developed. The embedding of 100 seeds at a time in a block of thermoplastic clay, in such a manner that the embryo is protected allows one to identify a single kernel among a large population of kernels and to germinate it thereafter. High intensity light is projected through an appropriate interference filter onto the block of clay containing the embedded and abraded kernels to excite the fluorescence of the antibody with the appropriate wave length. The kernels are viewed through the interference filter appropriate for the emmision maximum of the fluorescence employing a special video camera and a television screen. Mutants, rare recombinants or transformants elevating the content of a desired protein or lowering the amount of an undesired protein can be screened out in such a way and it is estimated that one person can screen 10,000 grains per day.

A cDNA gene encoding the mature form of a barley (1-3,1-4)- β-glucanase was constructed from two cloned cDNA segments representing complementary parts of the corresponding barley gene. This gene was fused in frame with a DNA fragment coding for an α-amylase signal peptide and inserted in replicating yeast plasmid behind the promoter region of the alcohol dehydrogenase I gene from Saccharomyces cerevisiae. Yeast cells carrying such plasmids synthesize (1-3,1-4) β-glucanase and the enzyme is exported to the culture medium. Small scale fermentation experiments with β-glucanase producing Saccharomyces cerevisiae have shown that the amount of β-glucanase released to the culture medium is sufficient to degrade up to 500mg/L β-glucan during a 7 day fermentation period at 10°C. The β-glucanase expression unit - promoter, β-glucanase cDNA gene and terminator - was inserted in an integration vector to transfer the barley β-glucanase gene into the genome of brewing yeast strains. After transformation of yeast to G418 resistance, β-glucanase activity can be detected in the culture medium. The integrating plasmids used to transfer the β-glucanase gene to the larger yeast genome have the potential of looping out by intrachromosoma 1 recombination leaving behind only the β-glucanase expression unit in the chromosome. This stable integration of a β-glucanase gene in the yeast genome opens the posibility for using a β-glucanase producing larger yeast for brewing and thereby to overcome the filtration and haze formation problems that may be encountered when wort with high β-glucan content is used.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

Rasmussen, S. K., A. Brandt.: Nucleotide sequences of cDNA clones for hordein polypeptides. Carlsberg Res. Commun. 51, 371- 379, 1986.

Jackson, E. A., G. Murray Ballance, K. K. Thomsen: Construction of a yeast vector directing the synthesis and release of barley (1-3,1-4)-β-glucanase. Carlsberg Res. Commun. 51, 445- 458, 1986.

Ullrich, S. E., U. Rasmussen, G. Høyer- Hansen, A. Brandt: Monoclonal antibodies to hordein polypeptides. Carlsberg Res. Commun. 51, 381- 399, 1986.

Ullrich, S. E., G. Høyer- Hansen: Monoclonal antibodies to hordeins for characterization and barley quality improvement. Abstract. 5th International Barley Genetics Symposium. Oct. 6- 11 1986, Okayama, Japan.

Ullrich, S. E., U. Rasmussen, G. Høyer- Hansen: Study and manipulation of barley seed proteins with immunological techniques. Abstract. American Society of Agronomy. Annual meeting. Dec 1-5 1986, New Orleans, USA.

Rasmussen, U.: The applicationm of immunofluorescence for detecting specific proteins in barley seeds. In: H. F. Linskens and J. F. Jackson (eds.) Modern Methods in Plant Analysis.

von Wettstein, D.: Genetic Engineering: Barley. 21th International European Brewing Convention Congress. Madrid may 9- 14 1987.

von Wettstein, D.: Missing Links from molecular biology to useful plants. Nato advanced study institute on plant molecular biology. Copenhagen, June 10- 19 1987.

Thomsen, K. K., E. A. Jackson, K. Brenner: Genetic Engineering of Yeast: Construction of strains which degrade β-glucans with the aid of a barley gene. American Society of Brewing Chemists. 53th annual meeting, Cincinnatti 1987.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	No
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Dipl. Ing. Klaus Brenner has been working on the project at the Carlsberg laboratory from April 1, 1986 through June 1987 and will now return to Andernach to develop biotechnological projects in barley and yeast at Frederich Weissheimer Maltzfabrik.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Ist. Sper. per la Cerealicoltura, Bergamo

Contract no.: BAP - 0214 - I

Project leader: M. MOTTO

Scientific staff: N. di Fonzo, H. Hartings, M. Maddaloni, G. Ponziani, M. Brembilla, N. Lazzaroni, C. Soave

Address:
Istituto Sperimentale per la Cerealicoltura
Sezione di Bergamo
Via Stezzano 24
P.O. BOX 164
I - 24100 BERGAMO

Telephone no.: +39.35.311422 or +39.35.313132

Telex no.:

Other contractual partners in the joint project:

W. Rohde, M. P. I. (Köln)

Title of the research activity:

Molecular studies of the high lysine genes opaque-2 and opaque-6 in maize.

Key words:

Maize, Opaque-2, Transposon tagging, Gene regulation, High lysine gene

Reporting period: March 1987 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The objective of the project is the elucidation at the molecular level of the mechanisms controlling the accumulation of the maize prolamin, zein, which represents the major storage protein of the maize endosperm (more than 60% of the total proteins of the grain at maturity). This will allow a better exploitation of breeding strategies for improving the nutritional value of maize seed proteins. Specific aims of the proposal are the molecular cloning of the two high lysine mutants opaque-2 and opaque-6. The two genes interact each other in controlling accumulation, O2 by controlling O6 through an unknown effector and by reducing the level of the 22 class of zeins. Both opaque-2 and opaque-6 map at loci different from the major zein structural gene loci.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

A. - Isolation and characterization of the Opaque-2 gene

Because the product of the O2 gene is unknown, the usual approach for the isolation of the gene by screening a genomic library with the corresponding cDNA clone is impossible. An alternative approach based on O2 tagging with transposons has been developed in this period. Strains bearing o2 mutations due to the insertion at the locus of the transposable element Ds or of the Bg(r) system are already available.

B. - Cloning of the gene encoding a protein related to the regulatory genes Opaque-2 and Opaque-6 (b-32)

The specific objectives of the laboratory in the reporting period was to isolate a cDNA clone from b-32 mRNA.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY

Transposon mutagenesis: The molecular product of the O2 locus is unknown. Gene tagging through transposable elements may be a valuable tool for the isolation of DNA sequences for which the gene product is unknown. Accordingly, we initiated projects dealing with the genetic properties of some spontaneous unstable o2 mutations and with the possibily of tagging O2 with an indirect procedure utilizing the cloned transposable controlling element Activator (Ac).

The source of Ac system of controlling element was a strain bearing the wx-m7 mutation. This strain carries Ac at the Wx locus. Homozygous plants from variegated wx-m7 kernels were crossed to a stable o2 tester line according to the following schemes: $\frac{wx\text{-}m7\,Ac}{wx\text{-}m7\,Ac}\ \frac{O2}{O2} \times \frac{Wx}{Wx}\ \frac{o2R}{o2R}$; $\frac{Wx}{Wx}\ \frac{o2R}{o2R} \times \frac{wx\text{-}m7}{wx\text{-}m7}\ \frac{Ac}{Ac}\ \frac{O2}{O2}$

Variegated F1 kernels, i. e., kernels showing vitreous and opaque sectors, were selected from mature ears. Plants originating from these kernels were selfed to confirm the presence of variegated phenotypes in the F2 generations. In fourteen cases the variegated phenotype observed in the F1 kernels was heritable giving rise to the mutable alleles termed o2-m5 to o2-m18.

Molecular analysis have been focused on the allele o2-m5. From the backcross of $\frac{o2\text{-}m5}{o2R}$ to $\frac{o2R}{o2R}$ variegated and opaque kernels were selected, germinated and

DNA were isolated from individual seedlings. The DNA samples were digested to completion with several restriction endonucleases, fractionated on agarose gel and transferred to nitrocellulose filters according to Southern analysis. The filters were ibridized with a probe prepared from the central 1.6-Kb HindIII fragment of Ac7 (MULLER-NEUMAN et al., 1986). The aim of these experiments was to identify a restriction fragment co-segregating with the mutable phenotypes which hybridizes to the Ac probe.

Genomic cloning. DNA was isolated from 6-week-old plants derived from the o2-m5 allele. The DNA was partially digested with BamHl and cloned into the BamHI site of the λEMBL4 vector. The resulting phages were screened by plaque hybridization (BENTON and DAVIS, 1977), using the Ac probe described above. Phage containing sequences with homology to the Ac7 element were isolated; at present phage DNA preparation and analyses for the presence of structures resembling the previously isolated Ac7 element are in progress.

Cloning of b-32 gene. Size-fractionated endosperm poly(A)$^+$ RNA (approx 5 μg) was used to prepare cDNA for cloning. The ds cDNA was cloned in the expression vector λgt11. The total cDNA library was screened using antibodies raised against the b-32 protein. The positive clones were subjected to hybrid-select translation experiments.
The b-32 clone (pb-32-54) has been subjected to restriction fragment analysis. The nucleotide sequence of the cDNA insert of the pb-32-54 clone according to the protocol of SANGER et al. (1977) are now underway.

2. RESULTS AND DISCUSSION

Induction and description of the unstable phenotypes. In order to induce instability at the O2 locus in the wx-m7 strain two experiments were performed in the summer nursery in 1985 and 1986. Approximately 760,000 (1985) and 250,000 (1986) F1 kernels were obtained by hand-pollination. In the two experiments 451 and 235 kernels, showing somatic instability at the O2 locus (i., e., with vitreous and opaque sectors) were identified. These kernels were sown and the resulting plants selfed. The majority of these plants segregated normal and opaque seeds in a 3:1 ratio, suggesting that the somatic instability observed in the F1 endosperms was not inherited. In fourteen cases, however, the variegated phenotype was transmitted to the next generation. They arose with a frequency of 1.5×10^{-5}.

Molecular analysis of the o2-m5 allele. Individual DNA from 10 plants 6-week-old, derived from variegated (o2-m5/o2R) and opaque-seeds (o2R/o2R) was extracted, digested with several restriction enzymes and submitted to Southern analysis with molecular probes corresponding to the central 1.6 Kb HindIII fragment of Ac7. Several bands hybridizing with the probes were observed in both genotypes. Only in plant DNAs digested with SstI, we detected co-segregation of the o2-m5 allele with a restriction fragments. It was clearly evident that a fragment of ~8.5 Kb was always present in the DNA from variegated plants and absent in the DNA extracted from opaque-seedlings.

Identification and analysis of cloned genome DNA fragment with Ac-like sequences. DNA was isolated from o2-m5/o2R plants, which appear to have an Ac element inserted at the O2 locus. BamHI fragments of the genomic DNA were cloned in the λEMBL4 vector, and the recombinant phages were screened with a 1.6 Kb HindIII fragment representing the center of Ac7 element. A total of 35 clones with homology to the center of the Ac element were identified. The structure of the Ac-like sequences in the cloned fragment are at present investigated by probing restriction endonuclease digests of the cloned fragments with probes derived from the Ac7 element.

Cloning of b-32 gene. b-32 protein is a polypeptide present in wild-type endosperms but absent in o2 and o6 endosperms. Genetic analyses showed that this protein is related to zein accumulation. Poly(A)$^+$ RNA isolated from developing normal endosperms was enriched in mRNA for b-32 by size fractionation on sucrose density gradients and then used to construct a cDNA library in the expression vector λgt11. About 500,000 recombinant clones were obtained. These were screened for sequences related to b-32 using as probe antibodies raised against purified b-32 protein. Thirty clones were identified with inserts ranging from 600 bp to 1400 bp. In hybrid-select translation experiments one of these recombinants (pb-3254) selected mRNA from endosperm poly(A)$^+$ RNA which translated to produce a major polypeptide of Mr 32,000 which comigrate in denaturating polyacrylamide gels with authentic b-32 protein purified from endosperm. Antiserum raised to b-32, immunoprecipitated this polypeptide. The sequency of the longest insert and the screening of a genomic library is now underway.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

None.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff	Yes	
Joint experiment(s)	Yes	
Joint meeting(s)		No

Descriptive information for the above data.

There is an active cooperation on research activity between the Bergamo laboratory and the Max-Planck laboratory in Cologne. The Bergamo laboratory has provided plant materials and plant genetic stocks, while the Cologne laboratory has provided cloning vectors and training in new method in genetic engineering.
In particular: W. Rhode (M.P.I.) has provided assistance in preparing cDNA libraries and nucleotide sequencing of b-32 cDNA clone. R. Thompson (M.P.I.) has provided assistance in preparing genomic libraries for the cloning of o2-m5 allele. Moreover R. Thompson (M.P.I.) and M. Maddaloni (Bergamo) have set up a joint experimental strategies for cloning the o2-m5 allele.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: M.P.I., Köln Contract no.: BAP - 0213 - D

Project leader: W. ROHDE
Scientific staff:

Address: Max-Planck-Institut für Züchtungsforschung
Abteilung Züchtungsforschung und
Ertragsphysiologie
Egelspfad
D - 5000 KÖLN 30

Telephone no.: +49.221.5062237

Telex no.: 8881028 MPIZ D

Other contractual partners in the joint project:

M. Motto, Istituto Sperimentale per la Cerealicoltura (Bergamo)

Title of the research activity:
Molecular studies of the high lysine genes opaque-2 and opaque-6 in maize.

Key words:
Maize, High-lysine, Opaque, Molecular, Regulation

Reporting period: June 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The project is intended to elucidate at the molecular level the mechanisms controlling the accumulation of the major maize storage protein,zein, with a view to improving the nutritional value of maize seeds. Strategies are proposed for the cloning of two genes giving rise to a high-lysine phenotype, opaque-2 and opaque-6.Both mutants reduce the level of zeins in maize endosperm. The opaque-6 locus will be cloned via the identification of a cDNA clone for b-32 polypeptide which is regulated at this locus. The opaque-2 locus will be cloned via transposon tagging due to the insertion of transposable elements Ac/Ds, Bg-(r), and mu. Further studies will be carried out to identify the gene product of the opaque-2 locus, and the way in which it controls the opaque-6 locus.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

1. Isolation of genomic clones from 02m-5 (Ac-induced) library
2. Isolation of genomic clones for the gene coding for b-32 polypeptide
3. Isolation of clones of the 02m(r) locus

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Methodology

Plant DNA was isolated from hand-dissected embryos(02m5) or etiolated shoots (02m(r)Bg) and purified via 2 cycles of CsCl-EtBr centrifugation. The DNA was digested with Sau3A and size fractionated by sedimentation through sucrose density gradients. Size fractions corresponding to 15-20 kb were cloned into EMBL4 arms prepared by double-digestion with SalI and BamHI. The libraries were screened on nitrocellulose filters using random oligonucleotide primed probes.

2.Results

1. Isolation of genomic clones from 02m5 (Ac-mutable)library. Using as a probe the central fragment of Ac over 130 positive clones were obtained. These are being further screened in Bergamo to identify clones carrying the complete element and therefore candidates for insertion at the opaque-2 locus (see Bergamo reports)

2. Isolation of genomic clones for b-32 coding sequences.
   Several putative b-32 genomic clones were identified also by screening the 02m5 (Ac) library. These are being further purified by Dr. DiFonzo (see Bergamo reports).

3. Isolation of clones for the Bg receptor sequence.
   A library of 02m(r)Bg was constructed and screened with a putative opaque-2 locus probe (provided by R. Schmidt, Brockhaven, USA)
   A total of thirteen homologous clones were identified. DNA from six of these has been examined and a preliminary restriction enzyme site map of the 02m(r) locus has been constructed.

3. Discussion

The identification of an Ac insertion within the 02 locus can be predicted from genetical data, but can only be verified from the isolation of corresponding clones. If Ac has inserted very near to but not in the locus, the locus would behave as though it was autonomous although having only a Ds insertion within the opaque gene. The current clone screening should indicate which of these possibilities is the case for 02m5.

The identification of genomic clones for the b-32 locus will be useful source of information about the function of this gene in maize endosperm development. The genomic clones should include sequences flanking the coding region which contain putative sites of regulation by the opaque-2 gene product.

The characterization of a series of mutations at the opaque-2 locus, such as those responding to the Bg transposable element will help us better define the locus and understand how its' effects can be modified.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

None.

TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	No
Exchange of staff	No
Joint experiment(s)	No
Joint meeting(s)	No

Descriptive information for the above data.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: V. U. B., Brussels
Contract no.: BAP - 0089 - B

Project leader: J.P. HERNALSTEENS
Scientific staff: H. de Greve, S. Renckens

Address: Vrije Universiteit Brussel
Laboratorium Genetische Virologie
Paardenstraat 65
B - 1640 ST-GENESIUS-RODE

Telephone no.: +32.2.3582194

Telex no.: 61051 B

Other contractual partners in the joint project:

A.G.M. Gerats, Vrije Universiteit Amsterdam

Title of the research activity:
Isolation of transposable elements from Petunia hybrida.

Key words:
Petunia hybrida, Transposable elements, T-DNA, Insertion mutagenesis, Auxin toxicity

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The common goal of the joint project is the isolation and characterization of transposable elements from Petunia hybrida. These elements transpose and induce new insertion mutations at a very high frequency. Therefore these transposons would be interesting tools for gene tagging.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Our aim is to clone the transposon by selecting its insertion in the T-DNA gene 2 which encodes an enzyme converting indole-3-acetamide (IAM) or α-naphthaleneacetamide (NAM) into the active auxins indole-3-acetic acid (IAA) or α-naphthaleneacetic acid (NAA) (Schröder et al., 1984; Inzé et al., 1984). As a first step it was necessary to introduce the gene 2 into Petunia plants containing the transposable element using a T-DNA vector. Plants harbouring a single copy of gene 2 are required for the selection of mutants and can be identified by Southern blot analysis.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY.

a. Construction of the binary vector pGV974.
The plasmid pGV974, harbouring in its T-DNA region both gene 2 and a chimeric nos-aph(3')II gene (Hain et al., 1985) conferring kanamycin resistance to plant cells, was constructed by introducing an AsuII fragment containing gene 2 into the HpaI site of pGV941 (Deblaere et al., 1987).

b. Transformation of Petunia.
The Petunia F1 hybrid "W138 x Mitchell" line was transformed with an Agrobacterium strain, harbouring the binary vector pGV974, using the leaf disc method (Fraley et al., 1984). Transformed shoots were selected on a shoot inducing medium supplemented with kanamycin (100 mg/l) and claforan (500 mg/l). Recently, a similar approach allowed the transformation of the Petunia line W138.

c. Test of the transformed *Petunia* plants.
The expression of the chimeric *nos-aph*(3')II gene was investigated by testing the ability of the clones to root on hormone-free medium containing kanamycin (50 mg/l) or to produce callus from leaf explants on geneticin (50 and 100 mg/l) containing medium.
Leaves of plants expressing gene *2* develop callus and roots on medium supplemented with 6-benzylaminopurine (BAP, 1 mg/l) and NAM (1 and 5 mg/l), while leaves of untransformed plants develop shoots.
Plants expressing gene *2* are also unable to root on hormone-free medium containing NAM (1 mg/l), while untransformed plants develop a normal root system.

d. Southern blot analysis.
Total DNA was isolated from the transformed plants as described by Dellaporta *et al.* (1983) and, after Southern transfer, hybridized with a nick-translated gene *2* probe.

e. Segregation of gene *2*.
Seedlings expressing gene *2* are unable to grow on hormone-free medium supplemented with NAM (1 mg/l), while untransformed seedlings develop normally.

f. Selection for inactivation of gene *2*.
The division of mesophyl protoplasts and the growth of protoplast derived cell clones, from plants expressing gene *2*, is inhibited in culture medium containing high levels of IAM or NAM (10-200 mg/l). This allows the selection for colonies no longer expressing gene *2*. These can regenerate into normal plants on zeatin (1 mg/l) containing medium.

2. RESULTS.

The *Petunia* line W138, which is the standard line harbouring the transposon, can only be transformed with a low efficiency because it regenerates poorly. In contrast, the *Petunia* line "Mitchell" (Mitchell *et al.*, 1980) regenerates efficiently. Therefore the F1-hybrid of W138 with *Petunia* "Mitchell" was used for the initial experiments. Leaf discs of the *Petunia* F1-hybrid line "W138 x Mitchell" were infected with an *Agrobacterium* strain harbouring pGV974. From the 59 independent plants tested, 51 were transformed and expressed both gene *2* and the chimeric kanamycin resistance gene.

The selection for the integration of the transposon into gene *2* and the further analysis will be facilitated by the use of transgenic plants containing only one T-DNA insert. Southern analysis of the T-DNA structure in 17 plants proved that 5 of them contain a single copy of gene *2*. This result is in accordance with preliminary data on the segregation of gene *2*: two of these 5 plants were tested and show a clear 3:1 segregation of gene *2*. The genetic analysis of other transformed plants is in progress.

The excellent tissue culture properties of the "W138 x Mitchell" F1-hybrid allow the use of protoplast techniques for the isolation of mutations. Selection against gene 2 in mesophyl protoplasts, isolated from transformed plants, was carried out on medium containing high levels of NAM or IAM. Normal plants were obtained from the calli selected on these media. In some of these plants gene 2 expression could not longer be detected.

3. DISCUSSION.

During this first year, gene 2 was introduced in the Petunia F1-hybrid line "W138 x Mitchell" and in the Petunia line W138. In the case of the hybrid line, plants harbouring and expressing a single copy of gene 2 were isolated, while the transformed W138 plants remain to be analyzed by Southern blot hybridization. Furthermore, plants no longer expressing gene 2 were obtained by selection for the inactivation of gene 2 in protoplasts isolated from transgenic plants expressing gene 2. These mutants will now be analyzed by Southern blot hybridization to determine if the inactivation of gene 2 is due to the insertion of a transposable element.

4. REFERENCES.

Deblaere R. et al., (1987) in "Methods in Enzymology", in press
Dellaporta S. et al., Plant Mol. Biol. Rep. (1983) 1, 19-47
Fraley R. et al., Plant Molecular Biology (1984) 3, 371-378
Hain R. et al., Mol. Gen. Genet. (1985) 199, 161-168
Inzé D. et al., Mol. Gen. Genet. (1984) 194, 265-274
Mitchell A.Z. et al., Z. Pflanzenphysiol. (1980) 100, 131-146.
Schröder G. et al., Eur. J. Biochem. (1984) 138, 387-391

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.2 SHORT COMMUNICATIONS.

De Greve H., Renckens S., Van Montagu M. and Hernalsteens J.-P. A strategy for the cloning of transposable elements from Petunia hybrida in "Genetic and cellular engineering of plants and microorganism important for agriculture". Books of abstracts - Biotechnology Action Programme meeting. Louvain-la-Neuve, March 23-26, 1987.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

EXCHANGE OF MATERIAL

The Petunia hybrida line W138 was supplied by Dr. A. Gerats (Vrije Universiteit Amsterdam). This line contains transposable elements that induce mutations at a very high frequency.

JOINT MEETINGS.

Two meetings were organized during this first year:
- In August 1986, we had a meeting with Dr. A. Gerats at the "Vrije Universiteit Amsterdam". We discussed the details of different strategies for the cloning of transposable elements from Petunia.
- In March 1987, a second meeting was held in our laboratory at the "Vrije Universiteit Brussel". The results obtained by both groups were communicated and the planning of the research was discussed.

Between these meetings the groups kept in contact by letters and telephone.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Vrije Universiteit Amsterdam

Contract no.: BAP - 0086 - NL

Project leader: A.G.M. GERATS

Scientific staff: A.G.M. Gerats, E. v.d. Berg, T. Stuitje, M. Beld

Address:
Vrije Universiteit
Dept. Applied Genetics
De Boelelaan 1087
NL - 1007 MC AMSTERDAM

Telephone no.: +31.20.548.57.54 or +31.20.548.47.75

Telex no.: 11329 dpvvu

Other contractual partners in the joint project:

J.P. Hernalsteens, Vrije Universiteit Brussel

Title of the research activity:

Isolation of transposable elements from Petunia hybrida.

Key words:

Petunia hybrida, Transposable elements, Alcohol dehydrogenase, Flavonoid synthesis

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

We try to isolate a transposable element from Petunia hybrida by selecting for insertion events into genes for which a probe is available. The Brussels group uses an unstable strain, which was transformed with a gene-2 construct. Insertion of an element into gene 2 will lead to resistance to high α-naphtalene acetamide concentrations; the nature of such a mutation can be recognized by Southern blot hybridization. A similar approach, using the alcoholdehydrogenase system and a flavonoid gene is followed in Amsterdam. Once an element is cloned, it can be used to isolate and characterize transposon induced mutant alleles of genes for which ns probes are available (for example developmental genes); besides such an element will be used for mutagenesis and tagging in both the homologous and heterologous systems.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

In the reporting period, we characterized ADH expression in different tissues of a number of Petunia strains. We did not detect any electrophoretic variation in pollen specific ADH activity for over 200 strains tested. Pollinations with allyl alcohol selected pollen of unstable strains gave rise to a low number of progeny, showing differential staining for ADH in their pollen; it could not be shown however, that this was due to a mutation in a pollen specific ADH gene (see section III). For technical reasons we now have abandoned the ADH approach to isolate a transposable element. A new approach, using a flavonoid gene, will be described in the discussion (III, 3).

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Materials and Methods

All tissues were collected from plants grown in a greenhouse.

ADH staining procedure

We essentially used the assay as described by Freeling (1976). Pollen of mature anthers of one flower was tipped into 500 µl 0.1 M sodium phosphate buffer (pH 7.3) in a Greiner replica dish. Samples were frozen at -20°C for 2-3 hours and then defrosted slowly at room temperature while rotating slowly.

The buffer was withdrawn and replaced with an ADH specific stain based on the ethanol-dependent reduction of p-nitrobluetetrazolium chloride: 86 mM sodium phosphate buffer pH 7.3, 0.3 mM p-nitrobluetetrazolium chloride (NBT) (stock in 100% methanol), 1.0 mM NAD^+ and 9.5% ethanol. 500 µl stain was added to the pollen and the staining was stopped by replacing stain with 100% methanol. ADH^+ pollen stain deep blue while ADH^- pollen remains uncoloured.

Polyacrylamide gel electrophoresis

Different tissues were extracted in 500 µl 0.1 M potassium chloride, 0.01 M DTT buffer pH 7.3 Vertical electrophoresis was carried out using

Desaga electrophoresis-containers.

6% Polyacrylamide gels were run in an alkalic system using 38 mM glycine, 5 mM Tris-base pH 8.3. A pre-run was carried out for 30 mins. with a constant current of 5 mA at 4°C. Afterwards 20 µl- 30 µl of the extracts were loaded on the gel and electrophoresis was carried out.

Allyl alcohol selection

Pollen samples were placed in a 1 liter jar, in which the athmosphere was saturated with allyl alcohol. Samples were allowed to be poisoned for 10, 20 or 30'. In all treatments we found around 30% seedset, when compared to non-treated pollinations.

Summary of the main results.

Results and discussion.

Three main variable bands were detected when samples from different tissues were electrophoresed (Figure 1). The fastest migrating band (1) is found in tissue culture-, callus-, root- and stem extracts, the slowest (3) in especially young leaves. The intermediate band (2) is found in anthers and shows a maximum intensity in mature pollen.

We did not find any electrophoretic variants in the pollen band for over 200 strains tested (Figure 2)

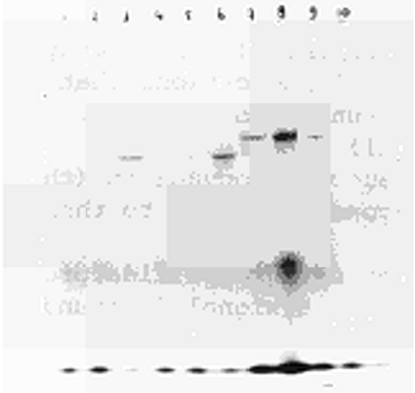


Fig. 1. Polyacrylamide gel, stained for ADH activity. Lanes 1, 2, 3: tissue cultures extract (1/100, 1/10, 1), 4, 5, 6: callus extract (1/100, 1/10, 1), 7 and 8: pollen extract (1/10, 1), 9 and 10 leaf extracts (1/10, 1) note the spill-over between lane 8 and 9 for the pollen band.

Fig. 2. Polyacrylamide gel, stained for ADH activity. Pollen extracts of 10 different Petunia hybrida cultivars.

Pollen from unstable strains was treated with allyl alcohol and subsequently used for pollination on a tester strain. Allyl alcohol treatment kills ADH^+ pollen and thus theoretically a mutant ADH allele is rescued if seed set is obtained with treated pollen. Since no variant ADH alleles were available to use in tester strains, the pollen of the resulting progeny was expected to be wildtype: mutant in a 1:1 ratio.

Indeed a number of such plants could be isolated (18 out of 852). Selfing of these putative mutants gave a 1:1 ratio of heterozygous: homozygous wildtype plants (as envisaged by staining the pollen of the progeny plants). This might indicate that ADH activity in pollen of Petunia hybrida is not dispensable as it is in corn. The fact that we did not find any variant alleles in our Petunia collection might point in the same direction.

As a control for specificity of the staining procedure we did not add ethanol to the assay mix. Since no difference was found in staining capacity of wildtype pollen, we assumed that there was a high amount of internal substrate. Following the directions of Freeling et al. ( ) we tried to eliminate the internal substrate by freezing and slowly thawing the samples. This did not lead to an improvement.

Moreover, when we tested the putative mutant plants with malate as a substrate, it appeared that they showed a segregation for malate dehydrogenase activity as well.

The hypothesis that we are dealing with dead instead of non-staining pollen seems attractive, the more since transmission of the mutation via the pollen appears to be virtually impossible. However, in vitro germination of pollen and subsequent staining indicated the presence of germinating non-staining pollen. The observation that staining begins at the tip of the protruding pollen tube might indicate that ADH activity has a function in pollen tube growth.

In view of the above mentioned difficulties, we decided not to pursue the ADH strategy, despite the fact that we isolated genomic clones for ADH in the mean time (results not shown). Instead, we have developed a new strategy.

The pallida gene (Antirrhinum majus) codes for the flavonoid enzyme dihydroglavonol reductase (DFR). There are several indications that gene An11 might be the structural gene for DFR in Petunia hybrida.:

1. dihydroflavonols are accumulated in an11/an11 mutants.
2. biochemical complementation is found when leuco-anthocyanidin (the product of the DFR conversion of dihydroflavonols) is fed to these mutants.
3. no mRNA signal is detected on a Northern blot using a pallida probe (made available by dr. C. Martin), whereas such a signal is found in the wildtype.

Since unstable alleles of An11 are present in our collection (Doodeman et al., 1984), it seems worthwhile to investigate the relationship between gene An11 and the structural (active) DFR gene. Genomic clones, of a wildtype strain, hybridizing to the heterologous pallida probe have been isolated and purified already.

References

Doodeman M, AGM Gerats, AW Schram, P de Vlaming and F Bianchi (1984). Genetic analysis of instability in Petunia hybrida 2. Unstable mutations at different loci as the result of transpositions of the genetic element inserted at the An1 locus. TAG 67: 357-366.

Freeling M, (1976) Intragenic recombination in maize: pollen analysis methods and the effect of parental ADH^+ isoalleles. Genetics 83: 701-717.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.2.

M. Beld, H. Huits, W. Veerman and AGM Gerats. The isolation of active transposable elements from Petunia hybrida. Abstract of a poster. BAP meeting. Louvain la Neuve, March 1987.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

AGM Gerats provided the Brussels group with plant material and seeds from unstable Petunia strains.

The Brussels group visited Amsterdam in August 1986, while AGM Gerats visited the Brussels group after the BAP meeting in March 1987. Dr. H. de Greve will visit Amsterdam in August 1987 again. We plan to have joint meetings every half year.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Max-Planck-Ges. zur Förderung der Wissenschaften e.V., Köln

Contract no.: BAP - 0087 - D

Project leader: J. SCHELL / F.M. KREUZALER

Scientific staff: J. Schell, F. Kreuzaler, R. Wingender-Drissen, H. Röhrig

Address: Max-Planck-Institut für Züchtungsforschung
Egelspfad
D - 5000 KÖLN 30

Telephone no.: +49.221.5062350

Telex no.: 8881028 MPIZ D

Other contractual partners in the joint project:

J.N.M. Mol, Vrije Universiteit Amsterdam

Title of the research activity:

Development of inducible gene expression systems for higher plants and plant cell cultures.

Key words:

Elicitor induction, Soybean chalcone synthase, Resistance genes, Induced transcription

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

a) Cloning and characterization of a plant gene that is induced upon infection by a pathogen

b) Cloning and characterization of potential resistance genes from bacteria or plants

c) Construction of a chimeric resistance gene; transformation of different plant species with this construct.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Our efforts are concentrated on the characterization of an elicitor induced chalcone synthase gene from soybean and on the establishment of a test system for different constructs carrying an NPTII gene under the control of different regulatory sequences of the chalcone synthase gene.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Introduction

Genetic engineering and tissue culture techniques have proven to be of potential value for crop improvement and plant cell productivity. For certain aspects of plant biotechnology not only the continuous expression of a foreign gene, but also tissue or time restricted expression is required. For example defense genes should be switched on only upon microbial infection due to possible pathogenic activity of substances related to the defense. The goal of this project is the identification of an inducible plant promotor for the specific regulation of foreign genes in transgenic plants. As a model system we chose soybean chalcone synthase (chs) that is known to be induced by light and fungal infection.

Results

Isolation of genomic chs genes

The soybean chs is encoded by a multi-gene family of about ten members. Evidence for this is deduced from the number of fragments in HindIII digested genomic DNA which hybridize with chs cDNA of parsley and from the number of gene products which can be detected after in vitro translation of poly A^+RNA isolated from elicitor treated cells. A genomic library in the phage λ 1059 was screened with parsley chs cDNA and four positive clones harboring inserts of 13-17 kb were selected for further characterization. BamHI, EcoRI and HindIII fragments of these clones that hybridized to chs cDNA were subcloned into the appropriate cloning site of pBR322. Interestingly most of the genomic clones contain more than one chs gene.

Structure of the genes

Three genomic clones were analyzed by R-Loop studies. The DNA was hybridized with poly A^+ RNA isolated from elicitor treated cells and examined using the electron microscope. Introns larger than 200 bp could not be detected in any of the genes. Two chs genes have been sequenced up to now and these data agree to the R-Loop studies. A comparison of these sequences with the parsley chs cDNA and the Antirrhinum genomic chs sequences revealed the presence of one intervening sequence of about 170 bp in size in the coding region of the soybean genes. The position of this intron might be conserved among chs genes since the first intervening sequence in chs of Petunia and Antirrhinum differ in size but have the same position as in the soybean genes.

The 5' and 3' untranslated region of gene 1 and 2 contain the genomic element that are to be postulated to be necessary for transcription. A putative TATA and CAAT box can be found for both genes, the transcription start however, has not been analyzed yet. In the 3' region of the genes two possible polyadenylation signals are found, but it is not yet known which one of them is used.

In order to analyze the 5' region of the genes we intend to construct chimeric promoters in a transcription enhancement system using NPTII as a marker gene and to test these constructs in transformed soybean cell cultures.

Testsystem

In order to analyze the 5' region of the genes we constructed chimeric promoters in a transcription enhancement system using NPT II as a marker gene. The vector we used for these constructs is a binary vector providing right and left borders from the Ti plasmid of Agrobacterium tumefaciens, an origin of replication for E.coli and A.tumefaciens, bacterial selectable marker genes, the plant selectable marker gene hygromycine-phosphotransferase and an expression cassette for NPT II under the control of a minimal promoter. In front of the minimal promoter cloning sites are available that enable us to test different regions of the 5' sequences of the chs genes for their influence on NPT II expression. The minimal promoter on its own is silent.
The system we choose for analysis is transient expression of these constructs in transformed soybean protoplasts. The protoplasts are transformed with naked DNA by the PEG-$CaNO_2$ method. After several unsuccessful experiments we are presently able to induce transient expression by addition of elicitor and we are testing different promoter regions for their inducible activity.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

None.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	No
Joint experiment(s)	No
Joint meeting(s)	No

Descriptive information for the above data.

Exchange of different elicitor preparations with the group of J. Mol

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Vrije Universiteit Amsterdam

Contract no.: BAP - 0086 - NL

Project leader: J.N.M. MOL

Scientific staff: R.E. Koes, A.R. Stuitje

Address:
Vrije Universiteit
Dept. Applied Genetics
De Boelelaan 1087
NL - 1007 MC AMSTERDAM

Telephone no.: +31.20.5482476 or +31.20.5484775

Telex no.: 11329 dpvvu nl

Other contractual partners in the joint project:

F.M. Kreuzaler, M. P. I. (Köln)

Title of the research activity:
Development of inducible gene expression systems for higher plants and plant cell cultures.

Key words:
UV-induction, Elicitor-induction, Flavonoid genes, Plant productivity, Plant cell culture

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Genetic engineering and tissue-culture techniques have proven to be of potential value for the development of better crop species and for the improvement of plant cell productivity. One prerequisite for such an approach is that newly introduced genes can be properly expressed in a specific tissue and in response to a specific stimulus. It is therefore necessary to isolate and characterize tissue-specific, inducible plant regulatory sequences. The goal of this project is to develop such a system based on the flavonoid-specific chalcone synthase (CHS) gene which has been shown in a number of plant systems to be strongly inducible by UV-light and phytopathogens.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

(i) A further characterization of the CHS multigene family with respect to gene structure and chromosomal localization,
(ii) An analysis of the expression of individual CHS members in different uninduced plant tissues,
(iii) An analysis of the expression of individual CHS members in UV-induced plant seedlings,
(iv) Testing the inducing capacity of UV-light and fungal elicitors in plant tissue cultures.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

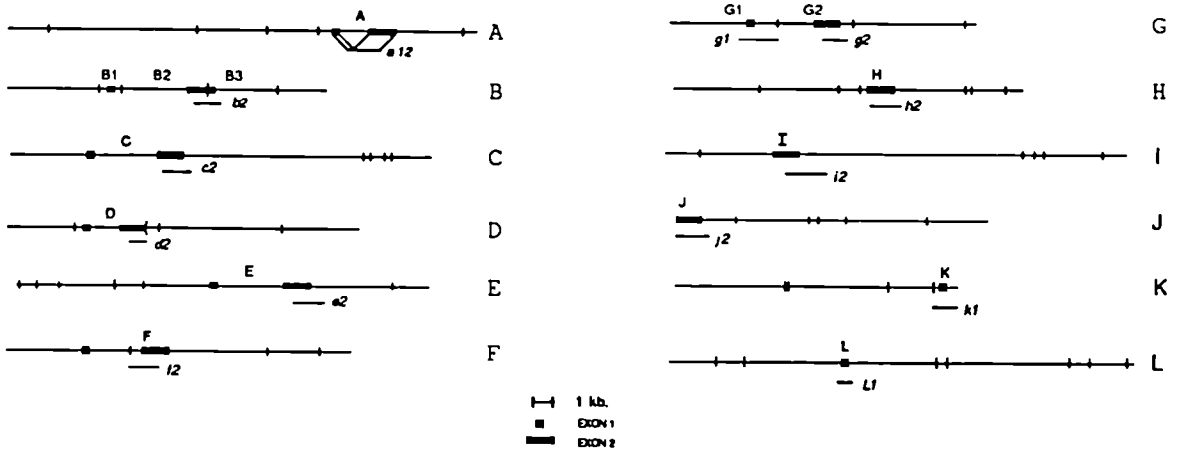
Methodology

1. CHS genes from Petunia hybrida (V30) are cloned in λEMBL-3.
2. The chromosomal location of CHS genes was determined using Restriction Fragment Length Polymorphisms (RFLPs). Briefly, two lines of Petunia (A & B) with distinct CHS Southern blot patterns are crossed (F_1 generation, superposition of patterns). The F_1 is then backcrossed to one of the parents (A). In the offspring the segregation of restriction fragments of parent B can be monitored. Using genetically-defined lines and scoring for phenotypic markers allows one to ascribe restriction fragments to a certain chromosome.
3. The expression of individual members of the CHS multigene family was studied either by cloned DNA x mRNA hybridization followed by S_1 nuclease digestion or by anti sense SP6 RNA x mRNA hybridization followed by RNAse treatment. The nuclease-resistant 'core' is analyzed by standard formaldehyde-formamide gel electrophoresis.

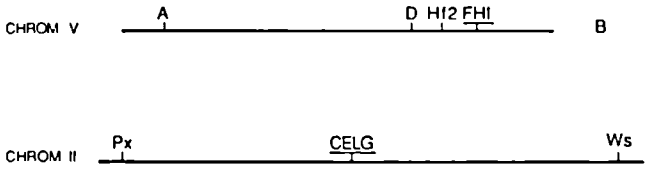
Results

We have recently shown that floral tissue of Petunia hybrida (V30) expresses only one member of the chalcone synthase (CHS) multigene family to a major extent (Koes et al. Nucl. Acids Res. 14, 1986, 5229-5239).

From a λ EMBL-3 library 7 distinct complete CHS-related genes (A-G) could be isolated including the one expressed in floral tissue (gene A). In addition some CHS gene fragments were isolated (Figure below).



All 7 complete genes show a similar overall architecture: one small and one larger exon separated by a variable (in size and sequence) intron. None of the clones contains more than one gene indicating absence of intimate linkage. However RFLP analysis shows tight clustering (fig. below).



CHS genes in Petunia hybrida V30 map on 2 separate chromosomes (II and V). Genes CELG and DHFI are in close proximity on chr. II and V resp., whereas gene A is far apart on chr. V.

The expression of CHS genes has been studied in different plant tissues and tissue cultures under normal and stress situations. The following Table summarizes the results:

	EXPRESSION					
Gene	petal	tube	anther	UV seedlings	UV suspension	elicitor suspension
A	+++	+++	+++	+++	+	
B	-	-	-	+/-		
C	-	-	-	-		
D	-	-	-	-		
E	-	-	-	-		
F	-	-	+/-	-		n.o.i.
G	-	-	-	+/-		
H	-	-	-	-		
I	-	-	-	-		
J	+	+	+	+	+	
K						
L	-	-	-	+/-		

+++, high expression; +, clear expression; +/-, doubtfull; -, no expression.
n.o.i., no overall induction

+++, high expression; +, clear expression; +/-, doubtfull; -, no expression; n.o.i., no overall induction

Several conclusions emerge from the table:

1) CHS genes A and J (to a lesser extent) are active in pigmented tissues as well as under UV-stress indicating that UV-inducibility is an intrinsic property of flower-expressed CHS genes,
2) UV-stress but not elicitor-stress (cellwall glucans from phytopathogens) induce CHS gene expression indicating that the fytoalexins of Petunia are non-phenyl propanoid compounds.

Discussion

Of the CHS multigene family only genes A/J show clear activity under normal and UV-induction conditions. The UV-effect in cell suspension cultures is still low compared to seedlings and non-irradiated pigmented tissues, and in addition white light seems to be essential for this effect! In the coming period this combined light effect will be further studied and optimized.

Chimeric genes (CHS-A promotor + CAT gene) have been constructed. The analysis of deletion mutants will possibly shed some light on the key-sequences involved in UV-inducibility. Furthermore the lack of elicitor-inducibility will be further studied by comparing the soybean CHS-chimeras constructed in Cologne at the MPI (Kreuzaler), which are elicitor-inducible, with our petunia chimeras in cross-transfer experiments.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

1. R.E. Koes, C.E. Spelt, J.N.M. Mol and A.G.M. Gerats: The chalcone synthase multigene family of Petunia hybrida (V30): sequence homology, chromosomal localization and evolutionary aspects (1987) submitted.

2. R.E. Koes, C.E. Spelt, A.G.M. Gerats and J.N.M. Mol. Cloning and expression of the chalcone synthase multigene family from Petunia hybrida. Proc. 4th European Congress on Biotechnology, Amsterdam, 1987 p. 379-382.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)	Yes	
Joint meeting(s)	Yes	

Descriptive information for the above data.

Phytopathogen elicitors have been made available to us by MPI and chimeric constructs are about to be exchanged. Exchange of staff is foreseen at a later stage of the joint project. Joint experiments are in progress concerning the phytopathogen induction of chimeric genes. In Aug./Sept. a new joint meeting will be held in Cologne/Aachen.

Outside BAP there exist a formal collaboration with Dr. Richard Dixon, Royal Holloway College, U.K. on elicitor-induction and phytoalexins and with Dr. Eckard Wellmann, Univ. Freiburg, FRG on light-induction

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: John Innes Institute, Norwich

Contract no.: BAP - 0097 - UK

Project leader: R. HULL

Scientific staff:

Address: John Innes Institute
Colney lane
UK - NORWICH NR4 7UH

Telephone no.: +44.603.52571

Telex no.: 975122 JIINOR G

Other contractual partners in the joint project:

J. Brunstedt, De Danske Sukkerfabrikker (Copenhagen)

Title of the research activity:

Molecular biological approach to the control of beet yellows virus.

Key words:

Beet yellows virus, Virus purification, Virus characterization, Plant viruses

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

To test if RNA complementary to beet yellows virus (BYV) genomic RNA will inhibit the replication of the virus. The cooperative study entails determining the genome structure and expression strategy of BYV, the production of constructs giving antisense RNA, the transformation of these constructs into beet protoplasts and the subsequent analysis of transformed cells.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

As above with the exception of development of mechanisms for transforming beet cells and the actual transformation using antisense constructs.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY

A considerable amount of effort has been put into obtaining reliable supplies of infected plant material and into developing a reliable method for purifying BYV from this material. The methods which have now been developed are described in the results section.

The studies on the translation of BYV RNA have used standard methods for the wheat germ and rabbit reticulocyte lysate *in vitro* translation systems (Davies, 1979; Pelham & Jackson, 1976). Total RNA was extracted from infected leaves using the method of Covey & Hull (1981).

Construction of cDNA probes to BYV was by methods described by Maniatis *et al*. (1982).

2. RESULTS

A method has been developed for obtaining reliable infection of Claytonia perfoliata and Tetragona expansa with BYV. Myzus persicae is fed on the source plant for 24h and 5-10 aphids are transferred to each host plant where they are fed for a further 24h. Infection rates of 85-95% are obtained if the host plants are grown at temperatures of 20-25^{o}C but this rate drops if the temperature goes higher.

The published methods for virus purification proved unsatisfactory as they gave low yields and did not maintain the integrity of the long flexuous virus particles. A new method was developed which gave increased yields and mainly unbroken particles. This method involves grinding infected plant tissue after freezing with liquid N_2, extraction with 0.1M ammonium acetate, 20mM EDTA, pH 7.0, low speed centrifugation, precipitation of virus with polyethylene glycol and centrifugation of virus on gradients of Cs_2SO_4. The various steps in the purification were monitored using electron microscopy and ELISA and the integrity of virus particles was assessed by gel electrophoresis of RNA extracted from them. Although this method is a considerable improvement on previous ones yields are still rather low.

The low yield and the very large size of the viral RNA (13Kb) has posed considerable problems in in vitro translation experiments. Using both the wheat germ and reticulocyte lysate systems various products were identified. However because of the problems it is not certain that these are full-sized viral products. In time-course experiments there was no evidence for the formation of polyproteins and their subsequent cleavage. There was some evidence for translation from sub-genomic RNAs but the interpretation of these observations was complicated by the possibility of fragmentation of the virion RNA.

Attempts to translate the RNA within virus particles by the use of the cotranslational disassembly phenomenon (Wilson, 1984) did not succeed.

In the hope of overcoming the need to purify virus

particles to provide material for translation we have started looking at the use of total RNA extracted from infected plants. Gel electrophoresis of total RNA reveals a high mol. wt. band, visible on staining with ethidium bromide which hybridizes with labelled cDNA probes to BYV RNA in northern blots. In the preparation of cDNA probes it was found that virion BYV RNA is not polyadenylated.

3. DISCUSSION

Much of the effort in this first year of the project has been directed at overcoming the problems of yield of virus and integrity of the particles and their RNA. The way forward is now much clearer and we will be using total RNA preparations as a source of genomic RNA (as it is much larger than other RNAs) and in the search for subgenomic RNAs. cDNA clones to BYV RNA, now being prepared, will be used in this work.

Various features of the virus have been revealed. These include the fact that the virion RNA is not polyadenylated and the lack of any evidence for a polyprotein strategy of expression.

References

Covey, S.N. & Hull, R. (1981). Virology 111, 463.
Davies, J.W. (1979). In "Nucleic acids in plants" (T.C. Hall & J.W. Davies, eds) Vol 2, p.114, CRC Press, Florida.
Maniatis, T., Fritsch, E.F. & Sambrook, J. (1981). Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory.
Pelham, H.R.B. & Jackson, R.J. (1976). Eur. J. Biochem. 67, 247.
Wilson, T.M.A. (1984). Virology 137, 255.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.2. EEC contractors report. BAP General Meeting, March 1987, Louvain la Neuve, Belgium.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	No
Exchange of staff	No
Joint experiment(s)	No
Joint meeting(s)	Yes

Descriptive information for the above data.

Meeting 1

Belgium, Louvain la Neuve, March 1987, EEC BAP contractors meeting.

Short presentation and poster on the project. This project is the only viral project in the group.

Meeting 2

Copenhagen, DDS April 1987.

Short oral presentation on the project and discussion with DDS collaborator on the work.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: De Danske Sukker-fabrikker A/S, Copenhagen

Contract no.: BAP - 0073 - DK

Project leader: J. BRUNSTEDT

Scientific staff: M. Jørsboe

Address: De Danske Sukkerfabrikker
Biotechnology Section
P.O. BOX 17
Langebrogade 1
DK - 1001 COPENHAGEN K

Telephone no.: +45.1.954100

Telex no.: 31436 DDSENG DK

Other contractual partners in the joint project:

R. Hull, John Innes Institute (Norwich)

Title of the research activity:
Molecular biological approach to the control of beet yellows virus.

Key words:
Electroporation, Antisense RNA, Virus resistance, Transient gene expression, CAT-activity

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The aim of this project is to see if RNA complementary to beet yellow virus (BYV) RNA will inhibit the replication of the virus. The work at Norwich will concentrate on determining the genome structures and the strategy of expression of BYV RNA, the production of constructs giving antisense RNA and the analysis of transformed cells. The part of the project at DDS in Copenhagen will concentrate on establishing a transformation system in sugar beet in order to test the feasibility of using antisense RNA to control gene expression.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

In order to study the transcription of antisense RNA-genes and their effect on replication of virus RNA, we have undertaken to evaluate electroporation as a tool for DNA delivery into sugar beet protoplasts. The strategy has been to optimize the electroporation procedure by dye uptake experiments, and to study transient gene expression, using a chloramphenicol acetyl transferase (CAT) gene expressed from the CaMV 35S promotor.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Methodology:

Protoplasts were isolated from rapidly growing sugar beet suspension cells by incubating 1 g of packed cells in 10 ml of 1% cellulase Y-C (Seishin), 0.3% pectolyase Y-23 (Seishin), 0.5% BSA and 0.4 M sorbitol in the growth medium for 4 h at 25°C in the dark on a rotary shaker at 50 rpm. Protoplasts were passed through a 60 µm sieve, washed once in CPW21% sucrose and twice in CPW13% sorbitol (1.5 mS/cm at 25°C), in which the protoplasts were resuspended for electroporation. Protoplast viability was determined by fluorescein diacetate and propidium iodide. The protoplast suspension (0,4 ml) was added 50 µl 0.1% phenosafranine in CPW26% sorbitol, or a plasmid solution to a final concentration of 25 µg/ml. The electrical pulses were rectangular and delivered from a TA 750 Transfection system (Kruess GmbH, FRG).
The protoplasts electroporated with phenosafranine were washed 3 times. The dye was extracted with EtOH and the absorbance measured at 530 nm. The protoplasts electroporated with plasmids were held on ice for 5 min, left at room temperature for 15 min, washed and cultured for 2 days in protoplast growth medium.

Cells were harvested by centrifugation and extraction buffer was added to 0.15 M Tris pH 7.8, 15% glycerol, 5mM EDTA, and 4 µg/ml leupeptin. Cells were sonicated (5 strokes), heated to 60°C for 10 min, and centrifuged 10 min. at 15.000 g. CAT activity in the supernatant was determined essentially after Sleigh, N.J. (1986) Anal. Biochem. 156, 251, using [^{14}C]Acetyl Coenzyme A and chloramphenicol. In addition some extracts were subjected to TLC analysis.

2. Results

In order to optimize the encapsulation of hydrophilic compounds into sugar beet protoplasts in respons to short high voltage electrical pulses, experiments on the uptake of the dye phenosafranine was undertaken.

The effect of field strength, pulse duration and number of pulses were investigated. A general trend was that the optimal uptake of dye (about 1.0-1.5 nmol/100.000 protoplasts) was seen at an immediate survival of 50-80%. The survival was gradually reduced during the next 24 h to a level 10-30% lower depending on the electrical conditions. Very few disrupted protoplasts were seen. In fig. 1 is shown the effect of the pulse duration on the uptake of dye. At 900 V/cm the uptake of dye increases proportionally to the pulse duration whereas the immediate survival is decreased. However, at 1500 V/cm long pulses reduces the viability and the optimal uptake is at shorter pulses (250 µs).

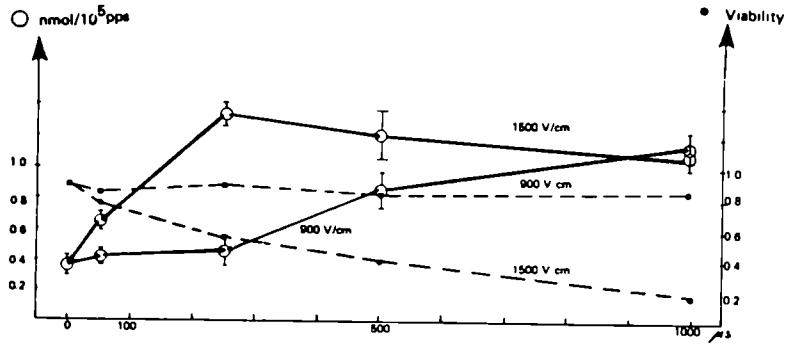


Fig. 1. Effect of pulse duration on uptake of phenosafranine ○ and on viability ✳ at field strength of 900 V/cm and of 1500 V/cm (1 pulse).

The CAT experiments were performed to define the optimal conditions for electroporation, leading to transient expression of introduced genes. Initially, low CAT-activities were found, but including a heat treatment and using the indicated extraction buffer lead to higher activities. About 1-5 mU was found routinely at different electrical conditions in extracts of 1-3 million protoplasts. However, some background from the protoplasts can still be seen (spot c in fig. 2).

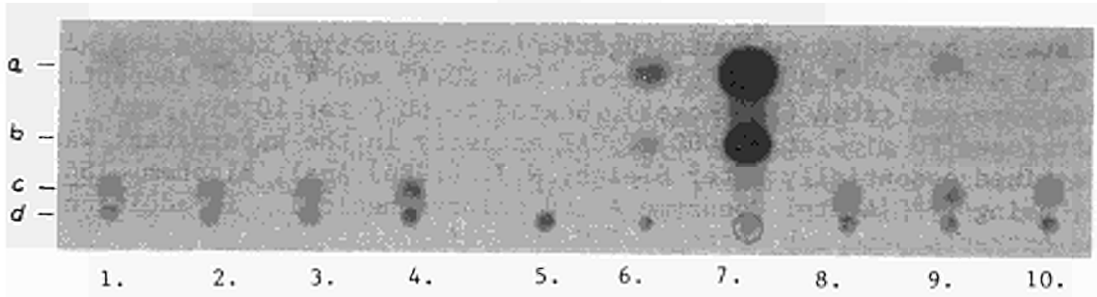


Fig. 2.

1. 600 V/cm, 999µs, 1 pulse, protoplasts.
2. 800 V/cm, 999µs, 1 pulse, "
3. 1000 V/cm, 999µs, 1 pulse, "
4. 1200 V/cm, 999µs, 1 pulse, "
5. Background from assay.
6. 10 mU of CAT.
7. Total conversion of 14-C-AcCoA.
8. Background from electroporated protoplasts.
9. 600 V/cm, 999µs, 1 pulse, crude protoplast preparation.
10. Background from electroporated crude protoplast preparation.

a. 3-AcChloramphenicol. b. 1-AcChloramphenicol. c. Unknown.
d. Site of application.

In fig. 2 is shown an experiment on the effect of the field strength on the CAT-activity. Highest activity is seen at 800 V/cm, 999 µs and 1 pulse (lane 2). Furthermore the CAT-activity of electroporated cells/protoplasts, unpurified after cell wall hydrolisis, is shown (lane 9).

3. Discussion

The dye uptake experiments showed that sugar beet protoplasts by electroporation could encapsulate significant amounts of phenosafranine without appreciable loss of viability. The uptake of dye is merely not caused by increased amounts of damaged protoplasts as the uptake declines at high mortalities at long pulses. This important feature is most often neglected in the litterature.
The experiments on transient expression showed that under appropriate conditions plasmids coding for CAT, under control of the 35 S promotor from CaMV, could be introduced and expressed in sugar beet protoplasts. Interestingly, unpurified protoplasts containing many cells having partially hydrolyzed cell walls also showed CAT-activity.

. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	No
Exchange of staff	No
Joint experiment(s)	No
Joint meeting(s)	Yes

Descriptive information for the above data.

Dr. U. Bissesar from the cooperating John Innes Institute, UK, participated in a joint meeting at DDS-Copenhagen 6th-7th April 1987 together with DDS-AGC research collaborators from 3 UK institutions.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: University of Durham

Contract no.: BAP - 0104 - UK

Project leader: R.R.D. CROY

Scientific staff:

Address: University of Durham
Plant Molecular Biology Group
Dept. of Botany
South Road
UK - DURHAM DH1 3LE

Telephone no.: +44.91.374.2433

Telex no.: 537351 DURLIB G

Other contractual partners in the joint project:

F. Stirpe, Istituto di Patologia Generale (Bologna)

Title of the research activity:

Lectins and ribosome inactivating proteins as pathogen and pest resistance factors in plants.

Key words:

Lectins, Ribosome inactivating proteins, Pathogen resistance, Insect resistance, RIPs

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

(a) The establishment of bioassays for a number of plant pathogens and pests to assay resistance factors.

(b) Isolation and characterisation of RIPs and lectin proteins from a range of species.

(c) Isolation of the genes encoding selected RIPs and lectins and their transfer to host plant species.

(d) Assessment of RIP and lectin gene function in transgenic plants against insect and pathogen attack.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

(a) To utilise our established insect nutritional bioassays to test the susceptibility of insect pests to selected RIPs.

(b) To establish other bioassays for insects and other pests and for pathogens.

(c) To develop methods for the isolation of genomic DNA from the selected RIP plant species for gene library construction.

(d) To obtain DNA probes suitable for screening genomic libraries and the isolation and analysis of RIP genes.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Methodology

(i) Methods have been developed for the micro-injection of very low volumes (1-10 µl) of Ribosome Inactivating Proteins (RIPs) and lectins into insect haemolymph as a convenient test of insect susceptibility.*

(ii) Insect nutritional bioassays have been adapted to allow toxicity testing of RIPs delivered by a nutritional route.**

(iii) Fungal bioassays based on a simple agar plate system have been developed to test the inhibition of mycelial growth by RIPs and lectins.

(iv) A number of isolation methods and insect tissues have been investigated in an attempt to isolate active polysomes in order to test the molecular effects of the RIPs on insect ribosomes.

(v) Techniques have been optimised for the isolation of genomic DNA from leaf tissues of the RIP plant species.

(vi) One highly purified RIP protein has been partially sequenced to yield peptide sequences from which gene probes can be prepared.

(vii) DNA and oligonucleotide probes have been obtained and tested for use as RIP gene probes in Southern blotting and gene library screening.

(viii) Gene libraries are currently under construction for the isolation of the genes.

We gratefully acknowledge the help of * the Insect Physiology Group in the Department of Zoology, and ** Dr. A.M.R. Gatehouse of the Department of Botany, University of Durham, in the development of these methods.

2. Results and Discussion

(a) Bioassays: Two RIPs have been selected for initial evaluation on the basis of their potent inactivation of mammalian ribosomes. The RIPs are ricin, a highly toxic type 2 RIP from Ricinus communis and saporin 6, a low toxicity type 1 RIP from Saponaria officinalis.

We have used both micro-injection (Fig. 1) and nutritional bioassays (Fig. 2) to test the effectiveness of these RIPs against insect pests and have shown conclusively that both ricin and saporin are very toxic to a range of different insect species (Figs. 1 and 2). Current experiments are aimed at quantifying this effect relative to other useful plant resistance factors such as lectins though it is clear that RIPs are potentially of great value.

Fig 1

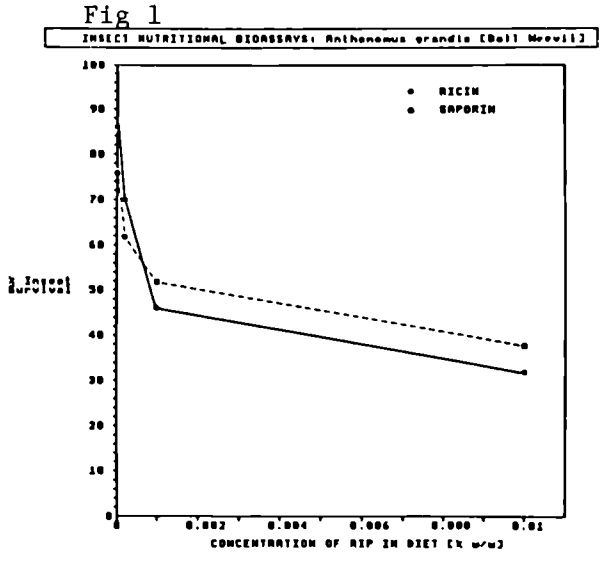
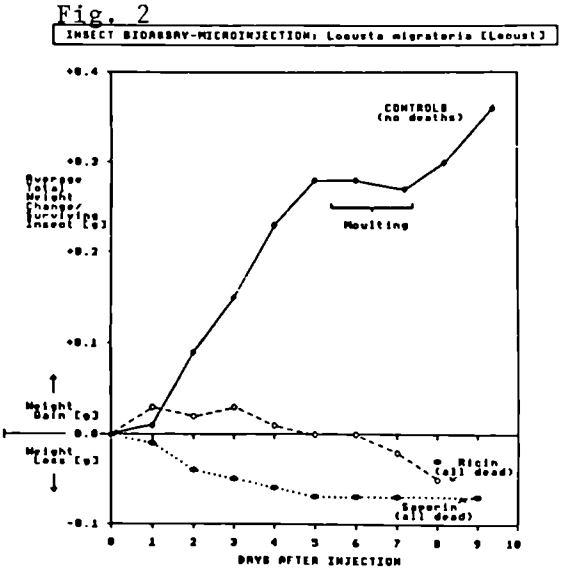


Fig. 2



The elucidation of the underlying molecular mechanism of this toxicity in the insects is of some importance for the development of future genetic engineering strategies. To this end we have isolated active polysomes from two insect tissues - testis and fat body, which are currently being tested directly with RIPs to show any inhibition of translation activity similar to that found with ribosomes of higher organisms. It is anticipated that these results will be published shortly.

Fungal bioassays employing simple agar plate assays have been used to evaluate RIPs as inhibitors of mycelial growth. So far none of the non-pathogenic fungal species investigated has shown any degree of inhibition by ricin or saporin compared with positive inhibition with known anti-fungal agents such as the barley anti-fungal protein and mycostatin.

(b) Molecular Biology: The plant species producing the selected RIPs have turned out to be difficult plants from which to extract genomic DNA. We have adapted and developed existing techniques with which we can extract DNA of reasonably high molecular weight and purity although somewhat low in yields. Both Saponaria and Ricinus DNAs digest well with most restriction enzymes and Southern blots are being used to analyse the RIP gene families using specific DNA probes.

A cDNA probe, pRCL59[+], encoding the Ricinus communis lectin and which is highly homologous to ricin, has been used to elucidate the ricin gene family. The results confirm that this cDNA probe cross-hybridises with the ricin gene family which comprises of five or six members. The 15-20 kb size fraction of partial EcoRI digests of Ricinus genomic DNA has been cloned in EMBL 3 to give a genomic library which is currently being screened with pRCL59 for the ricin genes.

Highly purified saporin 6 protein is currently being sequenced. The partial peptide sequences obtained have enabled us to design and synthesize three oligonucleotide probes of between 17 and 20 bases long and with minimum base degeneracy. These oligos are currently being labelled to high specific activity, by 5' phosphorylation with kinase or by overlap 3' extension using polymerase I (Klenow). It is anticipated that these methods will yield probes of very high specific activity (>10^9 cpm/μg) which will be used for hybridisation to Southern blots and in screening a library for the saporin genes (Fig. 3).

[+]By courtesy of J.M. Lord and L. Roberts, University of Warwick.

Saporin 6 Peptide Sequences

1. **N-terminal sequence** — **Oligonucleotides***

VTSITLDLVNPTAGQYSSPVDKIR[NNVKDP]NLKYGGTDR

AA(CT)AA(CT)GT(ACTG)AA(AG)GA(CT)CC

17-mer (x 64)

2. **Tryptic peptides** — **Oligonucleotides***

TALFPEATTANQKAL/I

INFQSSRGTRS

EVSKRKISTALYGDAKN

RYLQNLVTKN [FPNKFD] SDNKVL/IQF

TT(CT)CC(ACTG)AA(CT)AA(AG)TT(CT)GA

YLGTDIAVIGPPSKDF — 17-mer (x 64)

[NKDYDF] GFGKVRQVKDL

AA(CT)AA(AG)GA(CT)TA(CT)GA(CT)TT(CT)GG

GYTEDYQSIEKNAQITQGDK — 20-mer (x 64)

FRIAKYFRSEITSA

LAMDNTNVNRAY

3. **Peptic peptide**

MYLGKPK

*Synthesized using an Applied Biosystems 381 Synthesiser.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

(1) Highly purified ribosome inactivating proteins isolated from Ricinus communis and Saponaria officinalis have been supplied on several occasions by the Bologna group for use in insect and pathogen bioassays, development of PIP assays in Durham and for protein sequencing to produce oligonucleotide probes.

(2) & (3) The project leader from Durham will be visiting the Bologna group in July-August 1987 for the purposes of research seminars, information exchange, and joint experimental work possibly including assessment of RIPs on insect ribosomes, isolation of RIPs, isolation of RNA/DNA from RIP plant species. Future exchange visits of staff may be organised if sufficient funds are available.

(4) Several meetings have already been held between the project leaders in Durham and Bologna including the recent BAP meeting in Brussels.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Università di Bologna

Contract no.: BAP - 0094 - I

Project leader: F. STIRPE

Scientific staff: L. Barbieri, M.G. Battelli, A. Abbondanza, A. Bolognesi, P. Cenini, C. Stoppa

Address: Dipartimento di Patologia sperimentale
Via S. Giacomo 14
I - 40126 BOLOGNA

Telephone no.: +49.51.232484 or +49.51.235238

Telex no.:

Other contractual partners in the joint project:

R.R.D. Croy, University of Durham

Title of the research activity:

Lectins and ribosome inactivating proteins as pathogen and pest resistance factors in plants.

Key words:

Lectins, Ribosome-inactivating proteins, Pest pathogen resistance

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Production of genes of Ribosome-inactivating proteins for transfer to crop plants that will confer novel resistance to pest and diseases without undesirable side effects.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

1. Adaptation to large scale of chromatographic methods to produce large amounts of RIPs.
2. Identification and characterization of new ribosome-inactivating proteins.
3. Effect of RIPs on protozoan ribosomes.
4. Evaluation of the possibility of preparing RIPs from plant cell cultures.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. A chromatographic procedure for the purification of type I (single chain) ribosome-inactivating proteins from large amounts of starting material has been developed. The general procedure can be adapted with minor modifications to the purification of a variety of RIPs. The amount of time and work was substantially reduced as compared with previously described procedures, and reusable gels have been employed during all chromatographic operations. This methods allows the purification of large quantities of RIPs from plant material which contain them in high concentrations and also to prepare RIPs from materials in which the RIP activity is low. With this method RIPs could be purified from the seeds of Gelonium multiflorum, Momordica charantia, Phytolacca americana, Saponaria officinalis, from the leaves of Dianthus caryophyllus and from the roots of Bryonia dioica. The results obtained indicate that this method is probably applicable to every RIP type I .

2. A new ribosome-inactivating protein has been purified from the seeds of Trichosanthes kirilowii by ion-exchange and gel-filtration chromatography. It is a glycoprotein named trichokirin of 30,000 M_r with a highly basic isoelectric point. Sugar and aminoacid composition have been determined. Alignment of the N-terminal sequences shows a substantial degree of homology between trichokirin and other two RIPs type I already purified, trichosanthin and momordin. Biological activity has been evaluated in various systems. Activity on protein synthesis by cell-free and cell systems is comparable to that of other RIPs of type I. Toxicity to mice is lower then that of saporin or PAP, the LD_{50} being 8.1 mg/Kg. Dead animals showed signs of severe hepatic necrosis.

3. The effect of ribosome-inactivating proteins type 1 (single-chain) and type 2 (two-chains, toxins) on polyphenylalanine polymerization by protozoan ribosomes has been studied. Ribosomes were purified from Acantamoeba castellani, Tetrahymena pyriformis, Leishmania donovani infantum and Trypanosoma brucei rhodiense . Only some of the RIPs tested (10 of type I and 5 of type 2) inhibited polyphenylalanine synthesis by protozoan ribosomes. The effect was highly variable, with ID_{50}'s ranging by more than two orders of magnitude with significantly differnt spectra of activity on the various species. In particular RIPs type 2 with the exception of abrin were ineffective on all ribosomes tested. Amongst RIPs type 1 dianthin 32 (purified from Dianthus caryophyllus leaves) was the most effective. Some in vivo experiments have been performed on the toxicity of RIPs to intact Protozoa. Saporin 6 (purified from Saponaria officinalis seeds) added to the culture medium killed Acantamoeba castellani at high concentrations only (LD_{50} 0.6 mg/ml) but had no effect on Tetrahymena pyriformis, at concentrations up to 1 mg/ml. All other RIPs of either type, at the concentration of 1 mg/ml did not affect the growth of both protozoa examined.

4. Plant cell cultures have been grown and the evaluation of the possibility to purify RIPs from them is under investigation.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

Barbieri, L., Stoppa, C., Bolognesi, A. (1987)
Large scale chromatographic purification of ribosome-inactivating proteins.
J. Chrom. (in the press)

Casellas, P., Dussossoy, D., Falasca, A.I., Barbieri, L., Bolognesi, A. Cenini, P., Stirpe, F.
Trichokirin, a ribosome-inactivating protein from from the seeds of Trichosanthes kirilowii Maximowicz.
Submitted for pubblication.

Cenini, P., Battelli, M.G., Bolognesi, A., Stirpe, F., Villemez, C.L.
Effect of ribosome-inactivating proteins on ribosomes from Tetrahymena pyriformis and Acantamoeba castellani.
Submitted for pubblication.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)	Yes	
Joint meeting(s)		No

Descriptive information for the above data.

RIPs have been supplied to R.D.D. Croy group, Durham, U.K.
and plant cells cultured in vitro have been obtained by M. Buiatti group, Firenze, Italia.
Joint experiments are going on with the two mentioned groups.

INDUSTRIAL COOPERATIONS

SANOFI Recherche
Rue du Prof. J. Blayac,
F-34082 Montpellier Cedex, France.

Farmitalia Carlo Erba
Via Carlo Imbonati 24
20159 Milano, Italia

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Univ. of Cambridge

Contract no.: BAP - 0105 - UK

Project leader: D.S. INGRAM

Scientific staff: M.MacDonald, C. Simeti, A. Smith

Graduate students/Visitors : F. Aslam, R. Bennett, M. Gretenkort, M. Hadwiger, P. Loudon

Address: University of Cambridge
Botany School
Downing Street
UK - CAMBRIDGE CB2 3EA

Telephone no.: +44.223.333938

Telex no.:

Other contractual partners in the joint project:

M. Buiatti, University of Florence

Title of the research activity:

Development of methods for selection *in vitro* for resistance to pathogens.

Key words:

Brassica spp., Resistance, Selection, Tissue-culture, Pathogens

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Development of methods for the in vitro selection of novel disease-resistance factors generated by the genetic manipulation of plant cells grown in culture. The selection strategies to be evaluated are: exposure to toxic culture filtrates of pathogens; exposure to purified pathotoxins; challenge by pathogen spores or hyphae; and identification of biochemical or molecular markers.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Using Brassica napus and other Brassica spp., and the pathogens Alternaria, Leptosphaeria, Pyrenopeziza and Peronospora, as model host-pathogen systems, to: (1) continue studies of the generation in vitro of novel variation for resistance, particularly by haploid culture, somaclonal variation, mutagenesis and protoplast fusion; (2) to continue to develop and evaluate in vitro selection strategies involving fungal toxic factors, and (3) to initiate experiments for using pathogen spores and mycelium as in vitro selection agents and for employing molecular markers in selection.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Introduction

This is a large, ongoing and integrated programme of research, involving inputs from the European Community Biotechnology Action Programme, the University of Cambridge, The Nickerson Seed Co Ltd., Shell Research Ltd., and the Science and Engineering Research Council. We thank all these agencies for their support.

The support of the European Community is very important to the programme for a number of reasons. First, by providing research funds it has made possible the employment of an additional member of staff, thus increasing the speed and efficiency with which the research is being carried out. Secondly, by encouraging and funding the exchange of personnel, it is now possible to transfer techniques developed in one laboratory directly to the other. For example, methods for genome transformation developed in Florence are being transferred directly to Cambridge, and tissue culture techniques developed in Cambridge are being transferred to Florence. Thirdly, by involving two laboratories in the programme, the breadth of the research and the efficiency of its execution has been increased. For example, techniques for the use of fungal toxins and pathogen spores as selection agents is mainly being evaluated in Cambridge, while the use of

biochemical markers in selection is mainly being evaluated in Florence. Also, there is complementation between the two laboratories in the further development of tissue culture technology. Fourthly, as selected plant material is developed it will be exchanged, to increase the efficiency of its testing. Finally, but perhaps most important of all, the intellectual "cross fertilization" that has occurred and will continue to occur between Cambridge and Florence is of inestimable value in generating new ideas and new approaches to the research.

What follows is an extended summary of the work done to date. It attempts to integrate all the components of the programme being carried out in Cambridge and summarises the position at 30 June 1987.

2. Generation of variation

As a prerequisite for, and in parallel with, studies of in vitro selection, the generation of novel disease-resistance factors by the genetic manipulation of cultured Brassica cells is being studied, as follows.

2.1 Haploid culture A long term study of haploid production in Brassicas using rapid cycling species as models has been completed. Suitable protocols for haploid production by anther culture of rapid-cycling lines of B. napus and B. campestris have been devised. Selection experiments have indicated that the capacity for anther embryogenesis in B. campestris is heritable. During selection, inbreeding depression caused a significant decrease in pollen viability and germinability, but had no deleterious effects on anther efficiency. In contrast to the results for B. campestris, the anther culture potential of the plants of successive, selected inbred generations of B. napus remained uniform and poor. Techniques for the production of embryoids by microspore culture were therefore developed, and were found to be highly efficient as a means of producing haploids.

Dihaploid plants regenerated from microspore cultures of B. napus are being evaluated for novel disease resistance factors. Such screening of regenerants will continue into the future. Microspore culture is also being used as a component of research on mutagenesis (see 2.3).

2.2 Somaclonal variation In long-term experiments variation for morphological characters has been shown to be generated at frequencies of approximately 1-5% when Brassica tissues are grown in culture as callus, as secondary embryogenic clones and as protoplasts, and then regenerated. However, such variation is frequently unstable and is lost in the progeny of selfed plants. Stable variation for reaction to the pathogens L. maculans and Alternaria spp. has been detected at a low frequency, but so far this has been for increased susceptibility rather than increased resistance. Experiments to evaluate the potential of somaclonal variation for the generation of novel disease resistance are continuing.

2.3 Mutagenesis In long-term experiments techniques have been developed for the exposure of secondary embryoids, anthers within developing buds and microspores to gamma irradiation. Kill curves have been generated and optimum doses identified. Embryoids, anthers and microspores surviving irradiation have been cultured and plants regenerated. Laboratory and field tests for evaluating plants for novel resistance to Alternaria spp. have been devised and are being used to screen regenerants from

mutagenised tissues and their progeny. In further experiments irradiated seeds of B. napus have been shown to survive doses of 600 Gyes of gamma irradiation without loss of viability. Plant derived from such seeds were all fertile, and have been planted. These too are being screened for resistance to Alternaria spp.

An important additional finding arising from the work on mutagenesis is that doses of gamma irradiation which do not kill cells, induce a significant increase in the development of embryoids from cultured anthers.

2.4 Somatic hybridisation Protocols have been developed for the isolation of viable protoplasts from the six rapid-cycling Brassica spp. (B. oleracea, B. campestris, B. nigra, B. carinata, B. juncea and B. napus). Of these, only B. napus grew well in culture. Plant regeneration was very limited. It is concluded that rapid-cycling Brassica lines do not at present provide useful models for studies of somatic hybridisation. Protocols have also been developed for the isolation of viable protoplasts from normal agricultural cultivars of B. napus, B. oleracea and B. campestris, and for the regeneration of plants. Using chemical fusagens, efficient somatic hybridisation has been achieved, and experiments are now in progress on the genetic basis of somatic hybridisation and on the selection of hybrids carrying disease resistance-factors.

3. In vitro selection strategies

In parellel with, and using material generated by, the experiments outlined in (2) the following studies are now in progress on the prospects for the in vitro selection of novel disease resistance factors.

3.1 Selection using toxic factors produced by pathogens Long-term studies are in progress on the prospects for using culture filtrates and partially purified toxic factors produced by Alternaria spp., L. maculans, P. brassicae and S. sclerotiorum. With the exception of P. brassicae, all produce factors in culture which are highly toxic to cultured Brassica cells and tissues. However, pilot experiments with the factors from L. maculans and S. sclerotiorum suggest that while it may be possible to select lines of cells or tissues for resistance to toxic factors, plants regenerated from them do not exhibit any increased resistance to disease. In depth experiments carried out over several years with Alternaria spp. using partially purified and fractionated toxic materials, confirm this conclusion. Further experiments on the role of toxic factors in diseases of Brassicas caused by the pathogens, especially Alternaria spp. are now in progress, to provide a basis for further experiments on selection in vitro. Toxic factors are generated in Brassica leaves infected with Alternaria spp. and their precise role is now being evaluated. Also, the existence of a new group of toxins produced by very young cultures of Alternaria spp. has been identified, and the potential of these for use in in vitro selection is being explored.

3.2 Use of pathogen propagules in selection Experiments are being made to study the expression of known resistance factors in vitro. These will form a basis for devising methods for in vitro selection of novel resistance factors. In the case of the interaction between L. maculans and B. napus the following has been done: a detached leaf test has been devised for comparing the resistance of different cultivars to the pathogen; media have

been optimised for growing callus tissues from leaf discs and stem segments of B. napus cultivars said to carry differing levels of resistance to L. maculans; a chitin assay has been partially developed for quantifying fungal growth in infected tissues; and methods have been developed for inoculating cultured tissues with the pathogen. Experiments can now be conducted on the effect of the level of differentiation of cultured tissues, and environmental factors such as growth regulators and temperature,on the expression of resistance. A similar sequence of experimentation is in progress for the interaction between B. napus and P. parasitica and P. brassicae. In the case of P. parasitica particular problems are posed because the fungus is an obligate parasite and contains cellulose rather than chitin as a major component of its cell walls.

2.3 Use of biochemical and/or molecular markers known to be correlated with the expression of resistance. Most of the research on this topic is currently being carried out in the laboratory of M. Buiatti in Florence. However, in Cambridge, studies of the potential for using molecular probes for in vitro selection, especially in somatic hybridisation, are in progress.

Particular attention is being focussed on rDNA probes. A genomic library of B. oleracea was screened using a probe prepared from a complete wheat operon kindly supplied by S.M. Trick and R. Flavell. A strongly hybridizing clone was selected. It was not possible to isolate the whole insert intact, and the DNA was shotgun cloned into a plasmid vector. Analysis of the shotgun clones identified three clones which together constitute a complete rDNA operon. A map was produced by restriction site analysis which compares well with that already published for Raphanus. The relationships between the various clones have been confirmed by Southern blot analysis. The positions of the rDNA genes with respect to the various clones have been identified by northern blot analysis of total RNA.

Nuclear DNA has been isolated from the cotyledons of commercial varieties of B. campestris, B. oleracea and B. napus. This has been analysed for RFLPs by Southern blot analysis. Distinct RFLPs have been detected between the three species. The nuclear DNA used in the previous experiments was isolated from whole plant material. The next major step will be to develop methods for the isolation and comparison of DNAs from callus material. To this end, shoot and callus cultures are currently being grown. The potential of other probes for RFLP analysis of callus-derived DNA will be assessed. Initially work will be concerned with the development of probes from cDNA clones for the small sub-unit of Rubisco, 1HCP, and napin.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

None.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Exchange of materials Plans have been made for the exchange in the near future of seeds, tissue cultures, and genetically engineered material.

Exchange of staff Extensive co-operation has occurred, as follows.

1. D.S. Ingram made short visits to Florence for discussions, the exchange of ideas and the planning of the joint programmes in June 1986 and April 1987.

2. C. Simeti, who trained in the laboratory of M. Buiatti in Florence and graduated in November 1986, is now employed full-time in the laboratory of D.S. Ingram in Cambridge. She is a key person in the exchange of results and ideas. She made a short visit to Florence in April 1987, and will make manymore short visits.

3. M. Pellegrini, a member of the staff of the University of Florence, spent the period 23 May 1987 - 20 June 1987 learning techniques in the laboratory of D.S. Ingram in Cambridge.

Joint experiments All experiments of the two laboratories are joint in that M. Buiatti and D.S. Ingram and other staff consult and collaborate in the planning of the overall research programme and in the designing of individual experiments. C. Simetti is a key link-person in this process.

Joint meetings M. Buiatti and D.S. Ingram have already met informally several times. They are now planning a joint seminar on tissue culture and disease resistance, to include other BAP contractors and external collaborators and advisers.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Univ. degli Studi di Firenze

Contract no.: BAP - 0088 - I

Project leader: M. BUIATTI

Scientific staff:

Address: University of Florence
Dept. of Animal Biology and Genetics
Via Romana 17
I - 50125 FIRENZE

Telephone no.: +39.55.22.04.98

Telex no.:

Other contractual partners in the joint project:

D.S. Ingram, University of Cambridge

Title of the research activity:

Development of methods for selection in vitro for resistance to pathogens.

Key words:

Pathogen, Resistance, Tissue culture, Selection

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The general aim of the programme is to test the possibilities offered by plant tissue cultures for the development of efficient selection methods for resistance to pathogens. In this frame in vitro responses to pathogens and/or their cellular components will be analysed and compared with in vivo behaviour, using plants and tissue cultures from resistant and susceptible genotypes. The knowledge thus gained will then be used for the development of new in vitro selection methods.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

As stated in the programme, with the aim of obtaining new methods of selection for resistance to pathogens two main approaches have been used. On one hand the correlation between in vivo resistance or susceptibility to pathogens and in vitro response to pathogen components has been studied with the aim of devising reliable early screening methods and obtaining some useful information on the biochemical nature of plant response. Moreover, also on the basis of such informations, attempts were carried out to devise new direct selection methods.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

METHODOLOGY

The possible existance of a correlation between in vitro and in vivo response to pathogens was carried out on the system carnation-Fusarium oxysporum using susceptible and resistant cultivars and their F_1 progenies, and on Alternaria solani - potato. Calli of the different genotypes were grown on media containing different concentrations of fungal cultural filtrate and growth inhibition was recorded. The same genotypes were furthermore treated with fungal heat released cell wall components and screened for phytoalexin production both with a biological test previously described (Buiatti et al. Theor. Appl. Genetics 70: 42-47, 1985) and T.L.C. analysis. Selection expreriments were aimed at obtaining cell lines altered in the response to fungal cell wall components. For this purpose positive

selection was carried out on tomato suspension cultures (cv.Red River) using Fusarium heat released cell wall components as a selective agent. Negative selection for increased hypersensitivity was attempted by submitting cells of the same genotype to combined elicitor and BUdR treatments in the dark and then plating them under continuous light. Phytoalexin synthetic levels of selected clones were then measured with the previously mentioned methods in tissue treated with Fusarium and Phytophtora elicitors. The obtained variants were furthermore checked for resistance to fusaric acid induced ion leakage and inhibition of fungal growth in dual culture experiments in Petri dishes.

RESULTS

In the system Fusarium - carnation no significant correlation between in vivo resistance and in vitro tolerance to culture filtrates was observed while there was a good correlation in the case of phytoalexin production. Moreover, the character phytoalexin production in vitro behaved as a single dominant just as resistance in vivo.

On the other hand, in the system potato-Alternaria solani, although elicitation of flavonoid compound synthesis was shown after treatment with Alternaria cell wall components, no differences were found between susceptible cultivars and a supposedly resistant one (Chiquita).

In vivo infection experiments, however, did not confirm Chiquita's resistance and hence, research will be extended to another cultivar (Serrana Inta) whose resistance seems to be proven under our experimental conditions.

In selection experiments both high and low phytoalexin producing tomato cell clones were isolated. Further tests showed that these clones were also altered in the speed of reaction to elicitor treatment and that they reacted equally differently when challenged with Phytophtora infenstans elicitor, thus suggesting lack of specificity of the selected modification. These results were confirmed in dual culture experiments, experiments which showed this technique to be amenable to quantitative evaluation. Finally, selected cell lines were shown to differ from control ones also in the

resistance to fusaric acid measured as ion leakage. Attempts to regenerate plants from the selected cell lines and to extend the tests of the new selection methods to other systems are at present being carried out.

DISCUSSION

The results obtained point out to the possibility of using tests based both onphytoalexin synthesis and dual cultures for early screeining of crosses between resistant and susceptible genotypes and their progenies. Moreover they seem to offer a new in vitro selection method never exploited until now based on the obtention of variant (mutants) improved in phytoalexin production when challenged with the pathogen. This possibility however has to be further tested on regenerated plants and their progenies with in vivo infection experiments.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.I PUBLICATIONS IN SCIENTIFIC JOURNALS, MONOGRAPHS.

M. Buiatti, C. Simeti, S. Vannini, G. Marcheschi, A. Scala, P. Bettini, P. Bogani, M.G. Pellegrini: Isolation of tomato cell lines altered in the response to Fusarium cell wall components. Theor. Appl. Genetics, 1987, In the press.

M. Buiatti, G. Marcheschi, R. Venturo, P. Bettini, PK Bogani, R. Morpurgo, B. Nacmias, and G. Pellegrini: In vitro response to Fusarium elicitor and toxic substances in crosses between resistant and susceptible carnation cultivars J. Plant Breeding, 1987, in the press.

IV.II SHORT COMMUNICATIONS, INTERNAL REPORTS.

M. Buiatti, C. Simeti, G. Marcheschi, A. Scala, M.G.Pellegrini, P. Bettini, P. Bogani: Selection of tomato cell lines high and low producers of Phytoalexins after treatment with Fusarium oxysporum f.sp.lycopersici cell wall components 1986 Annual Meeting of the Italian Society for Agricultural Genetics

M. Buiatti, G. Marcheschi, M. Piro, M.G. Pellegrini, P. Bettini, P. Bogani, R. Venturo: Preliminary results on the genetic analysis of the character "in vitro phytoalexin production" , in crosses between carnation cultivars resistant and susceptible to Fusarium Oxysporum f.sp.dianthi, 1986 Annual meeting of the italian Society for Agricultural Genetics.

IV.IV DOCTORATE THESIS (Ph.D.) AND DEGREE THESIS AWARDED DURING THE PERIOD OF THE CONTRACT.

C. Simeti: Selezione e caratterizzazione di linee cellulari di pomodoro alterate nel meccanismo di interazione ospite-parassita (Selection and characterization of tomato cell lines altered in host-parasite interaction mechanisms) Thesis for the degree of "Dottore in Scienze Biologiche", 7 November, 1986.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Dr. Claudia Simeti who obtained her "Dottore in Scienze Biologiche" (see Publication list) is at present working in Dr. Ingram Laboratory.
Dr. M.G. Pellegrini has payed a two months to Cambrdidge Laboratory in May-June 1987.
Joint experiments are on the way on the systems Tomato-Fusarium, Brassica, Pyrenopeziza with the aim of studying the effect of hormone synthesis and concentration on the response to pathegens.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: University of Edinburgh

Contract no.: BAP - 0103 - UK

Project leader: S.C. FRY

Scientific staff: S. Green

Address:
University of Edinburgh
Dept. of Botany
Mayfield Road
UK - EDINBURGH EH9 3JH

Telephone no.: +44.31.6671081 EXT.3309

Telex no.: 727442 UNIVED G

Other contractual partners in the joint project:

P.J.G.M. de Wit, Agricultural University of Wageningen

Title of the research activity:

Apoplastic enzymes and biologically-active oligosaccharides as markers of early pathogenesis.

Key words:

Apoplast, Oligosaccharides, Pathogenicity, Glycosylhydrolases, Cell wall

Reporting period: October 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Any substances (glycosylhydrolases in the first instance) formed during infection of a plant by a potential pathogen will be identified. Such enzymes could be useful in the early detection of disease before visible symptoms appear and knowledge of their identity could help to predict and explain the outcome of specific race/cultivar interactions. The development of very sensitive assays for the detection of glycosylhydrolases will be required. These assays will then be exploited to distinguish between substances formed in compatible and incompatible interactions, and ideally they will indicate the race of fungus present. An experimental system will be used; however, the results of this project may also provide fundamental information transferable to other major crops.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Plant cell wall fragments appear to play a role in disease resistance, evoking phytoalexin synthesis and possibly inducing hypersensitivity in some way. Apoplastic enzymes produced either by the invading fungus or by the plant in response to the fungus are hypothesised to solubilise from wall polymers oligosaccharides which evoke these defence responses. Therefore, apoplastic enzymes and oligosaccharides will be explored as marker substances in disease detection and may highlight differences between compatible and incompatible interactions. The specific aim therefore is to identify these apoplastic enzymes and oligosaccharides.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Methods

Genetically defined infections of Lycopersicon esculentum and Cladosporium fulvum were made at Wageningen, tomato leaves of the cultivar Cf5 being infected with virulent (race 5) or avirulent (race 4) races of C.fulvum. These, and uninfected healthy controls of Cf5, were used as a source of apoplastic fluid (which was collected over a time course of 14 days following infection). This was then incubated with uniformly ^{14}C-labelled cell walls, and solubilised radioactive fragments were assayed.

The material solubilised after 7 hours' incubation was analysed by chromatography. Paper chromatograms were developed either in ethyl acetate: acetic acid: water 10:5:6 (EAW) to resolve oligosaccharides or in ethyl acetate:pyridine:water 8:2:1: (EPW) to resolve monosaccharides. Oligosaccharides and solubilised polysaccharides were also subjected to hydrolysis in trifluoroacetic acid (2M, 120°C, 1h) followed by paper chromato-

graphy in EPW.

Summary of Results

Following incubation of uniformly ^{14}C-labelled cell walls with apoplastic fluid from infected and uninfected sources, for seven hours, a considerable amount of material was found to be solubilised (see Fig. 1). The results showed that apoplastic hydrolases are naturally present in uninfected plants and that this enzymic activity is enhanced in infected plants, particularly during active defence. Although apoplastic fluid from healthy plants always solubilised the least amount of radioactive cell wall material, apoplastic fluid from the Cf5/race 5 infection (compatible) sometimes solubilised as much radioactivity as that from a Cf5/race 4 (incompatible) infection.

Solubilised material was analysed on Bio-Gel P2 and was found to consist of a range of poly-, oligo- and monosaccharides. Apoplastic fluid from infected plants liberated a higher percentage of mono- and disaccharides and a lower percentage of poly- and large oligosaccharides than that from healthy plants (see Fig. 2).

The solubilised ^{14}C-material was composed mainly of galactose, glucose and uronic acids. In addition, material solubilised by apoplastic fluid specifically from the Cf5/race 5 (compatible) interaction contained polysaccharide-bound arabinose.

Discussion

These initial results show that enzyme activity is present in the apoplastic fluid and that it is enhanced upon infection. Whether the enhancement is of a new or pre-existing enzyme(s) has yet to be ascertained.

The material solubilised from the radioactive cell walls by fluid from a) healthy, b) infected, resistant and c) infected, susceptible plants is being analysed for qualitative differences. Initial results have shown two major differences. Firstly, apoplastic fluid from infected, resistant plants can cleave from cell walls a very high proportion of monosaccharides and a very low proportion of large oligo- and polysaccharides suggesting exo-enzyme activity. Secondly, material solubilised by apoplastic fluid from infected, susceptible plants solubilised an arabinose-rich polysaccharide, suggesting the hydrolysis of an arabinose-containing polysaccharide from the cell wall by apoplastic enzymes specific to the Cf5/race 5 interaction.

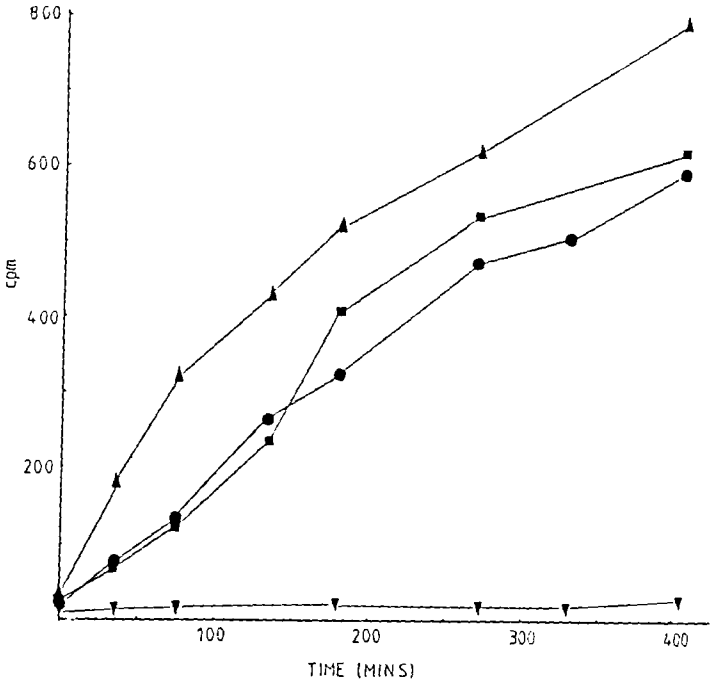
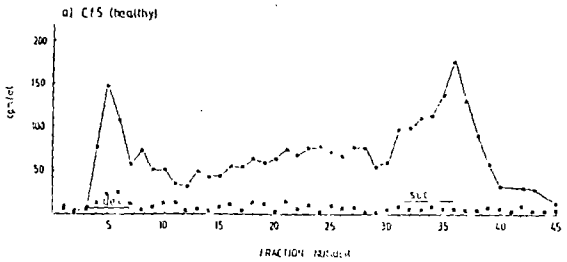
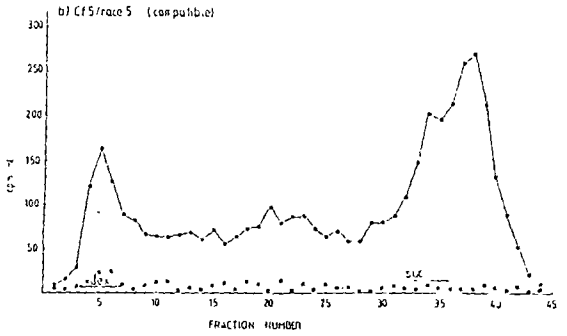


FIG 1 RADIOACTIVE MATERIAL SOLUBILISED FROM PLANT CELL WALLS BY APOPLASTIC FLUID

Key:

- Cf 5
- Cf 5 / race 5
- Cf 5 / race 4
- control





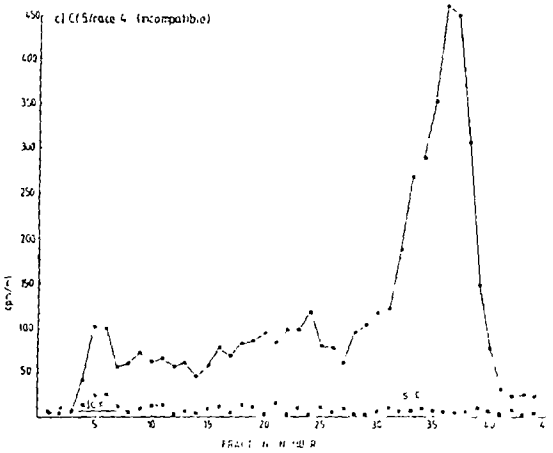


FIG. 2 ^{14}C-MATERIAL HYDROLYSED FROM PLANT CELL WALLS BY APOPLASTIC FLUID

Standards:

dex = dextran

suc = sucrose

KEY

- sample
- control

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1 Apoplastic glycosylhydrolases as markers of early pathogenesis. 'Food Hydrocolloids'. (In press).

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)	Yes	
Joint meeting(s)	Yes	

Descriptive information for the above data.

Exchange of material(s)

- apoplastic fluid, prepared at Wageningen after genetically defined infections of tomato plants had been made
- Cf5 and Cf4 tomato seeds supplied by Wageningen
- tomato cell cultures from Wageningen
- details of chromatographic methods used for analysis of mono-, oligo- and polysaccharides from Edinburgh to Wageningen
- assay method of enzyme activity developed at Edinburgh to Wageningen.

Joint experiments

- testing apoplastic fluid using complementary methods
- ^{14}C-labelling of tomato cell walls at Edinburgh, then used with apoplastic fluid from Wageningen.

Joint meetings

- group meeting during BAP meeting at Louvain-la-Neuve.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Landbouwhogeschool, Wageningen

Contract no.: BAP - 0074 - NL

Project leader: P.J.G.M. DE WIT

Scientific staff: M.H.A.J. Joosten

Address:
Agricultural University of Wageningen
Laboratory of Phytopathology
Binnenhaven 9
NL - 6709 PD WAGENINGEN

Telephone no.: +31.8370.83122

Telex no.: 45917

Other contractual partners in the joint project:

S.C. Fry, University of Edinburgh

Title of the research activity:

Apoplastic enzymes and biologically-active oligosaccharides as markers of early pathogenesis.

Key words:

Cladosporium fulvum, Apoplastic proteins/enzymes, Pathogenicity factors (race-specific) elicitors, mono/oligosaccharides

Reporting period: October 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The combination tomato-Cladosporium fulvum is used as a model system to study a plant-pathogen interaction. Identification of "marker substances" formed during infection and predicting the outcome of race/cultivar interactions, combined with the development of adequately sensitive assays for their detection is a major point of interest of the joint project. In view of the fact that cell wall fragments appear to evoke the hypersensitive defence response and phytoalexin synthesis, these "marker substances" are assumed to be apoplastic proteins/enzymes and oligosaccharides. The precise range of "marker substances" formed would predict, and ultimately explain, the outcome of specific race/cultivar interactions.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Intercellular washing fluid from inoculated leaf tissue (obtained by the vacuum infiltration technique) contains various substances (proteins, enzymes, (oligo)saccharides) of which the composition depends upon the type of interaction (compatible or incompatible). One of the points of research was to isolate and characterize a compatible interaction-specific protein, readily visible on polyacrylamide gels, run under low pH, non-denaturing conditions.

To obtain information about the carbohydrates present in the apoplastic fluid, fluids were analyzed for monosaccharides and attempts were made to isolate oligosaccharides for apoplastic fluids originating from compatible C. fulvum-tomato interactions.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Methodology

Tomato cultivars were inoculated with conidial suspensions of the various races of C. fulvum and for time-course experiments apoplastic fluids from the leaves were recovered at various times after inoculation. Leaves were harvested at random and the intercellular spaces were washed by infiltration of entire leaflets with distilled water in vacuo, followed by centrifugation.

A compatible interaction-specific protein, visible on low pH, nondenaturing polyacrylamide gels of apoplastic fluid, was purified by ion-exchange chromatography followed by chromatofocusing. About 140 µg of the protein was injected into a rabbit to raise antibodies.

To analyze intercellular fluids for monosaccharides a HPLC system equipped with a column for separating monosaccharides at 85 °C and a refraction index detector was used.

Some preliminary experiments were performed to isolate oligosaccharides from apoplastic fluids. For this purpose a Bio Gel P2 column (100 cm) was used, while distilled water was used for elution.

Results

The compatible interaction-specific protein eluted from the PBE column at pH 5.3 and migrated on SDS-polyacrylamide gels as one band with a molecular weight of 14 kD. Antiserum raised against complete apoplastic fluid obtained from the interaction Cf5 race 5, 14 days after inoculation of the plants (containing the compatible interaction-specific protein), did not interact with the protein on nitrocellulose blots, but antibodies were indeed produced when the purified protein was injected into rabbits. With use of the specific antiserum it was shown that the protein is neither present in mycelium of C. fulvum grown in vitro, nor in apoplastic fluid of healthy tomato leaves. More importantly the protein is not present in apoplastic fluid from incompatible C. fulvum-tomato interactions nor in compatible P. infestans-tomato interactions (Fig. 1). In time-course experiments of compatible C. fulvum-tomato interactions, the protein was detected on immunoblots about 8 days after inoculation. The protein appeared not to be a glycoprotein and was highly heat-stable.

The monosaccharide composition of apoplastic fluid originating from several C. fulvum-tomato interactions, obtained 14 days after inoculation, is presented in Table 1.

Apart from the sugars mentioned in Table 1, low amounts of arabinose and inositol could be detected in all apoplastic fluids. In compatible interactions a significant accumulation of glucose and fructose occurred, while the sucrose content of the apoplastic fluid was comparable with that of the control plants and incompatible interactions. In addition to the increase of the glucose and fructose contents, the compatible interactions show a marked accumulation of mannitol in the apoplastic fluid, with values up to 2 mg ml^{-1} for the Cf5/race 5 interaction.

Discussion

The unique occurrence of the compatible interaction-specific protein raises questions concerning its origin and function. As no other types of plant stress or fungal infections other than C. fulvum led to the accumulation of the protein, this protein is not related to the class of pathogenesis-related (PR) proteins. The absence of the protein in extracts of C. fulvum grown in vitro implicates that if the protein is of fungal origin, it is only produced in vivo, during the infection or colonization process. This specific synthesis of the protein suggests that it might be an important factor of basic-compatibility. As the production of the protein is not limited to specific compatible race-cultivar interactions, it is probably not involved in virulence of C. fulvum directed to certain cultivars.

The relatively low concentration of sucrose, in combination with the high glucose and fructose contents of apoplastic fluid originating from a compatible interaction, is probably the result of (induced) activity of an invertase. After the conversion of sucrose into fructose and glucose by invertase, the fructose is reduced into mannitol by mannitol dehydrogenase, of which the activity could be measured in the apoplastic fluid. In this way the fungus is able to accumulate mannitol, a monosaccharide that cannot be metabolized by the tomato plant.

Thus far we have not been able to isolate oligosaccharides from apoplastic fluid, probably because of the fact that their concentration is very low. A more sensitive assay procedure for their detection (probably by radio active labelling) will be necessary.

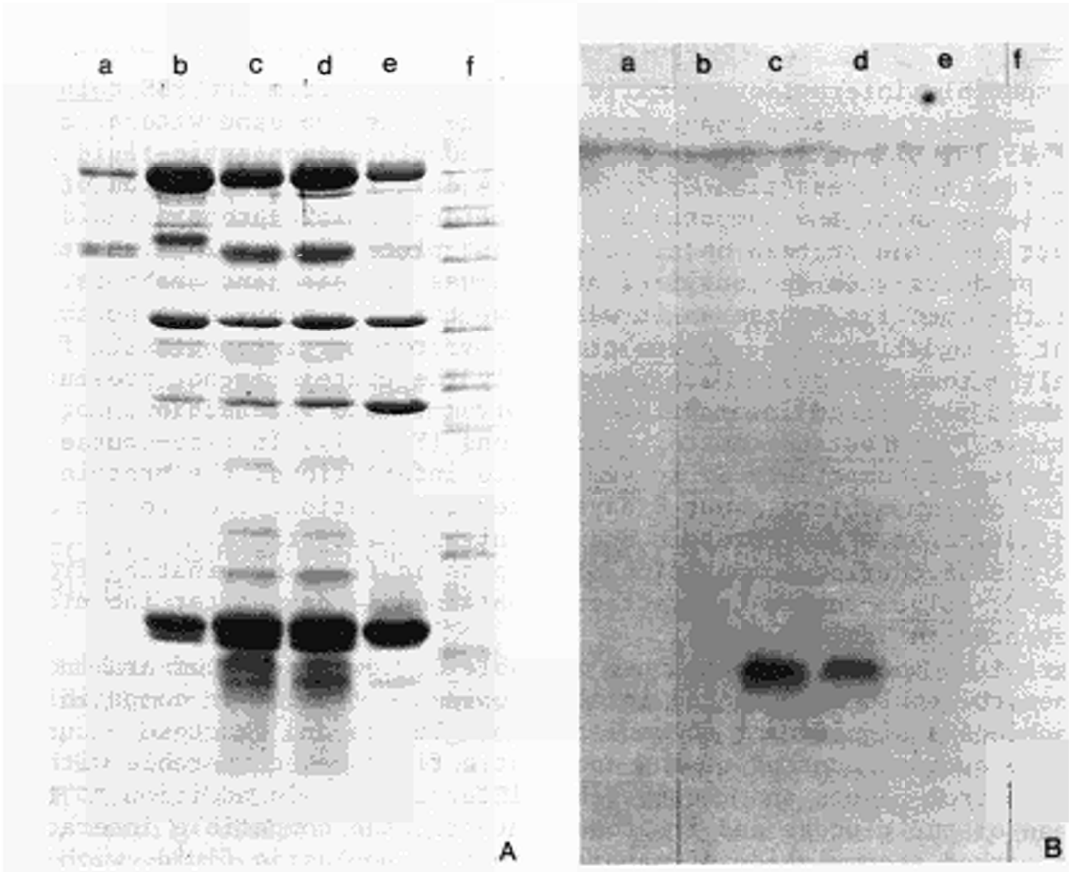


Figure 1. (A). Polyacrylamide gel electrophoresis profiles of proteins separated under SDS-denaturing conditions and stained with Coomassie brilliant blue R-250. Lanes a to e represent 200 µl of apoplastic fluid, originating from a non-inoculated control plant (Cf5, lane a), an incompatible interaction (Cf4/race 5, lane b), two compatible interactions (Cf5/race 5 and Cf5/race 2.4.5.9, lanes c and d, respectively) and a compatible interaction between P. infestans and tomato, 6 days after inoculation (lane e). Lane f represents the protein profile obtained from a homogenate of 50 mg mycelium of race 5, cultured in vitro.
(B). Autoradiographic immunoassay performed on a nitrocellulose blot of the gel presented in Fig. 1 A. The blot was incubated with antiserum raised against the purified compatible interaction-specific protein. Note that the protein is only detected in the two compatible C. fulvum-tomato interactions.

Table 1. A representative experiment showing the monosaccharide composition of apoplastic fluid originating from several C. fulvum-tomato interactions, obtained 14 days after inoculation.

Interaction	Monosaccharide content (µg ml^{-1})			
	sucrose	glucose	fructose	mannitol
Cf4/-	122	79	155	0
Cf5/-	100	54	90	0
Cf4/race 5	82	136	197	5
Cf5/race 4	89	122	184	20
Cf4/race 4	77	300	365	187
Cf5/race 5	91	829	1347	2011

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

Joosten, M.H.A.J. and De Wit, P.J.G.M. (1987).
Isolation, purification and preliminary characterization of a protein specific for compatible Cladosporium fulvum (syn. Fulvia fulva)-tomato interactions.
Submitted to Physiological and Molecular Plant Pathology.

V TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)	Yes	
Joint meeting(s)	Yes	

Descriptive information for the above data.

a) Exchange of material
- apoplastic fluid, prepared at Wageningen after genetically defined infections of tomato plants had been made
- Cf5 and Cf4 tomato seeds supplied by Wageningen
- tomato cell cultures from Wageningen
- details of chromatographic methods used for analyses of mono-, oligo- and polysaccharides from Edinburgh to Wageningen
- assay method of enzyme activity developed at Edinburgh to Wageningen.

b) Joint experiments
- testing apoplastic fluid using complementary methods
- ^{14}C-labelling of tomato cell walls at Edinburgh, then used with apoplastic fluid from Wageningen.

c) Joint meetings
small group meeting during BAP meeting at Louvain-la-Neuve.
In the autumn of 1987 a short visit will be paid to Edinburgh. In the spring of 1988 a mini-symposium on host-pathogen interactions will be organized in Wageningen. Possibly one or two other EEC contracted partners will be invited.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: INSA, Lyon Contract no.: BAP - 0210 - F

Project leader: J.M. ROBERT-BAUDOUY
Scientific staff: C. Allen, F. Chalet, G. Condemine, A. Godin, N. Hugouvieux-Cotte-Pattat, J. Ji, S. Reverchon, O. Shonukan

Address: Laboratoire de Microbiologie et biotechnologies
Institut National des Sciences Appliquées
20, Av. Albert Einstein
F - 69621 VILLEURBANNE CEDEX

Telephone no.: +33.78948331 or +33.78948088

Telex no.: 380856 INSALYN F

Other contractual partners in the joint project:

G.P.C. Salmond, University of Warwick (Coventry)
A. Toussaint-Pourbaix, U. L. B. (Bruxelles)
A. Kotoujansky, I. N. A. - P. G. (Paris)
J.P. Chambost, C. N. R. S. (Marseille)

Title of the research activity:
Analysis of the pathogenicity genes and mechanism of extracellular enzyme export in Erwiniae. Molecular biology of phytopathogenic Erwiniae.

Key words:
Pectinolysis, Erwinia chrysanthemi, Secretion, Pathogenicity, Regulation

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Plant pathology is an extremely complex field. Our group focusses on the soft rot *Erwinia* , a genus of economically important phytopathogenic bacteria whose close relationship to *E.coli* makes them amenable to existing physiological and genetic techniques. *Erwinia* species attack plants by means of a group of extracellularly secreted plant tissue-degrading enzymes including pectinases, cellulases, and proteases. There are additional poorly-understood factors essential to *Erwinia* pathogenicity. Most of the basic strains and techniques associated with this proposal have already been developed by our group, which has been collaborating for the past five years on studies of *Erwinia* disease. Each collaborating laboratory will analyze an aspect of pathogenicity (pectinolysis, cellulolysis, plant-bacteria interactions, protein secretion, etc...).

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

A genetic study of the catabolic pathway of pectin and hexuronates was attempted to establish a model of pectinolysis regulation. Structural and regulatory mutants were characterized and an investigation into the regulation of each gene involved in pectinolysis was carried out.
Fusions in each gene of the pathway were obtained. We wished to clone each gene of the pectinolytic pathway, beginning with the *ogl* and *kdgT* genes (encoding the enzyme oligogalacturonate lyase and a transport system for various intermediates, respectively).
A gene involved in secretory mechanisms was identified and cloned to gain a better understanding of the mechanims involved in the secretion of proteins, particulary pectates lyases.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1) Mu- lac insertion mutants of *E. chrysanthemi* affected in the degradative pathway of galacturonate and KDG were isolated. The mutations were characterized by the growth phenotype and assays of the different enzymes. Mutations were localized on the *E. chrysanthemi* chromosome. This work permitted us to determine the organization and the regulation of the genes involved in the galacturonate pathway. The eight genes constitute six independent transcriptional units and three regulatory units controlled by the products of the *exuR, uxuR* and *kdgR* regulatory genes. The genetic localization and the organization of the genes involved in hexuronate and KDG catabolism in *E. chrysanthemi* strain 3937 were determined and compared to that existing in *E. coli* K12 or in another *E. chrysanthemi* strain, B374 (HUGOUVIEUX-COTTE-PATTAT N. and J. ROBERT-BAUDOUY. 1987).

2) The gene *kdgT* encodes a transport system responsible for the uptake of ketodeoxyuronates. We studied the biochemical properties of this transport system. The 2-keto-3- deoxygluconate entry reaction displayed saturation kinetics with an apparent K_m of 0.52 mM (at 30°C; pH 7). 5-keto-4-deoxyuronate and 2,5-diketo-3-deoxygluconate appeared to be competitive inhibitors with Ki of 0.11 mM and 0.06 mM, respectively. The 2-keto-3-deoxygluconate permease can mediate the uptake of glucuronate with a low

affinity. *kdgT* was cloned on an R-prime plasmid formed by *in vivo* complementation of a *kdgT* mutation of *E. coli* . After subcloning, it was mutagenized with a mini-Mu *-lac* transposable element able to form fusions with the *lacZ* gene . We introduced a *kdgT-lac* fusion into the *E. chrysanthemi* chromosome by marker exchange recombination and studied *kdgT* regulation . *kdgT* expression was not induced by external 2-keto-3-deoxygluconate but by galacturonate and polygalacturonate; it is controlled by the *kdgR* regulatory gene (CONDEMINE and ROBERT-BAUDOUY 1987). The determination of the nucleotide sequence of the *kdgT* gene is in progress (ALLEN et al. in preparation).

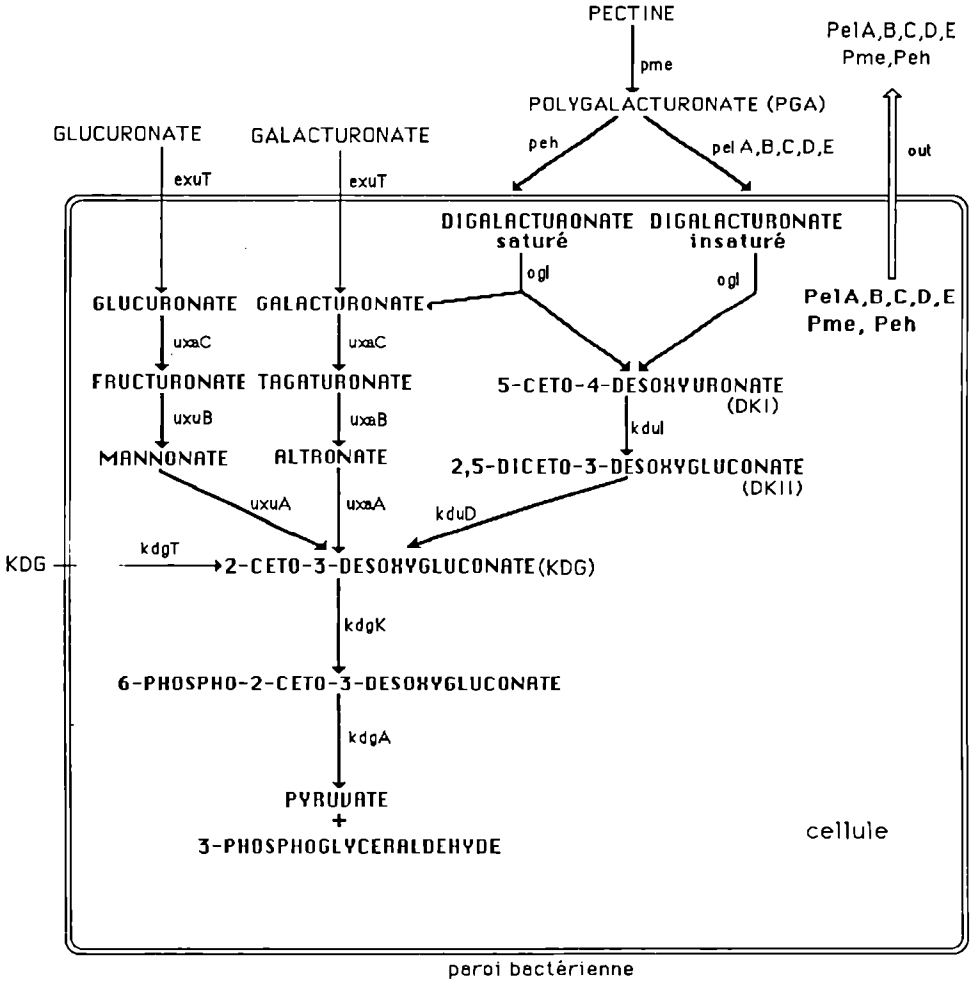
3) Mutants of *Erwinia chrysanthemi* 3937 deficient in the pectin catabolic enzyme oligogalacturonate lyase were isolated by chemical and phage Mud (Ap *lac*) insertion mutagenesis. The *ogl* mutation was biochemically characterized and localized near the *trp* and his markers on the *E. chrysanthemi* chromosomal map. Analysis of Mud (Ap *lac*) insertions, which generate polar mutations, indicated that the *ogl* gene probably forms a distinct transcriptional unit. We cloned the *ogl* gene by complementing the mutation using the RP4 :: miniMu plasmid. pR'*ogl* plasmids were analyzed for the presence of other unselected genes of strain 3937. One of them, called pROU2, also carried the *kduD* and *kdgR* genes encoding 2-keto-3-deoxygluconate oxidoreductase, an enzyme of the pectin catabolic pathway, and the KdgR repressor, governing the expression of several genes of pectin degradation, respectively. The plasmid pROU2 harbored a chromosomal DNA insert of about 35 kb indicating that *ogl*, *kduD* and *kdgR* are very closely linked. Structural analysis of the *ogl* gene was carried out in subcloning experiments. This gene was localized on a 3.5-kb *Pst*I fragment (REVERCHON and ROBERT-BAUDOUY 1987). The sequencing of the *ogl* gene is in progress.

4) The regulation of the *pelA*, *pelD* and *pelE* genes, encoding three of the five major pectate lyase isoenzymes (PLa, PLd and PLe) in *Erwinia chrysanthemi* B374, was analyzed using genetic fusions to *lacZ*. These three genes are clustered on a 5 kb DNA fragment, in the following order : *pelD-pelE-pelA* and constitute three independent transcriptional units. We localized the *pelDEA* cluster near the *pro-1* marker on the genetic map of B374 by chromosomal mobilization using the RP4::miniMu plasmid, pULB110.
Three classes of regulatory mutations responsible for constitutive PL synthesis have been described (*kdgR*, *gpiR*, and *cri*). We studied the effects of each mutation on *pelE*, *pelD* and *pelA* expression, independently. The mutations *kdgR* and *gpiR* mainly affect the expression of *pelE* and *pelD* , although PLa synthesis is sightly increased . The *cri* mutation results in a low level of constitutive expression of the three *pel* genes, but it is a pleiotropic mutation since other genes not involved in pectinolysis are also affected.The frequency of *gpiR* or *cri* mutations (about 10^{-8}) and the resulting constitutivity of pectate lyase synthesis suggest that these genes act as negative regulatory genes together with *kdgR*. Moreover, we found that expression of *pel-lac* fusions carried on pBR322 derivatives was higher in *E. chrysanthemi* than in *E. coli*; this fact suggests the existence of positive regulation of pectate lyase synthesis in *E. chrysanthemi* (REVERCHON and J. ROBERT-BAUDOUY. 1987).

5) A new type of mutation in the *kdgR* regulatory gene was isolated by Tn*5* insertion.Tn*5* mutagenesis was performed on the *Erwinia chrysanthemi* strain 3937 with the transposon vector pMO194. The vector is spontaneously lost from the cell and Tn*5* transposes randomly on to the chromosome. A mutant with a derepressed expression of a *kduD-lac* fusion was obtained. In this mutant, Tn*5* was inserted in the regulatory gene named *kdgR*, resulting in constitutive expresssion of genes of the polygalacturonate degradative pathway (*pelA*, *pelD*, *pelE*, *ogl*, *kdul*, *kduD*, *kdgT*, *kdgK* and *kdgA*). Inactivation of the *kdgR* gene by Tn*5* insertion suggests that *kdgR* is a negative regulatory gene (CONDEMINE and ROBERT-BAUDOUY 1987). The regulatory gene *kdgR* was cloned on a R-prime

plasmid bearing both the *ogl* and *kduD* genes. Subcloning of *kdgR* is in progress (REVERCHON et al, in preparation).

6) We studied the secretion of pectate-lyases by using Mu-*lac* insertion mutagenesis in the *E. chrysanthemi* strain 3937. Analysis of ß-galactosidase expression of the *out-lac* fusions, in different growth conditions, showed that the expression of the *out* gene is constitutive. Compartmentation of pectate lyases during growth suggests that pectate lyases are first exported to the periplasm with formation of an intracellular pool of active pectate lyases, and then released into the extracellular medium. In the *out* mutants, pectate lyases are retained in the periplasm. A 65 kd protein is absent from the periplasm of the *out* mutants; this protein could have a role in the secretory system of *E. chrysanthemi* (JI et al. 1987). The gene *outJ* was cloned on an R-prime plasmid formed by selection of a Tn5 insertion neighbouring the wild type *outJ* gene. Subcloning of this gene is in progress.



IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV .1 PUBLICATIONS IN SCIENTIFIC JOURNALS, MONOGRAPHS

- CONDEMINE G., HUGOUVIEUX-COTTE-PATTAT N. and J. ROBERT-BAUDOUY. "Importance of ketodeoxyuronate pathway in the degradation of pectin in *Erwinia chrysanthemi* ".1987. Plant Pathogenic Bacteria p. 172-181. Martinus Nijhoff publishers.

- J. JI, HUGOUVIEUX-COTTE-PATTAT and J. ROBERT-BAUDOUY . "Use of Mu-*lac* insertions to study the secretion of pectate lyases by *Erwinia chrysanthemi*". J. Gen. Microbiol. 1987. 133, 793-802.

- HUGOUVIEUX-COTTE-PATTAT, N and J. ROBERT-BAUDOUY." Hexuronate catabolism in *E. chrysanthemi*". J. Bacteriol. 1987. 169, 1223-1231.

- CONDEMINE, G. and J. ROBERT-BAUDOUY."*Tn5* insertion in *kdgR* a regulatory gene of the polygalacturonate pathway in *E. chrysanthemi* ". 1987 FEMS Microbiology Letters. 42, 39-46.

- CONDEMINE, G. and J. ROBERT-BAUDOUY. "2-keto-3-deoxygluconate transport system in *Erwinia chrysanthemi* ". 1987 J. Bacteriol 169, 1972-1978.

- REVERCHON, S and. J. ROBERT-BAUDOUY. "Regulation of expression of pectate lyases *pelA*, *pelD*, *pelE* in *Erwinia chrysanthemi* ". 1987 J. Bacteriol 169, 2417-2423 .

- REVERCHON, S and. J. ROBERT-BAUDOUY. "Molecular cloning of *Erwinia chrysanthemi* oligogalacturonate lyase gene involved in pectin degradation". 1987 Gene . 55, 125-133.

IV.2 - SHORT COMMUNICATIONS

- Meeting des contractants du programme BAP (Biotechnologie) Louvain la Neuve Belgique, mars 1987 : F. CHALET, G. CONDEMINE , J. JI , N. HUGOUVIEUX-COTTE-PATTAT, S. REVERCHON and J. ROBERT-BAUDOUY :"Molecular biology of phytopathogenic *Erwiniae*."

- Colloque de la Société Française de Microbiologie : Expression génétique chez les microorganismes. Institut Pasteur, Paris ,18-19 mai 1987 : J. ROBERT-BAUDOUY (invitée) : "Approches génétiques et moléculaires des bactéries phytopathogènes : le cas particulier d'*Erwinia chrysanthemi*."

- Colloque de la Société Française de Microbiologie : Expression génétique chez les microorganismes. Institut Pasteur 18 - 19 mai 1987 : COTTE-PATTAT N, CONDEMINE G, REVERCHON S et J. ROBERT-BAUDOUY : "Etude d'un gène régulateur de la pectinolyse chez *Erwinia chrysanthemi*. "

- Colloque de la Société Française de Microbiologie : Expression génétique chez les microorganismes. Institut Pasteur 18 - 19 mai 1987 : JI J, COTTE-PATTAT N, et J. ROBERT-BAUDOUY : "Expression des gènes impliqués dans la sécrétion des pectate-lyases chez *Erwinia chrysanthemi* ."

- INTERNAL REPORTS

- Centre National de la Recherche Scientifique, annual report 1987 UM 38002403

- Association Française pour la Maîtrise de l'Energie, final report 1987

- Ministère de la Recherche et de la Technologie, final report 1987

IV. 3 PATENTS DEPOSITED IN CONNECTION WITH THE RESEARCH PROGRAMME :
no patents

IV. 4 DOCTORATE THESIS (Ph. D)

- THESE de Doctorat es Sciences. Nicole HUGOUVIEUX-COTTE-PATTAT. 1987. "Etude génétique de la régulation de la dégradation de sucres hexuronates par les bactéries *Escherichia coli* et *Erwinia chrysanthemi.*"

- THESE de Doctorat, Guy CONDEMINE. 1987. " Etude de certaines étapes de la dégradation de la pectine chez la bactérie phytopathogène *Erwinia chrysanthemi."*

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	No
Joint meeting(s)	Yes

Descriptive information for the above data.

Exchange of material (s) :

The Lyon group has received Saintpaulia plants from Paris in order to conduct plant pathogenicity tests on mutant *Erwinia* strains. The Lyon group gave various *Erwinia* strains to the english group (M. Perombelon) as well as to the Brussels group (Vangijsegem).

Exchange of staff :

One person from the Lyon group went to Paris to learn Saint Paulia micro plant culture technique at the INA.

Joint Meeting :

At the end of January, all the five laboratories belonging to the BAP contrat n° 0210 had a meeting in Marseille (five oral communications were presented by the Lyon group).

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: University of Warwick

Contract no.: BAP - 0191 - UK

Project leader: G.P.C SALMOND

Scientific staff: J. Sidebottom, Technician Grade 4,
J. Hinton, Postdoctoral Research Assistant
M. Perombelon, Scottish Crop Research Institute
L. Hyman, Scottish Crop Research Institute

Address:
University of Warwick
Dept. of Biological Sciences
UK - COVENTRY, WARWICKSHIRE CV4 7AL

Telephone no.: +44.203.52.35.34

Telex no.: 31406

Other contractual partners in the joint project:

J.M. Robert-Baudouy, I. N. S. A. (Villeurbanne)
A. Toussaint-Pourbaix, U. L. B. (Bruxelles)
A. Kotoujansky, I. N. A. - P. G. (Paris)
J.P. Chambost, C. N. R. S. (Marseille)

Title of the research activity:

Analysis of the pathogenicity genes and mechanism of extracellular enzyme export in Erwiniae. Molecular biology of phytopathogenic Erwiniae.

Key words:

Erwinia, Pathogenicity, Transposons, Secretion, Cloning

Reporting period: March 1987 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The general aims of the joint project are to characterise the molecular bases of plant pathogenicity and extracellular enzyme secretion in Erwinia. In particular we aim to isolate and characterise avirulent Erwinia mutants, using transposons and phage Mu derivatives as mutagens. We will also investigate the structure, synthesis, regulation and export of the major phytopathogenicity determinants - extracellular enzymes. We will also clone the genes of the secretory apparatus and identify their protein products. Pathogenicity genes, other than enzyme structural genes, will also be cloned and used for a study of gene products and regulation.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The specific objectives for this period were to use Tn5 to produce random mutagenesis of Erwinia carotovora subsp. atroseptica (Eca), then screen these Tn5-containing colonies, on micropropogated potato plants, for avirulent mutants. In addition we wanted to isolate bacteriophages and/or bacteriocins effective against Eca and use these to isolate resistant mutants which can subsequently be screened for avirulence or secretion defects. We also intended to physiologically characterise any avirulent mutants and construct an Eca cosmid gene library for the eventual isolation of enzyme structural genes and other pathogenicity determinant genes.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Methodology

We have spent some time on the process of selecting the best strain(s) of Eca for pathogenicity and enzyme secretion studies. Our working definition of a "good" strain is one which is highly virulent in the plant test assays, and is amenable to a range of genetical tools that we currently employ i.e. the strain must be transformable; susceptible to transposon mutagenesis by conjugal mobilisation of suicide plasmids or after λ infection; and should preferably be sensitive to bacteriophages and/or bacteriocins which could be used to screen for mutants with defects in outer membrane components. The latter might prove to be defective in enzyme secretion or host recognition.

Transfer of pHCP2-to Eca. The plasmid pHCP2 carries the lamB gene of E. coli and was mobilised from E. coli to Erwinia, via helper plasmids, in simple patch matings using minimal agar plus ampicillin and raffinose (0.2%) for counterselection (1).

λ infection and transposition. Infection with λ::Tn5 (λ467) was as described previously (1) and kanamycin resistant transductants were selected on Oxoid nutrient agar plus kanamycin (50 μg/ml) after suitable

expression time (1).

Characterisation of auxotrophs, and reversion analysis. Tn5-containing colonies which grew on nutrient agar but failed to grow on minimal agar were putative auxotrophs. Auxotrophic requirements were determined by the pool plate assay method. Reversion analysis was done as previously described (1).

Transformation of Eca. Transformation via the previously described method (1) was effective, and produced 5 x 10^2 colonies per μg plasmid (pBR322) DNA.

Plant tests. Individual, small (approx. 30 cm high) potato plants, produced by micropropagation, were stem inoculated with individual bacterial colonies (1750) using sterile toothpicks. Infected plants were maintained at 20°C in a mist chamber to retain humidity. Avirulent mutants were identified as those which caused a reduced rot, or no systemic rot, after 3 days. The pathogenicity was scored from + (localised necrosis at site of inoculation) to ++++ (Total systemic rot and plant collapse).

Enzyme assays. Assay of pectate lyase, polygalacturonase, cellulase and protease were as described (2).

Construction of Eca genome clone bank. An EcoRI fragment gene bank of Eca1043 was made in the cosmid pHC79 essentially according to manufacturers instructions.

Cloning of Eca DNA flanking a Tn5 insertion. Total chromosomal DNA was isolated and digested with EcoRI before cloning into the EcoRI site of pBR322. Recombinant plasmid-containing transformants of E. coli were selected on nutrient agar plus kanamycin (50 μg/ml).

Screening for temperate phages and bacteriocins. Log-phase cultures of 80 strains of several Erwinia species were treated with mitomycin C (1 μg/ml) overnight before harvesting the supernatant. Supernatants were $CHCl_3$-sterilized and used in spot test assays against strain Eca 1043.

Enrichment of virulent phages. Sewage samples (raw sewage, activated sludge and final effluent) were added to log phase cultures of Eca 1043 and incubated at 25° overnight. Culture supernatants were removed and $CHCl_3$-sterilized before titration on Eca1043.

2. Results

Selection of the most appropriate Eca strain. Out of 50 Eca strains tested Eca 1043 gave the best and most reproducible results. It produced a ++++ score in virulence tests; was sensitive to conjugal mobilisation of pHCP2 - becoming λ-sensitive; was transformable; and was a good recipient for λ-based transposition (20 Kn^R colonies/10^7 pfu λ::Tn5).

Isolation of Tn5-containing mutants. Auxotrophs (five) of Eca 1043 (pHCP2) infected with λ::Tn5 arose at a frequency of 0.3%. The auxotrophic requirements were ura, arg, cysB, trp and an unknown. Reversion analysis showed that most were due to single Tn5 insertions.

Screening for, and characterisation of, avirulent mutants. Of 1750 Tn5-containing colonies screened five gave a reduced virulence phenotype in the stem inoculation assay. These mutants were tested for growth on 17 different sugar substrates and were as wild-type. Of the five, three were auxotrophic (arg, cysB and trp), one had a slightly reduced growth rate compared with wild-type and one (mutant 5) was phenotypically wild type for all substrate utilisation parameters, except plant stem attack. When the "avirulent" mutants were analysed for extracellular enzyme synthesis they were as wild-type except mutant 1 which had a reduced growth rate which affected enzyme production. All five mutants, except mutant 1, could multiply in potato tubers and cause a rot although they were avirulent on potato stems.

Search for temperate phages and bacteriocins. Of the 80 strain supernatants tested 21 produced an effect (either putative pin-point plaques or turbid lysis/reduced growth) on Eca 1043. This may be evidence for the presence of phage and/or bacteriocins plating on Eca 1043.

Enrichment for phage from natural environment. To date, 3 separate enrichments have been made, each one successful, producing 4 phages in all. An attempt has been made, with 2 phages, to test for generalized transduction, without success. The first phage produces large clear plaques with haloes at 25°C, but cannot form plaques at 30°. The reason for this temperature sensitivity is unknown but is probably due to a block in replication rather than adsorption. These phages are currently being characterised and used for the isolation of phage resistant mutants of Eca 1043.

Probing the chromosomes of mutants 1 and 5. Using a nick translated fragment of Tn5 as a probe it was shown that mutant 1 had two Tn5 insertions but mutant 5 had a single insertion. Using EcoRI digestion the Tn5, plus flanking sequences, were cloned into pBR322 for restriction mapping. This showed the Tn5 had inserted into a 6.5 Kb EcoRI fragment.

Discussion

We now have a strain of Eca (1043) which is highly virulent and genetically amenable. λ-based transposition is functional in this strain and generates random insertions (Tn5). Of the avirulent mutants isolated in the plant stem assay one, mutant 5, was like wild-type in all characteristics except virulence. The single Tn5 insertion has now been cloned and is currently being used to probe a wild-type bank for isolation of the wild-type gene. More plant tests are underway.

The phages we have isolated for Eca 1043 are being characterized and are being used to make resistant mutants which will be tested for avirulence or secretion defects.

References

1. Salmond, G. P. C. et al., Mol. Gen. Genet. 203, 524-528.
2. Hinton, J. C. D. and Salmond, G. P. C. Molec. Microbiol. (In press).

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

2. Short Communications, Internal Reports

a) Hinton, J. C. D., Perombelon, M. C. M. and Salmond, G. P. C.
"Pathogenicity of Erwinia carotovora subsp. atroseptica".

b) Hinton, J. C. D. and Salmond, G. P. C.
"Isolation of extracellular enzyme mutants of Erwinia using TnphoA" presented at the CEC/BAP "Erwinia Phytopathogenicity Group" meeting in Marseille (organised by Dr. J-P. Chambost).

c) Hinton, J. C. D., Gibson, M., Perombelon, M. C. M. and Salmond, G. P. C.
"Analysis of export and pathogenicity genes of Erwinia".
Poster presentation at the CEC/BAP sectoral meeting in Louvain-la-Nauve (see Abstract book) March (1987)

d) Salmond, G. P. C., Kotoujansky, A., Chambost, J-P., Toussaint, A. and Robert-Baudouy, J.
"Erwinia as a model for pathogenicity and protein secretion".
Oral presentation at the CEC/BAP sectoral meeting in Louvain-La-Neuve, March (1987).

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)		No
Exchange of staff	Yes	
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

We attended a research progress meeting of the CEC/BAP "Erwinia phytopathogenicity group", held in Marseille at the end of January (organiser - Dr. Jean-Pierre Chambost) and presented 2 papers as described in section IV.2. At the meeting, attended by representatives of all of the 5 collaborting centres, we discussed research progress, collaborative programmes and the direction of future research.

In addition, we had less formal discussions at the BAP meeting in Louvain-la-Neuve (March 23-26, 1987) and made contact with other BAP contractors for the exchange of ideas and information.

Dr. Martine Boccara (Laboratoire de Pathologie Vegetale, Institute National Agronomique, Paris) visited our Department at Warwick (13th and 14th July) and delivered a research seminar on "Molecular Genetics of Pathogenicity of Erwinia chrysanthemi". We had discussions on research strategies and the future exchange of relevant biological materials and methods, when appropriate.

We were also visited on 30th June by Mr. D. Lalo (a temporary worker who has also worked in the Paris Lab) who, under the supervision of Dr. Graham Plastow (Dalgety U.K. Ltd.), has been analysing the polygalaturonase gene of Erwinia carotovora subsp. carotovora strain SCRI193 - the one we use to study enzyme secretion mechanisms.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: U.L.B., Brussels
Contract no.: BAP - 0190 - B

Project leader: A. TOUSSAINT-POURBAIX
Scientific staff: F. van Gijsegem-Guilmot, M. Piecq-Said

Address: Université Libre de Bruxelles
Laboratoire de Génétique
67, rue des Chevaux
B - 1640 RHODE-ST-GENESE

Telephone no.: +32.2.3583530 EXT.240

Telex no.:

Other contractual partners in the joint project:

J.M. Robert-Baudouy, I. N. S. A. (Villeurbanne)
G. P. C. Salmond, University of Warwick (Coventry)
A. Kotoujansky, I. N. A. - P. G. (Paris)
J.P. Chambost, C. N. R. S. (Marseille)

Title of the research activity:

Analysis of the pathogenicity genes and mechanism of extracellular enzyme export in Erwiniae. Molecular biology of phytopathogenic Erwiniae.

Key words:

Pectinase, Cellulase, Secretion, Plant-bacteria-interactions, Phytopathogenicity

Reporting period: February 1987 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Soft rot Erwiniae are significant pathogens causing soft rot of stored crops and of plants in vegetation such as potato and Saintpaulia. One major trait in Erwinia pathogenicity is the production and secretion of several depolymerizing enzymes (pectinases, cellulases, proteases) which can digest the plant cell wall. However, other factors are certainly involved in pathogenicity. The aim of this project is, on one hand, to study the regulation and export of the depolymerizing proteins ; on the other hand, to identify and characterize some of the other factors which are involved in the plant-bacteria interactions and lead to a successful infection.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

During these five months, we continued the study of the pectinase and cellulase genes of Ech strain B374. Ech strains produce 5 pectinase(PL) and 2 cellulase isoenzymes. The genes coding for these different enzymes (pelA to E, celY and celZ) have been cloned in E. coli, some of them have been mutagenized and the mutated alleles have been reintroduced in the Ech chromosome. We currently analyze the chromosomal organization of the pel and cel genes on the B374 chromosome and we have undertook the determination of the MW and the processing of the individual pectate lyases.

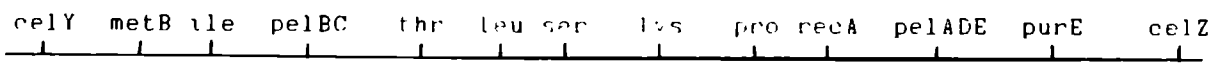
III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Methodology

B374 pel genes , mutagenized by insertion of Mud(lac,Km) derivatives (1,FVG, in prep), have been mapped by chromosome transfer mediated by the RP4mini-Mu. Mapping was also achieved by in vivo cloning in E. coli of different fragments of Ech chromosomal DNA and determination of the frequencies of cotransposition between the different markers tested. Physical mapping of the pel and cel genes in B374 was also analyzed by Southern hybridization, using different cloned pel and cel genes to probe total chromosomal DNA.
On the other hand, the proteins encoded by the different pel genes were characterized using SDS-PAGE. We compared the proteins produced either in the supernatant of different pel mutants of Ech B374 or in the supernatant of E. coli periplasmic leaky mutants (which release periplasmic proteins in the external medium) harboring pel genes cloned on pBR322 plasmids.

2 Results and Discussion

The results of the genetic mapping analysis are presented in Figure 1.



The pel and cel genes are located in two regions of the B374 chromosome ; as already reported, the pelBC genes and the celY gene map near the ile marker(2, Schoonejans, in prep). We now found that the pelADE cluster maps near the pro locus. Another cel gene, identified as celZ by its homology with Ech 3937 celZ gene by DNA hybridization, is also weakly linked to the pro marker.
Hybridization of different pel probes (pelB, pelC, pelD, part of pelE) with either total chromosomal DNA or pBR322 derivatives carrying different sets of pel genes has revealed a relatively strong homology between the pelB and pelC genes and a weaker homology between the pelD and pelE genes. This is in agreement with the immunological cross-reaction found between the PLb and PLc proteins on one hand, between the PLd and PLe proteins on the other hand (C. Ballas, pers comm.). Homology between the pelB and pelC genes has also been demonstrated at the DNA level in another Ech strain (3). These results open the possibility that pelB and C or pelD and E have evolved by duplication of two ancestral genes. Besides these duplications, there is no evidence of the existence of additional copies of homologous pel genes.
We have analyzed the protein content of supernatants of different B374 pel mutants of B374. We had shown earlier that growth of B374 in the presence of polygalacturonate (=demethylated pectin) induces the synthesis and secretion of 4 (or 5) polypeptides of 45, 42, 40, 38 (and 28) kDa (4). Analysis of the supernatants of pel mutants has now shown that the 45kDa protein is missing in pelE mutant, the 42kDa in pelD mutants and the 40kDa in pelC mutants. By comparing the supernatants of the pel mutants with those of E. coli periplasmic leaky mutants harboring appropriate pBR322-pel plasmids, we found that the molecular masses of the polypeptides present in the supernatant of B374 cultures are the same as those found in E. coli supernatants This suggests that, in B374, secretion of PL proteins from the periplasm in the external medium is not accompanied by processing of the protein.

1) Reverchon, S., van Gijsegem, F., Rouve, M., Kotoujansky, A. & Robert-Baudouy, J. (1986) Organization of a pectate lyase gene family in Erwinia chrysanthemi. Gene, 49, 215-224.

2) van Gijsegem, F., Toussaint, A. & Schoonejans, E. (1985) In vivo cloning of the pectate lyase and cellulase genes of Erwinia chrysanthemi EMBO J., 4, 787-792.

3) Schoedel, C. & Collmer A. (1986) Evidence of homology between the pectate lyase-encoding pelB and pelC genes in Erwinia chrysanthemi. J. Bacteriol., 167, 117-123.

4) van Gijsegem F. (1986) Analysis of the proteins secreted by three Erwinia chrysanthemi strains: B374, 3937.1 and 3665. J. Gen. Microbiol., 132, 617-624

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1

1) Breton A.M., Younes G., van Gijsegem F. and Guespin-Michel J. (1986) Expression in Mixococcus xanthus of foreign genes coding for secreted pectate lyases of Erwinia chrysanthemi. J. Biotechnol., 4, 303-311

2) Reverchon S., van Gijsegem F., Rouve M., Kotoujansky A. and Robert-Baudouy J. (1986) Organization of a pectate lyase gene family in Erwinia chrysanthemi. Gene, 49, 215-224

3) Schoonejans E., M. Faelen, L. Desmet and A. Toussaint (1987) Amber suppressors of Erwinia chrysanthemi. Ann. Inst. Pasteur/Microbiol. 138, 289-296

4) Van Gijsegem F., Toussaint. A. and Casadaban, M. (1987) Mu as a genetic tool. in The bacteriophage Mu. Symonds N., Toussaint A. and van de Putte P. ed. Cold Spring Harbor Laboratory, in press

TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff	Yes	
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

Exchange of material

We received :

- the plasmids pV008 and pMH7 which carry the celY and celZ 3937 genes respectively (from A. Guiseppi, CNRS, Marseille)
- Ech B374 chromosomal mutants (from INSA, Lyon)
- axenic Saintpaulia plants (from INA, Paris)

Exchange of staff

During a short stay in Paris laboratory, FVG learned the techniques of in vitro propagation of Saintpaulia plants.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: I.N.R.A., Paris Contract no.: BAP - 0212 - F

Project leader: A. KOTOUJANSKY
Scientific staff: Y. Bertheau, M. Boccara, A. Diolez,
D. Expert, E. Schoonejans, S. Bel, C. Enard

Address:
Laboratoire de Pathologie Végétale
I.N.A. - P.G.
16, rue Claude-Bernard
F - 75231 PARIS CEDEX 05

Telephone no.: +33.1.43371550 EXT.354

Telex no.:

Other contractual partners in the joint project:

J.M. Robert-Baudouy, I. N. S. A. (Villeurbanne)
G.P.C. Salmond, University of Warwick (Coventry)
A. Toussaint-Pourbaix, U. L. B. (Bruxelles)
J.P. Chambost, C. N. R. S. (Marseille)

Title of the research activity:

Analysis of the pathogenicity genes and mechanism of extracellular enzyme export in Erwiniae. Molecular biology of phytopathogenic Erwiniae.

Key words:
Erwinia chrysanthemi, Phytopathogenicity, Pectinase, Protein secretion, Molecular biology

Reporting period: April 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The project is aimed at an understanding of the pathogenicity of soft-rot *erwiniae* , at the molecular level (for review, see ref. 5). The laboratories of the "Erwinia group" are studying genes and gene products involved in the interaction of the bacterium with the plant. The potential applications of these studies are related to biological control of diseases caused by *E. chrysanthemi*, biomass valorization, and secretion of foreign proteins by a Gram-negative bacterium.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Whithin the general framework described above, the following advances were expected for the reporting period:

- construction of mutants to assess the role of the different pectinases in the virulence of *E. chrysanthemi* .
- purification and characterization of some pectate lyases .
- identification of the genes controlling the secretion of pectinases and cellulases.
- isolation of *E. chrysanthemi* mutants deficient for iron uptake, and isolation of the corresponding genes. Identification of the bacterial siderophores.
- selection of mutants to assess the role of the lipopolysaccharide in the virulence of *E. chrysanthemi* .

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Since the writing of the proposal, the following progresses have been achieved:

1. STUDY OF THE PECTINASE GENES AND THEIR PRODUCTS

a. Construction of Pel$^-$ mutants

In order to determine the role played by the different pectinases in the virulence of *E. chrysanthemi*, several *pel* mutants were constructed. The adopted strategy comprised two steps:

step 1: mutagenesis of individually cloned *pel* genes in *E. coli* (3, 6, 7). This was achieved *in vivo* by transposon insertion, or *in vitro* by insertion of a DNA fragment carrying a resistance gene within the target gene.

step 2: introduction of the mutated *pel* gene into the *Erwinia* chromosome by homologous recombination. By that way, mutants in all the five *pel* genes (*pelA* to *pelE*) were constructed (1, 3; see figure 1).

Recently, two double mutants, PelD$^-$ PelE$^-$ and PelB$^-$ PelC$^-$, have been obtained. Moreover, the construction of a mutant lacking the pectin methylesterase is in progress. Pathogenicity tests, performed on Saintpaulia ionantha plants and miniplants, indicated that the PelB$^-$, PelC$^-$, and PelB$^-$ PelC$^-$ mutants remained virulent, whereas all the others, PelA$^-$, PelD$^-$, PelE$^-$, PelD$^-$ PelE$^-$, were avirulent (1). Therefore, it seems that only the products of *pelA*, *pelD*, and *pelE* are compulsory for *E. chrysanthemi* virulence.

b. Characterization of the pectinases

Monoclonal antibodies raised against different PLs were obtained. Their efficiency for PL purification is under study. These antibodies have already helped to prove that *E. chrysanthemi* PLs are composed of at least two different families (9, 13). Indeed, no cross-

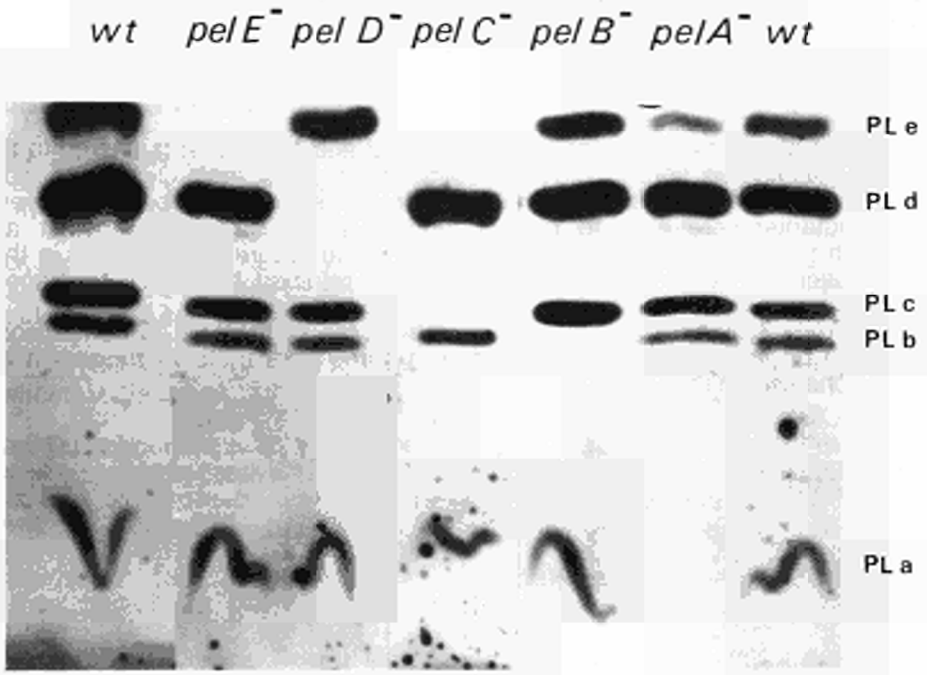


Figure 1. Electrofocusing of the pectate lyase isozymes produced by *E. chrysanthemi* wild-type and mutant strains. Presence of the isozymes was detected by activity staining. In the PelC⁻ strain, activity of PLe was low.

reaction was observed between the basic isozymes PLd and PLe, and antibodies raised against the neutral isozymes PLb and PLc, and conversely. Moreover, these monoclonal antibodies were used to trace the neutral isozymes PLb and PLc in infected tissues of Saintpaulia, by immunogold staining (12).

The comparison of the pectinases produced by different species, subspecies and pathovars of soft-rot Erwinias has been achieved by electrofocusing and cross-reaction with monoclonal and polyclonal anti-pectinase antibodies (11). This revealed a good match between the taxonomy and the pattern of pectinases, which suggests that the set of pectinases produced by a given strain of *Erwinia* is adapted to the plant host. Surprisingly, no cross-reaction was observed between antibodies raised against *E. chrysanthemi* pectate lyases (PL), and E. carotovora PLs.

The purification of PLa was achieved by chromatofocusing, followed by affinity chromatography (10). This enzyme has a molecular weigth of 36.000, an isoelectric point of 4.6, and a pH optimum of 8.5-9.0.

2. STUDY OF THE GENES GOVERNING THE SECRETION OF THE PECTINASES AND CELLULASES (*out* GENES).

Mutations were obtained in the *out* genes by insertion of a derivative of the bacteriophage Mu: MudII1734. Several of these mutations were subsequently cloned, and used as probes to detect the wild-type genes in a cosmid library. At least three different *out* genes (may be four) have already been identified and cloned. The location of these genes on the *E. chrysanthemi* chromosome has been determined, and their subcloning is underway.

Transcriptional fusions between the 5' part of one *out* gene, and the 3' terminal part of the *lacZ* gene have been constructed and should allow to study the regulation of that *out* gene.

3. OTHER BACTERIAL STRUCTURES AND FUNCTIONS INVOLVED IN THE INTERACTION WITH THE PLANT.

a. Iron assimilation

E. chrysanthemi produces siderophores, one of which being a phenolate . Many mutants, either spontaneous or induced by phage Mu derivatives, have been obtained (4). They are impaired in different steps of iron assimilation. All these mutants exhibited a markedly reduced aggressivness. By invivo cloning with the pULB113 vector, a 44 kb DNA fragment has been isolated, that complemented all the mutants for iron assimilation and

aggressivness. This cluster of genes seems to be located near *his* on the *Erwinia* chromosome. Subcloning of the genes and analysis of the system is underway.

Together, these data suggest that a competition for iron assimilation takes place between the pathogen and the infected plant.

b. Role of the lipopolysaccharide (LPS)

By selecting resistance to the phages PhiEC2 and Mu, mutants altered in their LPS were obtained (4, 8; see figure 2). The mutants having lost their O-antigen side chain remained virulent, whereas those also affected in the core were avirulent. Moreover, two of these core mutants were able to protect whole Saintpaulia plants against a further inoculation with the wild-type strain (8). The biochemical nature of the defect in these two mutants has been studied. All the avirulent mutants retained the ability to macerate cut leaves.

These data suggest that (i) integrity of the core of the LPS is compulsory for *E. chrysanthemi* to be virulent, and (ii) infection with some core mutants triggers a systemic resistance mechanism in the Sainpaulia.

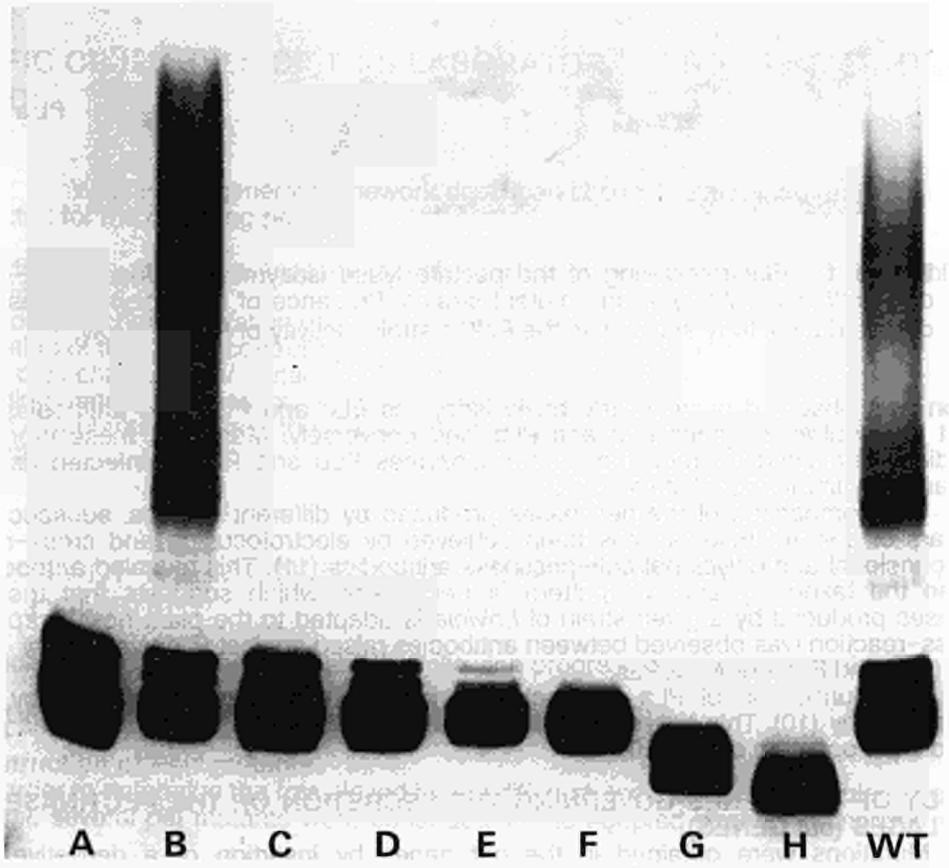


Figure 2. Silver-stained SDS-PAGE analysis of LPS obtained from *E. chrysanthemi* 3937 PhiEC2-resistant mutants. Migration was from top to bottom. The different phenotypes were: **B**: the only mutant with no visible change in LPS pattern (virulent); **A**, **C**, **D** and **E**: mutants lacking only the putative O-antigen (virulent); **F**: mutant lacking the O-antigen and slightly altered in the core structure (avirulent); **G** and **H**: mutants lacking the O-antigen and deeply affected in the core structure (avirulent and able to induce a systemic resistance in the plant); **WT**: wild-type.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1 PUBLICATIONS IN SCIENTIFIC JOURNALS

1. Boccara, M., Diolez, A., Rouve, M., Kotoujansky, A. Role of individual pectate lyase of *Erwinia chrysanthemi* strain 3937 in pathogenicity on Saintpaulia plants. *Physiol. Mol. Plant Pathol.* In press

2. Boyer, M.-H., Cami, B., Kotoujansky, A., Chambost, J.-P., Frixon, C., Cattaneo, J. 1987. Homology between *cel* genes of two strains of *Erwinia chrysanthemi* 3665 and 3937. Cloning of *celZ* and *celY* genes. *FEMS Microbiol. Lett.* In press

3. Diolez, A., Richaud, F., Coleno, A. 1986. Pectate lyase gene regulatory mutants of *Erwinia chrysanthemi*. *J. Bacteriol.* **167**:400-03

4. Expert, D., Schoonejans, E., Toussaint, A. 1986. Cell surface mutant of *Erwinia chrysanthemi*: possible involvement of iron acquisition in phytopathogenicity. In *Series A: Life Sciences. Iron, Siderophores, and Plant Diseases,* ed.T. R. Swinburne, **117**:261-71. Nato Asi Ser. New York: Plenum

5. Kotoujansky, A. 1987. Molecular genetics of pathogenesis by soft-rot erwinias. *Ann. Rev. Phytopathol.* **25**:405-30

6. Kotoujansky, A., Diolez, A., Rouve, M., Van Gijsegem, F., Reverchon, S., *et al.* 1987. Molecular cloning and mutagenesis in *Escherichia coli* of pectinase genes from *Erwinia chrysanthemi*. *Proc. 6th Int. Conf. Plant Pathogen. Bact., College Park, Maryland, 1985* , pp. 139-51. Dordrecht: Nijhoff/Junk

7. Reverchon, S., Van Gijsegem, F., Rouve, M., Kotoujansky, A., Robert-Baudouy, J. 1986. Organisation of a pectate lyase gene family in *Erwinia chrysanthemi*. *Gene,* **49**:215-24

8. Schoonejans, E., Expert, D., Toussaint, A. 1987. Characterization and virulence properties of *Erwinia chrysanthemi* lipolysaccharide-defective, PhiEC2-resistant mutants. *J. Bacteriol.* In press

9. Vergnet-Ballas, C., Bertheau, Y., Grosclaude, J. 1986. Production and potential uses of monoclonal antibodies to pectate lyases of *Erwinia chrysanthemi*. *Symbiosis* **2**:367-72

IV. 4. DOCTORATE THESIS AND DEGREE THESIS AWARDED DURING THE PERIOD OF CONTRACT:

9. Bel, S. 1987. Purification et analyses biochimiques de la pectate lyase acide d'*Erwinia chrysanthemi*. *Diplôme d'Etudes Approfondies,* Université Paris XI, Orsay. 32 pp.

10. Bouclet, V. 1987. Etude comparative des pectinases produites par différentes souches d'*Erwinia*. *Diplôme Universitaire de Technologie,* Institut Universitaire de Technologie, Créteil. 27 pp.

11. Manzanares, A. 1987. Essais en vue de la detection immunologique des pectate lyases dans des feuilles de Saintpaulia inoculées par *Erwinia chrysanthemi*. *Diplôme Universitaire de Technologie,* Institut Universitaire de Technologie, Créteil. 32 pp.

12. Vergnet-Ballas, C. 1986. Etude à l'aide d'anticorps monoclonaux des pectate lyases sécrétées par une entérobatérie phytopathogène: *Erwinia chrysanthemi*. *Thesis,* Institut National Agronomique Paris-Grignon, Paris. 94 pp.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

The collaboration between four of the five laboratories of the "Erwinia group" was established in 1981. This collaboration was extended to the british team (see list below) in 1985. Since then, many contacts have been organised, involving exchange of staff and material, as well as joint experiments and meetings. During the reporting period, the collaboration was materialized by:

with the C.N.R.S. in Marseille:

- use of anti-cellulases antisera
- test of virulence of Cel$^-$ mutants of *E. chrysanthemi*
- Dr. E. Schoonejans's stay in Marseille, then in Paris
- Dr. D. Expert and C. Enard's visit for doing P1 transductional mapping of genes controlling iron uptake

with the I.N.S.A. in Villeurbanne:

- sending to Lyon of an intermediate of the catabolic pathway of pectin
- teaching of techniques: electrofocusing of pectinases, pectin methylesterase assay, cultivation and inoculation of mini-Saintpaulia plants

with the University of Warwick:

- receipt of plasmids and techniques for transposon mutagenesis with phage Lambda vectors
- Dr. M. Boccara's visit to Warwick

with the U.L.B., in Rhode-Saint:-Genese:

- a joint experiment to detect bacterial genes induced *in planta* is underway

In addition, **joint meetings** were organised:

- April 1986 in Cork
- April 1986 in Paris
- January 1987 in Marseille
- March 1987 in Louvain-la-Neuve

List of the laboratories of the "Erwinia group", from North to South:

Dr. G. P. C. Salmond, Department of Genetics, University of Warwick, Coventry (U.K.)

Dr. A. Toussaint, Laboratoire de Génétique, U.L.B., Rhode-Saint-Genèse (Belgium)

Dr. A. Kotoujansky, Laboratoire de Pathologie Végétale, I.N.R.A., Paris (France)

Dr. J. Robert-Baudouy, Laboratoire de Microbiologie, I.N.S.A., Villeurbanne (France)

Dr. J. P. Chambost, Laboratoire de Chimie Bacterienne, C.N.R.S., Marseille (France)

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: C.N.R.S., Marseille

Contract no.: BAP - 0211 - F

Project leader: J-P. CHAMBOST

Scientific staff:

Address:
Laboratoire de Chimie Bactérienne / C.N.R.S.
31, Chemin Joseph Aiguier
B.P. 71
F - 13277 MARSEILLE CEDEX 9

Telephone no.: +33.91224000 EXT.4579

Telex no.: 430225 CNRSMAR F

Other contractual partners in the joint project:

J.M. Robert-Baudouy, I. N. S. A. (Villeurbanne)
G.P.C. Salmond, University of Warwick (Coventry)
A. Toussaint-Pourbaix, U. L. B. (Bruxelles)
A. Kotoujansky, I. N. A. - P. G. (Paris)

Title of the research activity:

Analysis of the pathogenicity genes and mechanism of extracellular enzyme export in Erwiniae. Molecular biology of phytopathogenic Erwiniae.

Key words:

Erwinia, Cellulase, Cloning, Sequencing, Enzyme export

Reporting period: January 1987 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT :

The project aim is to use biochemical, genetic and molecular biological approaches to investigate the mechanism of plant desease caused by Erwinia bacteria.

The genes responsible for each stage of interaction between the plant host and the bacterial pathogen are investigated.

Because the extracellular hydrolytic enzymes are major pathogenicity factors their synthesis, regulation and export of these enzymes are studied.

In addition to agricultural implications these investigations will have industrial applications including genetic manipulation of Erwinia strains already used for commercial productions and construction of secretion vectors for foreign polypeptide production and transfer of hydrolytic enzyme genes to or from other industrial microbes.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD :

The group's work is devoted to hydrolysis and use of plant cell wall cellulose in the pathogenic process caused by Erwinia chrysanthemi.

For the reporting period the investigations were devoted to the ennumeration of structural and regulation genes involved in :

Cellulose hydrolysis and the analysis of this genes by mutational insertions and sequencing.

The study of structural and regulation genes linked to cellobiose and other natural β-glucosides metabolism in the same approaches as above.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTACT :

The Erwinia celY and celZ genes encoding endoglucanases Y and Z respectively have been isolated.

Cloned in E. coli both genes have been mutated by mini-Mu phage transposition. In some of the celZ::MudII1734(Cm^r,lac) and celY::PR13(Cm^r,lac) fusions the lac genes were under the control of the cel promoters. Mu-mediated transduction was used to introduce the mutated cel genes into E. chrysanthemi; markers exchange by homologous recombinations gave chromosomal mutants which were proved to be $celZ^-$ and $celY^-$, respectively.

The cel genes were found to be far apart on the chromosome when mapped by RP4' formation using RP4::Mu3A conjugative plasmid. Due to the presence of the A gene of Mu, segregation of the cel genes and of the resistance markers was observed. An alternative method was then developped : RP4::mini-Mu conjugative plasmids were constructed ; by homologous recombination between the chromosomal and plasmidic Mu genomes, Hfr-like structures were formed, whose origin of transfer is at the site of the mutated gene ; celZ was accurately mapped between ura and pan and celY between xyl and met.

The regulation of the cel genes was studed with cel::lac fusions: for celZ, the activity varies with the growth phase and is maximum during the stationnary phase while for celY, the very low value of β-galactosidase activity observed indicated a very poor expression of the gene and not a high instability of the protein itself. Study of secondary mutations leading to a better expression of the celY::lac fusion revealed a complex control.

The celZ gene of Erwinia chrysanthemi has been sequenced and an open reading frame encoding a 428 amino acids protein was found. Since sequence from residue 44 to 63 of the translated sequence perfectly matches the first 20 amino acids of the purified endoglucanase Z, it was infered that the upstream 43 amino acids represent a signal peptide. A possible ribosome binding site (SD) sequence was found 7 bp upstream of the ATG initiator codon. In the 3' untranslated region, GC rich inverted repeats are present which may function as rho-dependent transcription termination signal. Examination of codon usage in the sequence indicated that rare codons are used to the extent of 14 % in the mature protein and 23 % in the signal sequence. Codon utilization is typical of weakly expressed genes of E. coli.

By comparing the protein sequence of endoglucanase Z with those of endoglucanases of Clostridium thermocellum, Cellulomonas fimi; Bacillus subtilis and alkalophilic Bacillus, significant homology was found only with endoglucanases of B. subtilis and alkalophilic Bacillus. In a highly conserved region of 118 amino acids located in the proximal half from the NH_2 terminus 70 % of homology is found. This region may be involved in substrate recognition or may be part of the catalytic site.

The breakdown of cellulose by bacteria leads to the production of cellobiose which can be used for growth. It has been demonstrated that cellobiose pathway in Erwinia works through a phosphate group translocation from PEP to sugar (PTS).

For bacterium which already possesses the general PTS enzymes, two proteins at least are required for β-glucosides utilization : one is a permease specific for the substrate while the other hydrolases the phosphorylated sugar. In E. chrysanthemi two different systems have been demonstrated : one inducible by cellobiose and the second by arbutin.

The clb genes of E. chrysanthemi had been previously cloned on a plasmid in E. coli. In vivo phage mini-Mu transpositions on the plasmid were performed. Accounting for their localization and physiological results, we have demonstrated that three genes, at least, are involved in the metabolism of cellobiose in E. chrysanthemi. Biochemical characterization provided evidences that one of them encodes the cellobiose permease. Some insertions have led to active hybride β-galactosidase proteins. After introduction by markers exchange into the E. chrysanthemi chromosome, some of these fusions are used to study the regulation of the clb genes.

In a genomic library constructed for this purpose, gene(s) allowing the utilization of arbutin and salicin have been isolated. Since they do not support growth on cellobiose, they are supposed to be different from the clb genes already mentionned.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

Publications in scientific journals :

- AYMERIC, J.L., PASCAL, M.C., GUISEPPI, A., CHIPPAUX, M.
Mapping and regulation of the cel genes in E. chrysanthemi.
Submitted to Mol. Gen. Genet.

- GUISEPPI, A., CAMI, B., AYMERIC, J.L., BALL, G., CREUZET, N.
Homology between endoglucanase Z of E. chrysanthemi and endoglucanases of Bacillus subtilis and Alkalophilic Bacillus.
Submitted to Mol. Microbiol.

Communications to meetings :

Genetic and Cellular Engineering of Plants and Microorganisms Importants for Agriculture. Louvain-la-Neuve, March 1987 :

- AYMERIC, J.L., CHIPPAUX, M., CHAMBOST, J.P.
Genetic Study of the cel and cbl genes in E. chrysanthemi.
- GUISEPPI, A., CAMI, B., BALL, G., CREUZET, N.
Nucleotide sequence of celZ, a gene encoding an endoglucanase from E. chrysanthemi.

Degree thesis :

- EL HASSOUNI, M., Diplôme d'Etudes Approfondies, Université d'Aix-Marseille II, 1987.
Construction d'une banque génomique d'E. chrysanthemi (souche 3665).

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS :

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)	Yes	
Joint meeting(s)	Yes	

Descriptive information for the above data.

Actually the work developped with this contract is the continuation of a former work based on close collaboration between 4 europeen groups. The collaboration has been extended to the U.K. group.

Exchange of materials as well as joint experiments have lead to joint publications. The last joint meeting was held in Marseille for 3 days last January with 43 attendants.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: I. N. R. A. Contract no.: BAP - 0079 - F

Project leader: P. BOISTARD
Scientific staff: J. Batut, M.L. Daveran, M. David, D. Kahn

Address:
C.N.R.S./I.N.R.A.
Laboratoire de Biologie Moléculaire des
Relations Plantes-Microorganismes
Chemin de Borde Rouge - B.P. 27
F - 31326 CASTANET-TOLOSAN

Telephone no.: +33.61.28.50.46

Telex no.: 520009 INRATSE F

Other contractual partners in the joint project:

P.R. Hirsch, Rothamsted Experimental Station, Harpenden
M. O'Connell, School of Biological Sciences (Dublin)
A. Pühler, Universität Bielefeld

Title of the research activity:

Comparison of late Sym genes in Rhizobium species and construction of improved strains.

Key words:

Rhizobium meliloti, fix genes, Regulation

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The project aims to a better understanding of Rhizobium genes controlling the functioning of the nitrogen fixing symbiosis between Rhizobium and legumes. This will allow to improve the efficiency of symbiosis by genetic engineering of the bacterial partner. The project involves three species important for European crops, R. meliloti (alfalfa), R. leguminosarum (pea) and R. phaseoli (bean and broad bean).

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The laboratory has focussed on nitrogen fixation (fix) genes from R. meliloti. Three lines have been followed: (i) genetic investigations on a fix cluster (1) described in the previous report (1986) in Magnien (ed.),Biomolecular Engineering in the European Community, pp701-714, Nijhoff, Dordrecht, for the C.E.C.; (ii) studies of the regulation of nif and fix genes in R. meliloti; (iii) sequence analysis of some fix genes as an attempt to elucidate their functions.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY.

Expression of fix genes from R. meliloti was monitored using DNA-RNA hybridization experiments and the measure of beta-galactosidase activity from fix-lacZ gene fusions. DNA sequencing was performed using shotgun-cloning into M13 derivatives followed by standard enzymatic sequencing using dideoxynucleotides. Sequence analysis was performed on the BISANCE package at CITI2 in Paris.

2. RESULTS AND DISCUSSION.

2.1. A fix gene cluster with a functional duplication.

Mutagenesis of the fix cluster indicated that two fix regions were separated by a 5kb spacer in which transposon or deletion mutagenesis produced a wild-type symbiotic phenotype (2). This was surprising considering the high transcriptional activity of this spacer region during symbiosis (3). This paradox was rationalized when this region appeared to be a functional duplication of fix genes on the pSym plasmid (4). The cluster encompasses 12.5 kb and consists of two single-copy fix operons bracketing a duplicated fix region.

2.2. Positive regulation of fix genes.
Symbiotic expression of the duplicated fix genes was independent of the nifA gene product (3). We therefore looked for potential regulatory genes within the fix cluster and found the expression of the duplicated fix genes to be dependent on two genes fixJ and fixL.
The sequence of fixJ and fixL strongly suggested that the two genes are organized in an operon fixLJ. The N-terminal domain of FixJ was homologous to the N-terminal domain of the OmpR protein from Escherichia coli, a transcriptional activator of outer membrane protein genes. This domain is conserved in other prokaryotic regulatory proteins, such as NtrC (5). The C-terminal domain of FixL was homologous to the C-terminal domain of the EnvZ protein from E. coli, a membrane protein necessary for the functioning of OmpR. This result suggested that the interaction of EnvZ with OmpR is mediated by their conserved C-terminal and N-terminal domains respectively. To test this hypothesis we compared the C-terminal domains of FixL and EnvZ with another regulatory protein, Klebsiella pneumoniae NtrB, functioning pairwise with NtrC. We found significant homology of the C-terminal domains of FixL, EnvZ and NtrB, which was strong indication of some conserved interaction between the conserved domains of the FixL/FixJ, EnvZ/OmpR and NtrB/NtrC regulatory couples. The same conclusion was reached independently by other authors (6). FixL is quite typical of the EnvZ family in that it clearly contains two transmembrane sequences (7) and is therefore probably inserted across the plasma membrane of bacteroids. We propose that FixL detects signals which modulate the expression of nitrogen fixation genes via the FixL/FixJ interaction.

2.3. Involvement of a cation-pump, FixI, in nitrogen fixation.
Sequence analysis of operon I (2) revealed three open reading frames, corresponding to fixG, fixH and fixI (8). An additional short ORF was very likely coding and was named fixY, which defined the fixGHIY operon. Analysis of the aminoacid sequences of the four gene products for potential transmembrane helices (7) predicted all four proteins to be membrane-bound. FixG contained two cysteine clusters characteristic of iron-sulfur centers from bacterial ferredoxins: CysxxCysxxCysxxxCys. Therefore we suggest FixG is a redox protein. FixI was homologous to the KdpB ATPase, the catalytic subunit of the K^+-pump from Escherichia coli (9). Thus FixI, as KdpB, belongs to the P-type ATPases, which include also eucaryotic cation-pumps such as the Ca^{2+}-ATPase, the Na^+/K^+-ATPase or the plasma-membrane H^+-ATPase (10). The involvement of such a pump in symbiotic nitrogen fixation had not been demonstrated previously and its exact function in the process remains to be established. Because the nucleotide sequence of fixGHIY suggested some translational coupling between the four genes, we propose that the four proteins participate in a transmembrane complex, so that the FixI cation-pump would be coupled to the redox process catalysed by the FixG subunit.

3. REFERENCES.

1. Batut J, Terzaghi B, Ghérardi M, Huguet M, Terzaghi E, Garnerone AM, Boistard P and Huguet T (1985) Mol. Gen. Genet. **199**, 232-239.
2. Batut J, Boistard P, Debellé F, Dénarié J, Ghai J, Huguet T, Infante D, Martinez E, Rosenberg C, Vasse J and Truchet G (1985) in Evans HJ, Bottomley PJ and Newton WE (eds), Nitrogen Fixation Research Progress, pp 109-115, Martinus Nijhoff, Dordrecht.
3. David M, Domergue O, Pognonec P and Kahn D (1987) J. Bacteriol. **169**, 2239-2244.
4. Renalier MH, Batut J, Ghai J, Terzaghi B, Ghérardi M, David M, Garnerone AM, Vasse J, Truchet G, Huguet T and Boistard P (1987) J. Bacteriol. **169**, 2231-2238.
5. Drummond M, Whitty P and Wootton J (1986) EMBO J. **5**, 441-447.
6. Nixon BT, Ronson CW and Ausubel FM (1986) Proc. Natl. Acad. Sci. USA **83**, 7850-7854.
7. Eisenberg D, Schwarz E, Komaromy M and Wall R (1984) J. Mol. Biol. **179**, 125-142.
8. Kahn D, Batut J, Boistard P, Daveran ML, David M, Domergue O, Garnerone AM, Ghai J, Hertig C, Infante D and Renalier MH (1987) in Verma DPS and Brisson N (eds), Molecular Genetics of Plant-Microbe Interactions, pp 258-263, Martinus Nijhoff, Dordrecht.
9. Hesse J, Wieczorek L, Altendorf K, Reicin AS, Dorus E and Epstein W (1987) Proc. Natl. Acad. Sci. USA **81**, 4746-4750.
10. Pedersen PL and Carafoli E (1987) Trends Biochem. Sci. **12**, 146-150.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1 PUBLICATIONS IN SCIENTIFIC JOURNALS, MONOGRAPHS...

1. David M, Domergue O, Pognonec P and Kahn D (1987) J. Bacteriol. 169, 2239-2244.
2. Renalier MH, Batut J, Ghai J, Terzaghi B, Ghérardi M, David M, Garnerone AM, Vasse J, Truchet G, Huguet T and Boistard P (1987) J. Bacteriol. 169, 2231-2238.
3. Kahn D, Batut J, Boistard P, Daveran ML, David M, Domergue O, Garnerone AM, Ghai J, Hertig C, Infante D and Renalier MH (1987) in Verma DPS and Brisson N (eds), Molecular Genetics of Plant-Microbe Interactions, pp 258-263, Martinus Nijhoff, Dordrecht.

IV.4 DOCTORATE THESIS (Ph.D) AND DEGREE THESIS AWARDED DURING THE PERIOD OF CONTRACT

Thèse présentée devant l'Université de Paris-Sud en vue de l'obtention du doctorat de 3ème cycle

Spécialité : microbiologie par Diogenes Infante Herrera

Etude de fonctions symbiotiques codées par le mégaplasmide pSym de Rhizobium meliloti

soutenue le 15 Avril 1987

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)	Yes	
Joint meeting(s)	Yes	

Descriptive information for the above data.

We exchanged DNA probes and sequence data with the groups of A.Pühler and P. Hirsch. We performed joint experiments with P. Hirsch on the conservation of operon I in fast growing Rhizobium. We held a joint meeting with our three partners in the BAP contract in Toulouse on November 24 and 25, 1986.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Rothamsted Experim. Station, Harpenden

Contract no.: BAP - 0100 - UK

Project leader: P.R. HIRSCH

Scientific staff: R. Snellgrove, A. Latham, M. White, D. Gibson

Address:
Rothamsted Experimental Station
Soil Microbiology Dept.
UK - HARPENDEN, HERTS AL5 2JQ

Telephone no.: +44.5827.63133 EXT. 2669

Telex no.: 825726 REXPST G

Other contractual partners in the joint project:

A. Pühler, Universität Bielefeld
M. O'Connell, School of Biological Sciences
P. Boistard, C. N. R. S. - I. N. R. A. (Castanet-Tolosan)

Title of the research activity:

Comparison of late Sym genes in Rhizobium species and construction of improved strains.

Key words:

Rhizobium, Nitrogen fixation, Symbiotic plasmid, Symbiotic genes, DNA homology

Reporting period:

July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

To identify genes important in the development of the late stages of the Rhizobium - legume symbiosis which may affect the efficiency of N fixation, and to construct inter-specific hybrids to investigate the possibility of improved symbiotic performance. Initially, the recently-identified late symbiotic genes in R. meliloti (Batut et al. MGG **199**, p.232-239, 1985) will be utilized.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

To investigate the extent of the occurrence of DNA homology between recently-identified late symbiotic genes in R. meliloti and other fast-growing Rhizobium species, and to examine complementation between the symbiotic plasmids of these rhizobia and R. meliloti mutants defective in their late symbiotic genes.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Gene probes from one part of the newly-identified region, region I, showed homology to the symbiotic plasmids of most fast-growing rhizobia investigated. In one case no homology was detected to any plasmid and in another to a plasmid that did not carry nif genes. Many strains also showed some homology to non symbiotic plasmids. Two R. leguminosarum sym plasmids showing DNA homology to the region, pJB5JI and pIJ1019 (from strains JI248 and JI300 respectively) could complement mutants of R. meliloti defective in Region I. Neither plasmid could complement mutants defective in region II, and a gene probe from this region had homology to the sym plasmid in JI300 but no homology to any plasmid in JI248. The hybrid strains did not appear to be improved in their symbiotic efficiency, in any combination tested so far.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.2

A new class of genes involved in symbiotic nitrogen fixation in Rhizobium R.C. Snellgrove and P.R. Hirsch Report to Sectorial meeting of contractants at Louvain-la-Neuve March 23-26, 1987

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)	Yes	
Joint meeting(s)	Yes	

Descriptive information for the above data.

Exchange of materials: Various Rhizobium strains and E. coli clones have been exchanged with Bielefeld and Tolouse and Bacteriophage samples have been sent to Dublin.

Joint experiments Plasmid blots of various Rhizobium strains have been sent from Rothamsted to Toulouse to be probed. With probes for the late symbiotic genes and R. meliloti mutants in these genes, generated in Toulouse, are being investigated for complementation by various Rhizobium plasmids at Rothamsted.

Joint meetings: A meeting between all the participants was held in Toulouse in November 1986 and a second meeting at the BAP contractors sectorial meeting in Louvain la Neuve in March 1987, both attended by Penny Hirsch and Bob Snellgrove.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: N.I.H.E., Dublin
Contract no.: BAP - 0080 - IRL

Project leader: M. O'CONNELL
Scientific staff: M. Hynes, D. Donnelly, G. Reigh

Address:
School of Biological Sciences
National Institute of Higher Education
Glasnevin
IRL - DUBLIN 9

Telephone no.: +353.1.370077 EXT.318/284

Telex no.: 30690 NIHE EI

Other contractual partners in the joint project:

P. Boistard, C. N. R. S. - I. N. R. A. (Castanet-Tolosan)
P.R. Hirsch, Rothamsted Experimental Station (Harpenden)
A. Pühler, Universität Bielefeld

Title of the research activity:
Comparison of late Sym genes in Rhizobium species and construction of improved strains.

Key words:
Rhizobium, Nitrogen fixation, Gene cloning

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The project aims to identify genes involved in the later stages of the Rhizobium-legume symbiosis and to study the expression of the bacterial genes. Symbiotic genes located on both the symbiotic plasmid and on the chromosome of different species are being cloned and analysed. The homologous genes are being compared especially in their promoter regions and their regulation will be studied in different genetic backgrounds. Novel strains will be constructed using combinations of genes from different backgrounds and these will be checked for improved symbiotic efficiency.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

A major role of this laboratory in the joint project is to isolate mutants of Rhizobium defective in the later stages of the symbiosis and to clone the corresponding wild type genes. We have concentrated on the isolation of mutants with an altered cell surface and altered exopolysaccharide. Mutants obtained have been checked on plants to determine the affect on their symbiotic efficiency. Mutated genes have been used to clone the corresponding wild type DNA and the genetic characterisation of these genes and their regulatory regions is underway.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Methodology

We are interested in identifying the recognition signals of Rhizobium that function in the later stages of the symbiosis. To identify recognition signals, we have isolated mutants in cell surface structures and tested these on plants to assess their symbiotic ability.

Rhizobium leguminosarum strain VF39 (originally isolated from Vicia faba) was mutagenised using the transposon Tn5 mob (Simon 1984). Rhizobium containing Tn5 mob inserts were selected on TY medium (a rich medium) and transferred to Vincent's minimal medium. The high content of mannitol in the minimal medium results in the production of excess expolysaccharide by Rhizobium. On the basis of the difference observed in colony morphology on minimal medium, mutants in exopolysaccharide production were isolated. Auxotrophs were also

detected by this method.

Mutants with altered exopolysaccharide were classified and assayed in plant tests on Vicia hirsuta. Biochemical analysis of the mutants to identify the precise chemical alteration has also been initiated. To aid the classification, Tn5 mob inserts were located in total DNA digests by Southern hybridisation to a Tn5 probe thus distinguishing inserts in the same fragment.

The wild type genes corresponding to some of the mutated regions have been cloned by complementation of the mutant phenotypes. A cosmid bank of strain VF 39 was introduced into an E. coli strain harbouring the broad host range plasmid RP4. The bank is constructed in a vector containing a segment of RP4 DNA and the formation of cosmid-RP4 cointegrates occurs at low frequency. Transfer of the cointegrates to Rhizobium can be selected using the resistance markers of the cosmid vector. Using this method the complementing cosmids were identified by visual appearance of normal exopolysaccharide production when cointegrates were introduced en mass into a mutant Rhizobium. The cosmids were reisolated from the cointegrate structures by transfer back to E. coli where they breakdown into cosmid/RP4 constituents.

2. Results

The efficiency of the mutagenesis procedure was confirmed by the isolation of auxotrophs at a frequency of 0.5%. Mutants altered in exopolysaccharide production were also observed and these were classified into 2 groups.

a) Overproducers : The visual appearance of excess exopolysaccharide was confirmed by the use of an anthrone assay to quantify exopolysaccharide produced.

b) Non producers : One mutant was isolated that produced small rough colonies with an atypical appearance for Rhizobium. Plasmid analysis confirmed the identity of the strain.

In addition, some mutants were identified as having abnormal morphology and these were also tested on plants.

Plant tests revealed that both the overproducers and the non producers were capable of nodulation and nitrogen fixation. The efficiency of nitrogen fixation is presently being assayed.

From the group showing abnormal morphology one Nod^+ Fix^- mutant was

identified. It flocculated when grown in rich broth. This phenomenon has been observed for lipopolysaccharide mutants. Cosmid clones containing the wild type genes corresponding to the mutants have been obtained.

3. Discussion

The initial period has facilitated the isolation of mutants. The biochemical and genetic characterisation of these mutants is now in progress.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.2

O'Connell, Michael; Hynes, Miriam; Donnelly, Dan; and Reigh, Geraldine. Analysis of late symbiotic genes in Rhizobium. Report to the sectorial meeting of contractants. Louvain la Neuve, March 1987.

Priefer, N. B.; O'Connell, M and Puhler A. Identification and cloning of Rhizobium leguminosarum genes involved in cell surface polysaccharides and symbiotic nitrogen fixation. Presentation to the sectorial meeting of contractants. Louvain la Neuve, March 1987.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Exchange of materials : The Tn5mob mutagenesis system was obtained from Bielefeld University and used to generate the mutants described. Mutants and cloned genes have been supplied to Bielefeld University to facilitate a comparative analysis with cloned genes available there. Bacteriophage samples have been obtained from the Rothamsted laboratory for use in the analysis of lipopolysaccharide alterations in the mutants isolated in Dublin.

Exchange of staff : During August and September 1987, Michael O'Connell spent a 6 week working visit at Bielefeld University to initiate the contract research.

Joint experiments : A comparative analysis of lipopolysaccharide mutants is being undertaken at Bielefeld and Dublin. Bacteriophages supplied by Rothamsted are also being used to aid the characterisation of the mutants available. Cosmid clones isolated in Dublin are being used by the Bielefeld group in complementation studies to compare lipopolysaccharide mutants from the two laboratories.

Joint meetings : Two joint meetings have been held during the reporting period with participants from the four groups. The first was held in Toulouse on the 23rd November 1986 and the second was held during the sectorial meeting of BAP contractants at Louvain la Neuve in March 1987.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Univ. Bielefeld Contract no.: BAP - 0081 - D

Project leader: A. PÜHLER / U.B. PRIEFER
Scientific staff: P. Grönger, G. Ruhl, C. Schmidt

Address:
Universität Bielefeld
Biologie VI/Genetik
Postfach 8640
D - 4800 BIELEFELD 1

Telephone no.: +49.521.1065607

Telex no.: 932 362 UNIBI D

Other contractual partners in the joint project:

M. O'Connell, School of Biological Sciences (Dublin)
P.R. Hirsch, Rothamsted Experimental Station (Harpenden)
P. Boistard, C. N. R. S. - I. N. R. A. (Castanet-Tolosan)

Title of the research activity:

Comparison of late Sym genes in Rhizobium species and construction of improved strains.

Key words:

Interspecific complementation, Rhizobium meliloti, Rhizobium leguminosarum, Late symbiotic genes, Cell surface polysaccharides

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The aim of the joint project is to identify and study bacterial genes involved in late symbiotic steps and in maintenance of effective nodules, mainly in the organisms *R.meliloti* and *R.leguminosarum*. Genes homologous in both species will be compared, their regulation studied in different genetic backgrounds and the symbiotic efficiency of new genetic combinations assayed.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The aim of this project is the characterization of late symbiotic genes in *R.leguminosarum* (strain VF39) with special emphasis on genes functionally homologous to *R.meliloti*.

Specifically for the first year, work has concentrated on the identification and isolation of clones of *R.leguminosarum* VF39 encoding functions involved in nodule development and persistence.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY

Complementation studies: Interspecific DNA transfer into *R.meliloti* is achieved by individual conjugative introduction of VF39 indigenous plasmids or of VF39 cosmid clones by cointegrate formation with RP4. Complementation is assayed via plant tests on alfalfa. For intraspecific complementations, cosmid clones are introduced either via RP4-cointegrates or by integration into the mutant genome. For VF39, *Vicia hirsuta* is used as the testplant.

Test for changes in cell surface polysaccharides: Criteria for EPS mutants were slime morphology on yeast-mannitol medium, precipitation of acidic EPS with cetylpyridinium-chloride, staining with calcofluor white; LPS mutants were tested by selfagglutination in rich medium, motility, SDS-PAGE of cell extracts and subsequent $AgNO_3$ staining.

Plant tests and microscopy: Sterilized seeds were inoculated after germination. Plants were grown on N-free salt-medium. After three to four weeks, nitrogen fixation was assayed by acetylene reduction. Semi-thin sections of the nodules were stained with toluidin blue and analyzed by light microscopy.

2. RESULTS AND DISCUSSION

Complementation and hybridization studies with *R.meliloti*

Since a series of *R.meliloti* mutants, defective in infection or nodule morphology, as well as the corresponding genes have been characterized in P. Boistard's (Toulouse) and in our group, we have attempted to isolate homologous *R.leguminosarum* VF39 genes by interspecific complementation and/or hybridization analyses. Preliminary data suggest, that one of the *R.meliloti* mutants obtained from Toulouse (GMI 181, mutated in the *fix*I gene cluster) could be complemented upon introduction of the VF39 cosmid bank. Reisolation and physical characterization of three complementing cosmids revealed that they all share common fragments. The same mutant appeared to be complemented by the VF39 plasmid pVF39c (cryptic plasmid). Hybridizations using a probe specific for the *R.meliloti fix*I gene cluster (also obtained from Toulouse) are currently being carried out. These studies include hybridization to the complete VF39 plasmid profile, to total DNA and to the putative complementing cosmids described above.

R.leguminosarum VF39 mutants defective in nodule development

Isolation of mutants

In addition to the complementation studies outlined above, strain VF39 was subjected to a random Tn5 mutagenesis. This part of the project is being carried out in strong collaboration with M. O'Connell (Dublin). Since it has been substantiated in the meantime, that rhizobial cell surface polysaccharides play a role during the normal infection process, the mutants were primarily screened for altered colony morphology (see methodology) and then tested for their symbiotic properties.

Both, mutants affected in EPS and LPS production could be identified. According to their symbiotic phenotype, the mutants can be grouped into four major classes: (1) apparently normal in symbiosis, (2) no nodule formation, (3) reduced levels of nitrogen fixation and (4) no nitrogen fixing activity.

Representatives of the latter two classes have been analyzed in more detail.

Morphology of mutant nodules

Some of the nodules which showed reduced or no nitrogen fixation activity have been analyzed by light microscopy. They all showed nodule morphologies different from wild type, although to various degrees. Many of

them are poorly infected, contain only few infection threads and a reduced number of bacteria and show premature senescence. One mutant produced nodules obviously completely devoid of bacteria.
Therefore it appears that many of the VF39 mutants isolated as cell surface mutants are simultaneously blocked in some stage of nodule development.

Genetic characterization

Hybridization studies with total DNA revealed that all mutants contained only one *Eco*RI fragment carrying Tn5 and that the hybridizing band was different from strain to strain. This implicates that the various phenotypic effects observed (i.e. altered colony morphology and failure to form effective nodules) are due to different single Tn5 insertions. Moreover, complementation experiments with the VF39 cosmid bank resulted in the identification of cosmid clones able to restore not only wild type colony morphology but at the same time also the ability to form normally developed nodules.
A number of complementing cosmids has been purified and compared with each other on the basis of their *Eco*RI restriction pattern. Since some of them share common fragments, it is likely that different mutants can be complemented by the same cosmid clone. These cross-complementation studies are being carried out at the moment. We also obtained mutants and corresponding cosmids from Dublin which are included in the comparative analysis.

For some of the cosmids, fractions still sufficient for complementation have been subcloned. These fragments are currently being subjected to saturated Tn5 mutagenesis in order to study their genetic organization.

3. SUMMARY AND PROSPECTS

The interspecific complementation studies already carried out indicate the existence of late symbiotic genes functionally homologous between *R.meliloti* and *R.leguminosarum*. The second approach led to the isolation of *R.leguminosarum* mutants defective in both cell surface polysaccharides and nodule morphogenesis. *R.leguminosarum* phages provided by P. Hirsch (Harpenden) or isolated in Dublin will help characterize the surface alterations in more detail. Complementation between these *R.leguminosarum* and analogous *R.meliloti* mutants may identify symbiotic genes common to both organisms and involved in development and maintenance of a functional *Rhizobium* nodule.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

P. Grönger, S. S. Manian, H. Reiländer, M. O'Connell, U. B. Priefer and A. Pühler (1987):
Organization and partial sequence of a DNA region of the *Rhizobium leguminosarum* symbiotic plasmid pRL6JI containing the genes *fix*ABC, *nif*A, *nif*B and a novel open reading frame.
Nucleic Acids Research, *15*:31-49

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Mutant strains of *R.meliloti* as well as the corresponding hybridization probes were obtained from Toulouse; a number of VF39 Tn5 mutants and cosmid clones were exchanged with the group in Dublin. We provided other groups with *nif*A-*lac*Z fusions, strains and vector plasmids and passed the whole VF39 cosmid bank on to the Dublin group. We also exchanged sequence data and unpublished information concerning experimental strategies and methodologies.
M. O'Connell spent 6 weeks in our laboratory (28. July - 15. September, 1986) in order to screen and complement Tn5-Mob mutants of VF39. Further exchanges of staff are planned.
The project under study in Dublin is closely related to our work and therefore, joint experiments, such as cross-complementation analyses using mutants of one and complementing cosmids of the other group, are being carried out especially with this group; mutants obtained in one laboratory and of special interest to the other are forwarded for further analysis.
In order to discuss results and problems more extensively and to plan future strategies and experiments, all group leaders meet regularly approximately every 6 months. Apart from the first meeting in March 1986 in Paris (which was to set up the overall strategy of the research project) all members met in Toulouse in November 1986 (23.11-25.11). Since there was an opportunity to discuss in Louvain-la-Neuve in March 1987, we did not arrange an extra meeting for spring 1987. The next group session is being organized by M. O'Connell and will take place the end of October.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Aarhus Univ. Contract no.: BAP - 0173 - DK

Project leader: K.A. MARCKER
Scientific staff:

Address:
Aarhus University
Dept. of Molecular Biology
and Plant Physiology
C.F. Mollers Allé 130
DK - 8000 AARHUS C

Telephone no.: +45.6.125177

Telex no.: 64767 AAUSCI DK

Other contractual partners in the joint project:

A. Pühler, Universität Bielefeld
M. Van Montagu, Rijksuniversiteit Gent
J. Schell, Max-Planck-Institut, Köln

Title of the research activity:

Regulation of expression of genes involved in biological nitrogen fixation.

Key words:

Nitrogen fixation, Leghemoglobin, Nodulin, Regulation

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

1) Identification of the DNA sequences present on nodule specific plant genes which are responsible for the activation of such genes.

2) The components which interacts with the said sequences in such a way that the genes are activated.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Deletion mutagenesis of leghemoglobin and nodulin gene promoters.

Identification of transacting factors interacting with leghemoglobin and nodulin gene promoters regions.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

In all legumes the interaction between Rhizobia and the plant leads to the formation of root nodules in which the fixation of atmospheric nitrogen takes place. During nodule development a number of plant genes is specifically expressed. The specific gene products are the leghemoglobins and the nodulins which are involved in nodule formation and function. Recently we have developed a transformation and regeneration system for the legume Lotus corniculatus, using a derived Ri plasmid as vector. Transferred chimeric genes containing nodule specific promoters are expressed in transgenic plants in a nodule specific way. The detailed description of the transformation/regeneration system is publisted in Mol. Gen. Genet. 1987 207, 245-250 and 251-255.

Recently we have transferred an entire soybean leghemoglobin gene to Lotus corniculatus. It was shown that the gene is expressed in Lotus nodules to an extent comparable to that of the endogenous leghemoglobin genes. Our results so far strongly suggest that the induction mechanism for leghemoglobin genes in legumes most likely is conserved. A chimeric soybean nodulin 23 - CAT gene is also specifically expressed in the root nodules of Lotus corniculatus and T.repens formed after inoculation with their respective microsymbionts Rhizobium loti and Rhizobium trifolii. The soybean N23 promoter appears to be as well recognized in Lotus and T.repens as in soybean. It is therefore tentatively concluded that the molecular mechanisms responsible for activation of af least some of the nodulin genes are also conserved throughout the various Rhizobium legume associations.

Identification of regulatory DNA sequences present in nodule specific plant genes.

The soybean leghemoglobin *lbc*$_3$ gene promoter was analyzed in transgenic *Lotus corniculatus* plants. Hybridpromoter constructions and 5' deletions were studied using chimeric genes composed of the various promoters, the chloramphenicol acetyl transferase (CAT) and *lbc*$_3$ 3' region. The 5' *Bal31* deletion series mapped a strong posivive regulatory element to the -1000 region, a weaker element to the -200 region, and further defined the minimum 5' region required for detectable promoter activity.

Activation by the constitutive CaMv 35S enhancer of inactive promoter deletions terminating between positions -230 and the transcription initiation site located a *cis* element sufficient for nodule specific expression beyond position -139. A 37 bp region which includes sequences conserved in the leghemoglobin and nodulin genes was required for organ specific expression. No indispensable control elements were found on the *lbc*$_3$ 3' region.

A 5' region of less than 1 kb was sufficient for the organ-specific expression of a chimeric N23-CAT gene in root nodules formed on Lotus corniculatus and Trifolium repens after infection by their respective Rhizobium symbionts. Expression was regulated at the level of RNA in both species of transgenic plants. Promoter deletion analysis defined the 5' region required for high level expression and delimited two putative regulatory sequences involved in positive control of the N23 gene in L. corniculatus.

Identification and characterization of nodule specific transacting regulatory factors involved in soybean leghemoglobin gene induction.

We have analyzed the 1b promoter for the presence of cis-acting (regulatory) elements and trans-acting factors which may be involved in regulating the expression of the 1b genes in the nodule. The promoter region was subcloned to yield specific fragments of up to 150 bp. Enriched nuclear extracts were prepared from leaves, roots and nodules and used to carry out gel retardation assays with the isolated DNA fragments. Specific binding of (a) nodule extract factor(s) to two distinct restriction fragments in the region -50 to -300 upstream of the first exon could be demonstrated. Using Bal 31 deletion analysis, putative binding sites were delimited and oligonucleotides corresponding to these regions were synthesized. Both binding site I and II oligonucleotides, are capable of binding the nodule specific factor(s) and can outcompete each other for binding.

Isolation of nodule specific genes from soybean.

The primary structure of two nodule specific soybean genes has been determined. The two genes code for primary products of 20.0 (nodulin 29) and 22.7 (nodulin 22) kdaltons, respectively. Both genes are related to the nodulin 23 and 44 genes. Alignment of the deduced amino acid sequences of all four genes revealed threee domains of high homology interrupted by highly diverged regions due to numerous duplication and insertion events. The first conserved domain codes for a putative signal peptide, while the two others each contain four Cys residues that can be arranged in a way reminiscent of the metal binding domains present in some enzymes and in several DNA binding proteins.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

A small family of nodule specific genes from soybean.
Niels N. Sandal, Kirsten Bojsen and Kjeld A. Marcker. Nucleic Acids Research (1987) 15: 1507-1519.

Transformation and regeneration of legume Lotus corniculatus: A system for molecular studies of symbiotic nitrogen fixation.
Annik Petit, Jens Stougaard, Astrid Kühle, Kjeld A. Marcker, and Jacques Tempé. Mol. Gen. Genet (1987) 207: 245-250.

The Agrobacterium rhizogenes pRi TL-DNA segment as a gene vector system for transformation of plants.
Jens Stougaard, Dorte Abildsten, and Kjeld A. Marcker. Mol. Gen. Genet (1987) 207: 251-255.

Expression of a complete soybean leghemoglobin gene in root nodules of transgenic Lotus corniculatus.
Jens Stougaard, Torben E. Petersen, and Kjeld A. Marcker. Proc. Natl. Acad. Sci. USA. (August 1987).

TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff	Yes	
Joint experiment(s)		No
Joint meeting(s)		No

Descriptive information for the above data.

Several Soybean nodule specific cDNA clones sent to Bielefeld.

Lic.Scient Erik Østergaard Jensen spent the period 1/11-1986 to 1/6-1987 in professor J. Schell's laboratory working on transacting factors.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Univ. Bielefeld Contract no.: BAP - 0173 - DK

Project leader: A. PÜHLER
Scientific staff: A. Perlick

Address: Universität Bielefeld
Biologie VI / Genetik
Postfach 8640
Universitätstrasse
D - 4800 BIELEFELD 1

Telephone no.: +49.521.1065607

Telex no.: 932362 UNIBI D

Other contractual partners in the joint project:

K.A. Marcker, Aarhus University
M. Van Montagu, Rijksuniversiteit Gent

Title of the research activity:
Regulation of expression of genes involved in biological nitrogen fixation.

Key words:
Nodulin genes, cDNA clones, Soybean, Broadbean, Conserved regulation signals

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

A coordinated expression of specific legume and *Rhizobium* genes is involved in the symbiotic interaction which leads to the development of nitrogen fixing root nodules. In this project it is planned to study the regulation of nodule specific gene expression. In particular, the identification of regulatory signals as well as the identification of transacting factors involved in nodule specific gene expression is planned.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Recent experiments suggest that the regulation of nodulin genes might be conserved in different leguminous species (13). Our part in the joint project is to identify by hybridization nodulin genes homologous in soybean and broadbean and to compare their sequences. We hope to find conserved motifs in non-coding regions which might serve as signal sequences. Our recent work has dealt with the isolation of nodule specific cDNA clones of broadbean which show homology to soybean nodule specific cDNA clones.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY:

cDNA clones: Soybean nodule specific cDNA inserts were obtained from K.A. Marcker, Aarhus. The broadbean nodule cDNA library was constructed in Bielefeld (1).

Southern blot analysis: DNA from broadbean cDNA clones was digested with *PstI*, separated on 2 % agarose gels and transferred onto nitrocellulose after alkaline treatment according to Southern (2). Hybridization of filters was carried out as described by Maniatis et al. (3). Washing stringency was 2xSSC and 0.1% SDS at 62°C, for increasing stringency 1x, 0.5x and 0.2x SSC and 0.1% SDS at 62°C was used. Radioactive labelled single stranded probes were generated by primer extension (4) using M13mp18 clones containing the soybean cDNA inserts (5).

Sequencing: cDNA inserts were cloned into the phage vector M13mp18 and sequenced by the dideoxy chain termination method (6). In addition, cDNA inserts were cloned into the newly constructed plasmid pSVB21 (Arnold, pers. comm.), and the chemical method according to Maxam and Gilbert (7) was applied.

2. RESULTS:

Using Southern hybridization, we compared 12 soybean (sb) nodule specific cDNA clones with about 120 selected broadbean (bb) cDNA clones. Under low washing conditions we found hybridization with clones sb46 and sb107. Clone sb46 appears to be of special interest, since it hybridizes, with different strengths, to a number of bb clones. A subgroup of 13 relatively strong hybridizing bb clones was analyzed in detail. Upon increasing the stringency of the washing procedure to 1x SSC, we observed that clone bb92 hybridized markedly stronger than the other 12 clones. Three other clones (bb23, bb67, bb115) showed signal intensities of medium strength. Under washing conditions less than 1xSSC no hybridization was detected.

To verify these results we intend to compare the sequences of the hybridizing clones. The inserts of clones bb92 (*Pst*I fragment of about 1200 bp) and sb46 (*Bam*HI fragment of about 400 bp) are currently being sequenced. Both contain polyA stretches of approximately 180 and 60 bp, respectively. To examine the influence of these polyA tails on hybridization we constructed a deletion derivative of clone sb46 (sb46.D) by eliminating a 3' terminal *Hae*III-*Bam*HI fragment of less than 100 bp. Hybridization experiments using clone sb46.D as a probe showed the same hybridization intensities as clone sb46. These results indicate that the polyA tail of clone sb46 is not responsible for the observed hybridization.

3. DISCUSSION:

Nodule specific gene expression involved in symbiotic nitrogen fixation has been studied in several leguminous plants. To date, the best studied system is that of soybean-*Bradyrhizobium japonicum* (8,9,10), but also alfalfa-*Rhizobium meliloti* (11) and pea-*Rhizobium leguminosarum* (12) systems have been investigated.

Recently, Jensen et al. (13) were able to demonstrate a conserved induction mechanism in legumes for at least one nodulin gene. They transferred a chimeric gene with the non-coding 5'

and 3' regions of the soybean leghemoglobin gene Lbc3 (5'Lbc3-chloramphenicol acetyltransferase coding sequence-3'Lbc3) to *Lotus corniculatus* plants and observed development and tissue specific gene expression in the heterologous host.

To investigate whether other nodulin genes show conserved coding sequences and regulatory elements it is planned to compare soybean and broadbean nodulin genes. In our experiments we could demonstrate crosshybridization between broadbean and soybean at the cDNA level. These results were obtained under low washing stringencies. The sequence analysis currently being carried out could prove the homologies between the investigated clones.

References:

1) Kuhse J. and A. Pühler (1987): Plant Science **49**, 137-143
2) Southern, E.M. (1975): J. Mol. Biol. **98**, 503-517
3) Maniatis, T., E.F. Fritsch and J. Sambrook (1982): Molecular Cloning, A Laboratory Manual, Cold Spring Harbour Laboratory, Cold Spring Harbour
4) Hu, N.T. and J. Messing (1982): Gene **17**, 271-277
5) Yanisch-Perron, C., J. Vieira and J. Messing (1985): Gene **33**, 103-119
6) Sanger, R., A.R. Coulson, B.Q. Barrell, A.J.H. Smith and B.A. Roe (1980): J. Mol. Biol. **143**, 161-178
7) Maxam, A. and W. Gilbert (1977): Proc. Natl. Acad. Sci. USA **74**, 560-564
8) Verma, D.P.S., M.G. Fortin, J. Stanley, V.P. Mauro, S. Purohit and N. Morrison (1986): Plant Mol. Biol. **7**, 51-61
9) Sandal, N.N., K. Bojsen and K.A. Marcker (1987): Nucl. Ac. Res. **15**, 1507-1519
10) Sengupta-Gopalan, C., J.W. Pitas, D.V. Thompson and L.M. Hoffman (1986): Mol. Gen. Genet. **203**, 410-420
11) Lang-Unnasch, N. and F.M. Ausubel (1985): Plant Physiol. **77**, 833-839
12) Bisseling, T., C. Been, J. Klugkist, A. van Kammen and K. Nadler (1983): EMBO **2**, 961-966
13) Jensen, J.S., K.A. Marcker, L. Otten and J. Schell (1986): Nature **321**, 669-674

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

none

TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	No
Joint experiment(s)	No
Joint meeting(s)	No

Descriptive information for the above data.

see methodology

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Rijksuniversiteit Gent

Contract no.: BAP - 0173 DK

Project leader: M. VAN MONTAGU

Scientific staff:

Address:

Rijksuniversiteit Gent
St.-Pietersnieuwstraat 25
B - 9000 GENT

Telephone no.: +32.91.227821 EXT. 542

Telex no.: 11995 GENGEN B

Other contractual partners in the joint project:

K.A. Marcker, Aarhus University
A. Pühler, Universität Bielefeld

Title of the research activity:

Regulation of expression of genes involved in biological nitrogen fixation.

Key words:

Gene expression, Nodulation, Sesbania rostrata, Azorhizobium, Nitrogen fixation

Reporting period:

July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The identification in Azorhizobium caulinodans strain ORS571 of genes involved in nodulation and symbiotic N_2 fixation; the regulation of expression of these symbiotic genes will be studied by means of lacZ gene fusions.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The isolation of Fix^- ORS571 mutants and the cloning of fix loci, coding for functions that are specifically required for the symbiotic N_2 fixation.
The construction of lacZ fusions in an essential nodulation region of ORS571.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

III.1. Methodology

For the isolation of symbiotic mutants we introduced the plasmid pSUP2021 in ORS571 and selected for ORS571 derivatives resistant to kanamycin (Km) and streptomycin (Sm).
To isolate clones from a *fix* region of the ORS571 genome, DNA from Tn*5* induced Fix$^-$ ORS571 mutants was digested and cloned in a pBR322 cloning vector and Km^R clones were selected.
These clones from mutated *fix* regions were subsequently used as a hybridization probe against an ORS571 gene library in the phage λ vector EMBL3.
MudIIPR13 mutagenesis was carried out as desribed by Ratet *et al.* (1987). Tn*3*HoHo1 mutagenesis was carried out as described by Stachel *et al.* (1985).

III.2. Results

1. We isolated several Fix$^-$ ORS571 mutants after random Tn*5* mutagenesis. These mutants are defective only in symbiotic N_2 fixation; they can still fix N_2 in the free living state.
   We isolated clones from the mutated regions and from the corresponding wild type regions of the genome and constructed physical maps.
2. A previously identified essential nodulation region of ORS571 (Van Den Eede *et al.*, 1987) was studied in more detail. This region, ORS571 *nod* locus 1, contains genes homologous to the common *nodC* and *nodA* genes of *Rhizobium meliloti*. We have constructed *lacZ* fusions in this region, using either a MudIIPR13 or a Tn*3*lac*Z*HoHo1 transposon. Insertions in the *nodC* and the *nodA* gene gave, in one orientation, expression of β-galactosidase activity when the bacteria were grown in the presence of *Sesbania rostrata* root exudate.

III.3. Discussion

The cloning of a *fix* region of ORS571 will allow the study of the expression of *fix* genes during the symbiotic interaction. It will be interesting to determine whether the expression of these *fix* genes, which are essential for the symbiotic N_2 fixation to occur without being directly involved with the nitrogenase assembly or activity, is regulated and if so, at which stage of the interaction, these genes are expressed, and which signals induce expression.
By means of lac*Z* fusions in the ORS571 *nod* locus 1 we have learned that the regulation of expression of essential nod genes of ORS571 is similar to that of the fast growing rhizobia. The expression is induced by *Sesbania rostrata* root exudates We are at present analysing these exudates in order to purify and identify the inducing molecule.

References

1. RATET, P., SCHELL, J., and DE BRUIJN, F.J.
Mini-Mu-lac transposons with wide host-range origins of conjugal transfer and replication for the construction of hybrid proteins in Escherichia coli and gene regulation studies in Rhizobiaceae.
Gene, in press.
2. STACHEL, S.E., AN, G., FLORES, C., and NESTER, E.W.
A Tn3 lacZ transposon for the random generation of β-galactosidase gene fusions : applications to the analysis of gene expression in Agrobacterium.
EMBO J. 4, 891 - 898 (1985).
3. VAN DEN EEDE, G., DREYFUS, B., GOETHALS, K., VAN MONTAGU, M., and HOLSTERS, M.
Identification and cloning of nodulation genes from the stem-nodulating bacterium ORS571.
Mol. Gen. Genet. 206, 291 - 299 (1987).

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

Nihil

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	No
Exchange of staff	No
Joint experiment(s)	No
Joint meeting(s)	Yes

Descriptive information for the above data.

J.S. Jensen and K.A. Marcker have visited the laboratory in Ghent in December 1985 and July 1986 respectively to discuss various aspects of the research work. Also, additional joint meetings were held in Montreal - Canada, 1986 at the occasion of the "Third International Symposium on the Molecular Genetics of Plant-Microbe Associations" with K.A. Marcker and R.Pühler and their collaborators and at the "Workshop on the Molecular Biology of bacterium-plant interactions" in Amalfi - Italy, in April 1987.
Exchange of materials for nodulin identification can start during the next year now the necessary constructs have been characterized.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: P.G.S., Gent Contract no.: BAP - 0095 - B

Project leader: M. DE BLOCK
Scientific staff:

Address:
Plant Genetic Systems N.V.
J. Plateaustraat 22
B - 9000 GENT

Telephone no.: +32.91.242525 or +32.91.255473

Telex no.: 11361

Other contractual partners in the joint project:

J. Schell/P. Schreier, M. P. I. (Köln)

Title of the research activity:

The transformation of chloroplasts with Agrobacterium tumefaciens and naked DNA.

Key words:

Plastids, Transformation, Marker genes, Agrobacterium

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Development of technology to introduce reproducibly DNA in a stable manner into the plastid genome.

II. SPECIFIED OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

During the first grant period experiments were designed to settle following questions:
Can the Agrobacterium T-DNA transfer system be used to deliver DNA into the plastid genome?
Are the chimaeric PpsbAbar (see note) and PpsbAaphII genes usable as plastid specific reporter genes? Or more specific: Does the psbA element exhibit a transcription initiation activity when located into the nucleus? Does a plastidial expression of the bar and aphII coding sequences provide a selectable phenotype?

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Methods

DNA manipulations were performed basically as described by Maniatis et al.,1982, Molecular cloning, C.S.H.L.. Mobilisations and cocultivations were carried out as described by van Haute et al., 1983, EMBO J. 2, 411-417, and De Block et al., 1984, EMBO J. 3, 1367-1372. Plant enzyme assays were done as described by the Block et al., 1987, EMBO J. in press.

2. Results

a. Construction of T-DNA vectors containing plastid specific reporter genes.

The following T-DNA vectors were constructed: pGSC1700/54D, pGSC1700/54LSD and pGSC1700/65LS. The parental T-DNA vector was pGSC1700, a pGV1500 (De Blaere et al., 1987, Methods in Enzymology, in press) derived binary vector. Vector pGSC1700/45D carries between the T-DNA borders the chimaeric PpsbAbar and PpsbAaphII genes in a direct orientation. pGSC1700/45LSD is identical to pGSC1700/45D but carries in addition 3' to the aphII coding sequence a 1kbp plastid sequence from the tobacco rbcL gene. This sequence is included assuming it may direct the T-DNA to a defined site on the plastid genome by a recombination event. pGSC1700/65LS carries a nuclear marker gene comprising the CaMV 35S promoter and the aphII coding sequence as well as the PpsbAbar gene and the rbcL homology region; the two promoter regions are oriented in a head to head configuration. The three constructs were conjugated to Agrobacterium tumefaciens C58 C1 rif+ (pGV2260) (De Blaere et al., 1985, Nucl. Acids Res. 13, 4777-4788).

b. Transformation of protoplasts with the Agrobacterium strains mentioned under a.

Cocultivation of Nicotiana tabacum SR1 protoplasts with the three Agrobacterium strains was carried out twice. After one week a selection pressure of 100 mg/l kanamycin was applied to the dividing plant cells. The efficiency with which kanamycin resistant calli were obtained depended on the Agrobacterium strain used. In the experiments with the pGSC1700/54D vector only 0.01% of the cocultivated protoplasts developed into resistant transgenic calli, whereas in both the pGSC1700/54LSD and pGSC1700/65LSD experiments this percentage was approximately 8%. A control experiment with an Agrobacterium strain containing a promoterless aphII gene (Herman et al., 1986, Mol. and Cel. Biol. 6, 4486-4492) yielded 0.01% of kanamycin resistant transformants.

c. Analysis of transgenic plant material exhibiting the reporter phenotype

All plants obtained from the cocultivation with C58 C1 rif+ (pGV2260;pGSC1700/54D) died upon continued selection pressure after about 4 months and could not be analysed. Anyhow, the frequency with which resistant calli in this experiment had been obtained was at the level at which promoter tagging of abberant T-DNA's occurs (Herman et al.,1986, Mol. and Cel. Biol. 6, 4486-4492). Plants obtained from the other cocultivations were assayed for production of PAT protein. From both experiments 9 plants were analysed and without exception they contained a detectable level of biologically active PAT protein in their total extracts but not in extracts prepared from purified plastids. DNA analysis of these plants is still under way.

3. Discussion

T-DNA's containing transcription units which may be functional in a plastid genetic background were introduced into Nicotiana tabacum SR1 protoplasts. A selection pressure was applied to the dividing plants cells which only permitted cells expressing the foreign DNA to survive. A significant number of resistant calli was obtained in the experiment involving the pGSC1700/54LSD construct. However, protein analysis of transgenic plants obtained from these calli revealed a nuclear location of the T-DNA. This suggests that the chimaeric PpsbA genes are not plastid specific, although the frequency with which resistant calli were obtained clearly depends on the presence of the rbcL sequence on the T-DNA. The role of the rbcL homology region remains unclear. Transgenic plants carrying the pGSC1700/65LS T-DNA in the nucleus do also express the chimaeric PpsbA gene. This favours the idea that PpsbA may support nuclear transcription initiation.
In conclusion: the experiments decribed show that plastid transformation by T-DNA transfer is not a straight forward technique, possibly it may not be feasible. Experiments are in progress which may give insight in the efficiency of DNA uptake by plastids.

Note

The bar gene (Streptomyces hygroscopicus) encodes a phosphinotricin acetyltransferase (PAT) which detoxifies the herbicide phosphinotricin (PPT). PPT inhibits glutamine synthetase which in tobacco is plastidially located.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

None

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)	Yes	
Joint meeting(s)	Yes	

Descriptive information for the above data.

Gene constructs, vectors and resulting data were exchanged. Regular meetings were held in Köln and in Gent to discuss progress and decide on complmentary strategies.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: M.P.I., Köln Contract no.: BAP - 0096 - D

Project leader: J. SCHELL / P. SCHREIER
Scientific staff:

Address: Max-Planck-Institut für Züchtungsforschung
Abt. Genetische Grundlagen der
Pflanzenzüchtung
Egelspfad
D - 5000 KÖLN 30

Telephone no.: +49.221.5062373

Telex no.: 8881028 MPIZ D

Other contractual partners in the joint project:

M. de Block, Plant Genetic Systems (Gent)

Title of the research activity:
The transformation of chloroplasts with Agrobacterium tumefaciens and naked DNA.

Key words: Chloroplast, Chimeric genes, Chloroplast transformation

Reporting period: April 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

To develop methods allowing either the introduction of new genes in chloroplast genomes or the exchange of resident chloroplast genes for modified genes (reversed genetics). The restraints to genetic exchanges involving the chloroplast genome are investigated and strategies are devised to circumvent these restraints.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

To test various genes and vector constructions that can be used to select for rare chloroplast genome transformants one strategy was based on hypothetical circular T-DNA intermediates capable of inserting in the chloroplast genome without disruption of any resident gene. The other involves the exchange of a resident gene by a mutated gene conferring resistance to antibiotics.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Plant cells are of particular importance, since they contain an organelle responsible for photosynthesis: the chloroplast. It would be of major scientific interest, to be able to manipulate the chloroplast by reverse genetics. Such experiments, if successful, would lead to a broader understanding of plant development and energy conversion from light into organic matter.

Two ways of genetic engineering of chloroplasts appear feasable: the introduction of genetic material directly into the chloroplast genome or the transport of protein products encoded by nuclear genes, upon transformation. Both ways have been described recently. Van den Broeck et al. (1985) and

Schreier et al. (1985) have demonstrated successful transport of the bacterial enzyme neomycin-phosphotransferase II into the chloroplast with the aid of the transit peptide from the small subunit of ribulose-bisphosphate-carboxylase after nuclear transformation of a chimearic gene with an Agrobacterium tumefaciens Ti-plasmid. On the other hand De Block et al. (1985) gave an example of direct transformation of the chloroplast. The latter experiments suffered from instability of the transformed genetic marker in the chloroplast. The transformed chloramphenicol resistance marker was lost after selection was released, indicating that not all plasmid genomes were transformed. Since these experiments were originally not designed to select for chloroplast transformation, the chloramphenicol gene was under the direction of the nopaline synthase promoter, known to be most active in the nuclear compartment. To improve the experimental approach, chimearic genes were constructed at PGS (collaborating institution) containing promoter fragments isolated from known chloroplast genes such as the psbA and the rps4 gene fused to the NPTII coding sequence of Tn5. An intact 5 or 3 part of a defined plastid transcription unit was included in some of the T-DNA vectors. Assuming a circular T-DNA intermediate in the plastid, these sequences may offer a site for recombination with the plastid genome. The constructs were used in "leafdisc" and "cocultivation" experiments and yielded significant numbers of kanamycin resistant transformants. None of these calli turned out to contain stably transformed chloroplasts (Cornelissen et al. 1987).

The same T-DNA constructs were also tested in Cologne in direct DNA transformation experiments which also did not yield stable chloroplast transformants. Furthermore, we have made intermediate Agrobacterium vectors containing a complete mutant psbA gene which confers resistance to triazin herbicides. We intend to select stable chloroplast transformants, which could have been the result of gene conversion between the incoming mutant and the resident sensitive allele. We have

established a procedure allowing regeneration from leaf disks and protoplasts under photoautotrophic or photomixotrophic conditions respectively, in order to be able to use triazin herbicides as selective agents. Under these conditions regeneration from protoplasts to plantlets is prolonged to 6 months. We have performed 4 independent transformation experiments but were not able to obtain material surviving selection with the herbicide so far.

At the same time we are developing other selectable markers for chloroplast transformation experiments on the basis of marker exchange. We have choosen streptomycin (Maliga et al. 1973) and lincomycin resistance (Cseplö and Maliga, 1982) since the genes responsible for these phenotypes are located on the chloroplast genome. As a first result we have determined a mutation in the 16S RNA gene of tobacco SR1 chloroplasts most likely to be responsible for the streptomycin resistance phenotype (Etzold et al., 1987). Experiments are in progress to determine the mutation or mutations causing lincomycin resistance.

References:

Cornelissen et al. in press (see preprint)
Cseplö and Maliga (1982), Curr. Genet. 6: 105-109
De Block et al. (1985), EMBO J. 3: 1681-1689.
Etzold et al. (1987), FEBS Letters in press
Maliga et al. (1973), Nature 244: 28-30
Schreier et al. (1985), EMBO J. 4: 25-32
Van den Broeck et al. (1985), Nature 313: 358-363.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)	Yes	
Joint meeting(s)	Yes	

Descriptive information for the above data.

Gene constructs, vectors and resulting data were exchanged. Regular meetings were held in Köln and in Gent to discuss progress and decide on complementary strategies to overcome unexpected problems.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: University of Edinburgh

Contract no.: BAP - 0102 - UK

Project leader: C.J. LEAVER

Scientific staff: C. Bachem, EEC supported Postdoctoral Research Associate since 1.1.87
I. Moore, SERC Biotechnology Directorate supported Postgraduate Student

Address: University of Edinburgh
Dept. of Botany
The King's Buildings
Mayfield Road
UK - EDINBURGH EH9 3JH

Telephone no.: +44.31.6671081 EXT.3304

Telex no.: 727442 UNIVED G

Other contractual partners in the joint project:

A.P. Czernilofsky, M. P. I. (Köln)

Title of the research activity:

Genetic transformation of plant mitochondria : development of a general strategy.

Key words:

Plant mitochondria, Genetic transformation, Mitochondrial genes

Reporting period: July 1986 - June 1986

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The major aim of the collaborative research programme conducted in the laboratories of Prof. C.J. Leaver (Department of Botany, University of Edinburgh, Scotland) and Prof. J. Schell (Max-Planck-Institut fuer Zuechtungsforschung, Cologne, FRG) is to develop a general strategy for the genetic transformation of plant mitochondria, and the use of this technique to study mitochondrial biogenesis and genetics with respect to agronomically important traits such as cytoplasmic male sterility (CMS).

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The major aim of the Edinburgh group has been to continue the use of isolated and characterised plant mitochondrial DNA sequences involved in initiation of transcription and translation from a number of plant mitochondrial genes for the construction of selectable transformation vectors. Tissue culture and transformation techniques have been used in collaboration with the Cologne group to develop a selection procedure for the recovery of mitochondrial transformants.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1) Methods:

The methods being used to obtain mitochondrial transformation are largely standard published protocols, involving DNA technology for the construction of the plasmids described below. Both naked DNA transformation ($CaNO_4$ method) and Ti-plasmid mediated transformation are being used to transfer the constructs, carrying the chimaeric genes, into SR1- and Samson NN-tobacco lines. A variety of assay procedures are planned and being used to analyse transformed plant material including NPTII tests, cat-assays and in-organello protein synthesis.

2) Results:

a) Plasmid construction involving the mitochondrial regulatory DNA sequences and the cat gene from Proteus mirabilis have been completed. These constructs contain either i) approximately 320 bp of the up-stream region from the cytochrome c oxidase subunit I (coxI) gene of N-type maize (AvaII fragment) or ii) approximately 700 bp of the same sequence from S-type CMS maize. The latter has a sequence (S-TIR) which is thought to be actively involved in genomic recombination events. Each of these two sequences contain two possible transcription initiation sites and 5 nucleotide triplets coding for the 5 amino-terminal residues of coxI (including the ATG). The selectable marker gene chloramphenicol acetyl transferase (cat) is fused in-frame to the 5'-section of coxI. The authentic mitochondrial transcription and translation signals are thus installed in the chimaeric gene construct. In addition to the basic construct several other plasmids have been produced carrying different promoters and/or sites for promotion of homologous recombination.

b) The coxI-cat fusion protein has been shown to retain enzyme activity in tobacco protoplasts when expressed from the CaMV 35S promoter in transient assays, demonstrating that the extra 5 amino acids at the N-terminus are 'tolerated' by the enzyme.

c) When stably integrated into the nucleus of tobacco cells and in preliminary transient expression studies, these constructs alone give rise to no detectable cat activity, suggesting that the mitochondrial regulatory DNA sequences do not promote expression of the cat gene in the nucleus. This will allow the specific expression of the gene in mitochondria without a 'background' of chloramphenicol resistant nuclear transformants. In bacteria, expression of the cat gene from these constructs is also very weak, suggesting that a low level of expression in chloroplasts may also be expected.

d) Homologous recombination in mitochondria of several plant species has been investigated by this and other laboratories, and appears to be a regular feature of plant mitochondrial genomes. Thus 3 kb of tobacco mitochondrial DNA has been additionally included in the initial constructs to potentially promote integration and maintenance of the foreign DNA, by homologous recombination.

e) Initial experiments have been performed to develop selection conditions with chloramphenicol for SR1 and Samson NN tobacco varieties using introduced nuclear marker genes as a model system. Nuclear transformants expressing cat can be regularly recovered and attempts to improve the efficiency of recovery of transformants are underway.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

None

TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

DNA constructs and transformed material generated in both Cologne and Edinburgh are transferred between the two laboratories for further analysis. Christian Bachem and Ian Moore are based in Edinburgh but have spent extended periods of time working in Cologne Experiments are planned at joint meetings of the two groups and telephone discussions are held at frequent intervals.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: M. P. I., Köln

Contract no.: BAP - 0075 - D

Project leader: A.P. CZERNILOFSKY

Scientific staff:

Address: Max-Planck-Institut für Züchtungsforschung
Abt. Genetische Grundlagen der Pflanzenzüchtung
Prof. Dr. J. Schell
Egelspfad
D - 5000 KÖLN 30

Telephone no.: +49.221.5062336 or +49.221.5062350

Telex no.: 8881028 MPIZ D

Other contractual partners in the joint project:

C.J. Leaver, University of Edinburgh

Title of the research activity:
Genetic transformation of plant mitochondria : development of a general strategy.

Key words:
Mitochondria, Transformation, Homologous recombination, Chloramphenicol selection

Reporting period: April 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Genetic transformation of plant mitochondria: development of a general strategy

Recombination of selectable marker DNA in Nicotiana tabacum

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Construction of selectable marker DNA to transform specifically plant mitochondria
Development of selection conditions with chloramphenicol for SR1 and Samsun tobacco
Transformation with those constructs of SR1 and Samsun

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Plasmid constructions involving the mitochondrial regulatory DNA sequences and the CAT gene of Proteus mirabilis have been completed.

2. The fusion protein has been shown to retain enzyme activity in tobacco protoplasts when expressed from the CaMV 35S promoter in transient assays.

3. When stably integrated into the nucleus of tobacco cells, and in preliminary transient expression studies, these constructions direct no detectable CAT activity suggesting that this mitochondrial DNA does not promote expression of the CAT gene in the nucleus. This will allow the specific expression of the gene in the mitochondria without a "background" of chloramphenicol resistant nuclear transformants. In bacteria, expression of the CAT gene from these constructs is also very weak suggesting that it will be similarly poorly expressed from

chloroplasts.
4. 3 kb of homology to the tobacco mitochondrial DNA has been additionally included in the initial constructs to potentially promote integration and maintenance of the foreign DNA by homologous recombination (see also enclosed summary, Wirtz et al.).
5. Initial experiments have been performed to develop selection conditions with chloramphenicol for SRI and Samsun tobacco varieties using nuclear marker genes as a model system. Attempts to improve the efficiency of recovery of transformants are underway.
6. Initial transformation with the mitochondrial plasmids have been started.

Recombination of selectable marker DNA in Nicotiana tabacum

Uwe Wirtz, Ian Moore, Jeff Schell, Armin P. Czernilofsky

Max-Planck-Institut f. Züchtungsforschung, D-5000 Köln 30, FRG
Tel. 221-5062336

Homologous recombination of foreign non viral DNA in cells of a higher plant was studied. These studies will hopefully provide the necessary basic information to achieve target specific integration into the nuclear genome or into the genome of organelles such as mitochondria.
A chimeric neomycin phosphotransferase II (NPTII) gene, which provides a selectable kanamycin resistance in transformed plant cells, was inactivated by in vitro deletions. Repair plasmids not containing plant specific transcription signals but only the NPTII coding region, or parts of it, were used in co-transformation experiments involving direct DNA uptake into protoplasts isolated from Nicotiana tabacum. Recombination, or gene conversion, mediated by homologous sequences produced active NPTII genes in about 1% of transformants, rendering these cells resistant to kanamycin. Analysis of the size of the active enzyme indicates that recombination processes have occured producing a NPTII gene indistinguishable from the wild-type gene. Southern blot analysis revealed that the bulk of co-transformed donor plasmid DNA has suffered structural modifications, however, Km resistance was inherited in a Mendelian fashion indicating that at least one functional and structurally intact copy of the regenerated NPTII gene is integrated into the host genome (2).

Presently we are exploring the possibility to improve the chances for transformation of mitochondria by inserting homologous mitochondrial sequences into the vectors used for transformation (1; Cross-reference to joint project see:
Genetic Transformation of plant mitochondria: Development of a general strategy
Ian Moore*, Armin P. Czernilofsky, Jeff Schell, and C.J. Leaver*
*Dept. of Botany, University of Edinburgh, Mayfield Road, Edinburgh, UK, EH9 3JH, Tel. 31 667 1081 (3304)

(1) A.P. Czernilofsky, B. Baker, B. Gronenborn, R. Hain, C. Leaver, V. Matzeit, I. Moore, J. Schalk, U. Wirtz, and J. Schell (1987). Fate and expression of vector DNA in plant cells. In: Tailoring Genes for Crop Improvement: An Agricultural Perspective, G. Bruening, T. Kosuge, J. Harada, A. Hollaender (eds.), Plenum Press, New York, pp. 189-195.
(2) U. Wirtz, J. Schell, and A.P. Czernilofsky (1987). Recombination of selectable marker DNA in Nicotiana tabacum. DNA, in press.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

None.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Exchange of material and staff for mitochondrial transformation experiments

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: U. C. L., Louvain-la-Neuve
Contract no.: BAP - 0019 - B

Project leader: M. BRIQUET
Scientific staff: A. Vassarotti, E. Selva

Address: Université Catholique de Louvain
Faculté des Sciences Agronomiques
Laboratoire d'Etude de l'Hérédité Cytoplasmique - Place Croix du Sud 1
B - 1348 LOUVAIN-LA-NEUVE

Telephone no.: +32.10.433615

Telex no.: 59037 UCL B

Other contractual partners in the joint project:

A. Cornu, I. N. R. A. (Dijon)
D.R. Davies, John Innes Institute (Norwich)
H.J.J. Nijkamp, University of Amsterdam
F. Quétier, Université de Paris XI

Title of the research activity:
Mitochondrial molecular genetics in relation to crop improvement.

Key words:
Cytoplasmic male-sterility, Plasmids, Mitochondrial transformation, Protoplast culture, Faba bean, Wheat

Reporting period: January 1986 - July 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The specific aims of the present joint proposal are to characterize the mitochondrial genomes of different species to clone and compare sequences, to identify particular genes, ori and enhancer sequences, to characterize mitochondrial plasmids and to establish homology between part of different genomes within the mitochondrial and within the cell. The expression of certain sequences will be studied with particular reference to differences between CMS and fertile forms. The transfer of mitochondria by microinjection and somatic cell fusion techniques will be attempted. Transformation of mitochondria by using constructed new hybrid vectors will be attempted also.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Sequencing of the mitochondrial plasmids of faba bean and analysis of the expression products in both fertile and sterile lines.

Construction of mitochondrial DNA library. Preparation of antibodies against the variant polypeptides. Culture of protoplasts of Vicia faba and regeneration.

Analysis of transcription products of CMS genes from faba bean and wheat.

Construction of a vector for mitochondrial transformation.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Mitochondrial plasmids of faba bean

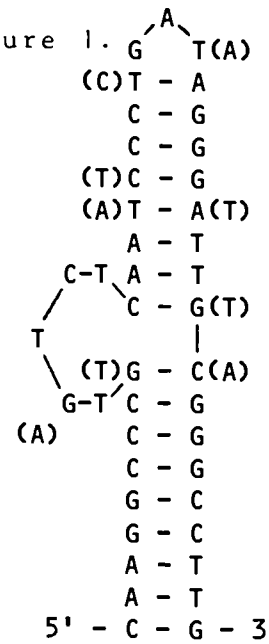
1.A. Nucleotide sequences

Methodology

The mitochondrial plasmids were cloned in pEMBL8 plasmid (Dente et al., 1983, Nucl. Ac. Res. 11, 1645-1655) and sequenced by the method of Sanger et al., 1977, P.N.A.S. 74, 5463-5467).

Results and discussion

Figure 1.



In faba bean, male fertile and male sterile lines possess mitochondrial minicircular plasmids in addition to the main mitochondrial DNA. The complete nucleotide sequences of six plasmids have been determined.

Computer analysis revealed that the 1700S (1704 bp) plasmid, specifically associated with the male sterile 447 and 350 cytoplasms, cannot come from the 1700F (1695 bp) plasmid, found in both fertile and male sterile cytoplasms.

Additional plasmids of 1657, 1559 and 1576 bp observed in some lines bearing the male sterile 350 cytoplasm seem to result from recombination event between the two 1700 bp plasmids.

Direct repeats containing inverted repeats are present in all the mitochondrial plasmids including the 1478 bp plasmid found in fertile and male sterile cytoplasms. This secondary structure folded into hairpin structure is reminiscent of origin of replication of mitochondrial genome (Fig. 1).

1.B. Transcripts

A major RNA product of approximately 490 nucleotides for the 1700 F plasmids, two major products of 430 and 360 nucleotides for the 1478 bp plasmid and a RNA product of 400 nucleotides for the 1700 S plasmid are found.

2. Cytoplasmic male sterility in faba bean and wheat : RNA transcripts

Methodology

Northern hybridization are used to identify differences in the mitochondrial RNA transcripts in both male sterile and fertile cytoplasm. The mitochondrial library of faba bean were cloned in the pTZ19R vector and the wheat library in the cosmid pHC79 (Orsay, F. Quetier and A. Lejeune).

Results

Preliminary results indicate almost one difference in RNA transcript between the fertile and the male sterile cytoplasm (Timopheevi) of Triticum aestivum.

3. Mitochondrial transformation

Methodology

A genomic bank of N. plumbaginifolia mitochondrial DNA was constructed and probed with the maize gene for the alpha subunit of the mitochondrial F1-ATPase. We isolated a clone with a 4.2 Kb HindIII insert containing the entire ATP1 gene and his 5' region. After subcloning and RNA analysis (to assess the level of expression as well as the size(s) of the message(s), the ATP1 region corresponding to the structural part of the gene was sequenced.

Results

The N. plumbaginifolia F1-ATPase alpha subunit was highly homologous to the corresponding protein from maize. Gene fusions of the 5' region with the bacterial lac z gene were constructed. The corresponding hybrid protein was expressed in E. coli transformants showing that a mitochondrial promotor is active in an heterologous system. In the near future we are planing to employ the ATP1 promoter in our attempt to express foreign genes into plant mitochondria.

4. In vitro culture of faba bean protoplasts

The first effort undertaken toward V. faba protoplasts culture was to overcome the polyphenol oxidation reactions that take place in donor tissue during enzymatic digestion. This oxidation didn't enable protoplasts isolation but affect their viability in culture.

A short time washing (10 minutes) of tissue with thiols reagents in osmoticum, just before the incubation in the enzymatic mixture can avoid this oxidation. Cell wall digestion was also speeded up by this procedure without affecting protoplasts viability.

Protoplasts isolated from in vitro cultured shoots showed a good ability to sustain first divisions within 4 days but they failed to give rise to colonies. Attempts to isolated protoplasts from cell suspension culture failed due to the incapacity of the different enzymatic mixtures tested to digest the cell walls.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

Short communications, abstracts, thesis

- Goblet, J.P., Kunze, N., Grimard, A., Boutry, M. and Briquet, M. (1987) Nucleotide sequence of the mitochondrial plasmids from faba bean. Arch. Int. Physiol. Biochim. 95, B72.

- Goblet, J.P., Flamand, M.C., Kunze, N., Boutry, M. and Briquet, M. (1987) Nucleotide sequence and transcription analysis of mitochondrial plasmids from fertile and CMS lines of Vicia faba L.. BAP-Meeting on "Genetic and Cellular Engineering of Plants and Microorganisms important for Agriculture" Louvain-la-Neuve, March 23-26, Abstract pp. 144-145.

- Leterme, S., Scheepers, D., Gilson, P. and Briquet, M. (1987) Cytoplasmic male sterility and variant mitochondrial polypeptides. BAP-Meeting on "Genetic and Cellular Engineering of Plants and Microorganisms important for Agriculture" Louvain-la-Neuve, March 23-26, Abstract pp. 151-152.

- Vassarotti, A., Chaumont, F., Di Antonio, C., Selva, E., Flamand, M.C., Boutry, M. and Briquet, M. (1987) Towards mitochondrial transformation. BAP-Meeting on "Genetic and Cellular Engineering of Plants and Microorganisms important for Agriculture" Louvain-la-Neuve, March 23-26, Abstract pp. 117-118.

- Goblet, J.P. (1987) Caractérisation moléculaire des plasmides mitochondriaux dans le cadre de la sterilité mâle cytoplasmique de la féverole (Vicia faba L.). Doctoral thesis. Faculty of Agronomy. University of Louvain, Louvain-la-Neuve, Belgium.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Exchange of material(s):

- A mitochondrial library (SalI partial and total digest in Cosmid pHC79 and pBR322 received from the laboratory of Dr. F. Quetier (Orsay).
- Mitochondrial genes cytochrome oxidase (sub-unit I and II) and of apocytochrome of Maize received from Dr. C. Leaver (Edinburgh).
- Nucleotide sequence of mitochondrial plasmids of sugar beet received from Dr. C. Thomas, Dr. R. Davies (Norwich).
- Nucleotide sequence of mitochondrial plasmids of Vicia faba sent to Dr. C. Thomas for computer analysis.
- Exchange of manuscripts between C. Thomas (Norwich) and J.P. Goblet (Louvain-la-Neuve), and between Dr. M. Briquet and P. Pfeiffer (Strasbourg-Dijon).

Exchange of staff:

Dr. F. Quetier (Orsay) was member of the jury of J.P. Goblet's doctoral thesis (Louvain-la-Neuve).

Joint experiments

Dr. P. Tuduri joined the laboratory of Ph. Lebacq (Orsay) for a joined experiments on non-radioactive method for probing mitochondrial DNA of wheat and rice.

Joint meetings

Joint meeting between the staffs of the 7 laboratories working on CMS in September 17-18 in Norwich (organized by Prof. R. Davies).

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: I. N. R. A., Dijon Contract no.: BAP - 0016 - F

Project leader: A. CORNU
Scientific staff: A. Bervillé, H. Dulieu, G. Duc, P. Nicolas

Address: Station de Génétique et
d'Amélioration des Plantes
I.N.R.A.
B.P. 1540
F - 21034 DIJON CEDEX

Telephone no.: +33.80397221

Telex no.: 350507 INRADJ F

Other contractual partners in the joint project:

M. Briquet, U. C. L. (Louvain-la-Neuve)
D.R. Davies, John Innes Institute (Norwich)
H.J.J. Nijkamp, University of Amsterdam
F. Quétier, Université de Paris XI

Title of the research activity:
Mitochondrial molecular genetics in relation to crop improvement.

Key words:
Organelle DNA, Cytoplasmic male sterility, Transfer of mitochondria, Virus-like particles, Nuclear atp genes

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The specific aims of the present joint proposal are to characterise the mitochondrial genomes of several cultivated species, to clone and compare sequences, to identify particular genes, to characterise certain mitochondrial plasmids and to establish homology between parts of different genomes within the mitochondria and within the cell. The expression of certain sequences will be studied in vitro, in vivo and in expression vectors with particular reference to differences between cms and fertile forms and to the effects of nuclear genes on mitochondrial gene expression.

The transfer of mitochondria as well as the generation of variant forms will be attempted by microinjection and somatic cell fusion techniques as well as by sexual crosses.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

1. Sugar Beet and relative species : research on origins of CMS, using chloroplastic-DNA's variability. (A. Bervillé, Dijon).
2. Study on nuclear genes coding for mitochondrial proteins in Helianthus (P. Nicolas, Clermont-Ferrand).
3. Attempts to transfer mitochondrial information by the pollen parent (H. Dulieu and A. Cornu, Dijon).
4. Cytoplasmic male sterility in Vicia faba : characterization of RNA containing particles (RCP) concentrated in C.M.S. tissues (P. Dulieu and H. Dulieu, Dijon).

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Studies on cp-DNA variability in sugar beet CMS and relative species

The Owen source of sugar beet CMS is characterized by :

. a specific set of mitochondrial plasmids ; a specific profile of the restricted mt DNA ; a specific profile of the restricted chloroplastic DNA - most of the labs work on the mtDNA ; however we proposed to used the cpDNA as tracer of the cytoplasmic history of the Owen CMS and some wild species.

Methodology

The cpDNA was prepared from leaves of plants 2 to 3 month old.
A collection of wild species was collected and about 3 ecotypes for each species were studied.

A relationship between the botanical classification and the cpDNA variation was looked for.

Results

The genus Beta is divided into 3 sections : vulgares, corollinae and patellares. We found 3 types of cpDNA with a slight variability within each group. Obviously some ecotypes were mis marked because of the shape of the plants and by their cpDNA. Obviously the cpDNA of the CMS form was closely related to the vulgares section. About 30 origins of beets (sugar, table and forage) were collected and compared to the CMS one by their cpDNA The cpDNA of the vulgares section appeared unvariable for several restriction endonuclease. B. maritima and most of the sugar beet, forage beet and the table beet carry the same cpDNA. Nevertheless B. macrocarpa gave a specific diagram. Some ecotypes of B. macrocarpa could be contaminated by B. maritima as judged by the cpDNA. On the other hand, a table beet "crapaudine" carries a cpDNA with the same restriction profile as the Owen's CMS. Since crosses between table beet and sugar beet were carefully avoided for two hundred years it is reasonable to think that "crapaudine" is probably the donor of cytoplasm to the Owen source. Crosses are in progress to verify this hypothesis.

2. Study on nuclear genes coding for mitochondrial proteins in Helianthus

Methodology

Nuclear DNA was prepared from isolated nuclei.DNA was digested with restriction endonucleases. Digests were fractionated by agarose electrophoresis and transferred to nitrocellulose filters according to Southern. Blots were hybridized according to Jeffreys and Flavell. A cDNA library was prepared from poly $(A)^+$ mRNA, using an oligo dT primer and cloning with λ GT 11.

Results and discussion

We have entered upon the study of the restriction polymorphism of the nuclear genes among the Helianthus family ; the work was centered on the nuclear atp 2 gene coding for the β subunit of the mitochondrial ATP synthase. DNA fragments were hybridized using a cDNA coding for the Nicotiana gene (obtained from Dr. Boutry). This probe also cross-hybridizes with the chloroplast atp B gene, coding for the β subunit of the chloroplast ATP synthase. Among the different lines of species studied within the Helianthus family, no polymorphism was detected for the chloroplast atp B gene, whereas some variability appears for the nuclear atp 2 gene. In order to obtain the homologous probes for the study of the variability of the nuclear genes, and to allow the sequencing of the atp 2 gene, we have undertaken the construction of a cDNA library. The better conditions for the obtention of the poly $(A)^+$ mRNA have been investigated. A cDNA library was obtained by cloning in λ GT II. Inserts of this library are presently studied.

3. Attempts to transfer mitochondrial information by the pollen parent

Methodology

a. Plastome and plasmone introduction by recurrent backrosses. Progeny-tests. Use of marker genes.

b. cp-DNA and mt-DNA isolation from green leaves. Purification ; restriction endonuclease incubation ; agarose gel electrophoresis and fluorophotography of the ethidium-bromide colored gels.

Results and discussion

a. The line Tbl-3 of Petunia hybrida Hort. was shown to transfer plastid-DNA through the male gametophyte in spite of the strict maternal inheritance of plastids expected in Petunia.

b. A genetic construction of a Tbl-3 nuclear genome with a cytoplasmone from Petunia parodii used as pollen tester showed that this pt-DNA can be recovered in green sectors from chimeral plants segregating virescent (TV) and green plastids.

c. A maternal receptor of both plastids and mitochondria was obtained by exceptional transfer of virescent (TV) plastids through the pollen into embryosacs carrying cms cytoplasm and green plastids.

d. Partial reversion towards partial fertility has been found among the progenies between with TV plastids and cms mitochondria and paternal carrying Parodii plastids and mitochondria.

e. Identification of gene factors governing transfer is tempted using pollen from Fl hybrids between Tbl-3 and non-permissive lines carrying marker genes.

4. Cytoplasmic male sterility in Vicia faba

Methodology

a. Cell fonctionation. Sucrose gradient centrifugation. Column chromatography (gel filtration ; immuno-affinity). PAGE and agarose electrophoresis.

b. Rabbit immunization. Immunoblotting. Elisa.

c. Electron microscopy.

Results and discussion

a. Further purification of RNA containing particles was successfull. The presence of a double-stranded RNA around 12.10^6 daltons of molecular weight was steadily associated with the particles.

b. Antiserum against highly purified RCP was obtained. The response was of the classical optimum type when reaction was made against increasing concentrations of purified RCP.

c. The serum exhausted against extracts from maintainer fertile plants yielded very specific responses against post mitochondrial extracts from RCP-containing tissues.

d. The -Elisa test allowed to detect, very early in development, individuals carrying particles ; the correlation between the positive response and sterility equals 1 until now in the "447" RCP containing cytoplasm.

e. Antigenic determinants are under investigation.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

Several publications are in progress or submitted.
A complete list with be provided in final report.

. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	No
Joint experiment(s)	No
Joint meeting(s)	No

Descriptive information for the above data.

. The cDNA coding for the Nicotiana atp 2 gene was a gift from Dr. Boutry (Prof. Briquet Laboratory, Louvain-la-Neuve, Belgium) to Dr. P. Nicolas (Université de Clermont-Ferrand).

. Achieved preparation of new genetic material of Vicia faba (from G. Duc, Dijon) for mt-DNA analysis (Dr. Briquet, Louvain-la-Neuve).

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: John Innes Institute, Norwich
Contract no.: BAP - 0017 - UK

Project leader: D.R. DAVIES
Scientific staff: C. Thomas, W.A. Lawrence, G. Hussey, N. Ellis, I. Bedford

Address: John Innes Institute
Colney Lane
UK - NORWICH NR4 7UH

Telephone no.: +44.603.52571 EXT.347

Telex no.: 975122 JIINOR G

Other contractual partners in the joint project:

M. Briquet, U. C. L. (Louvain-la-Neuve)
A. Cornu, I. N. R. A. (Dijon)
H.J.J. Nijkamp, University of Amsterdam
F. Quétier, Université de Paris XI

Title of the research activity:
Mitochondrial molecular genetics in relation to crop improvement.

Key words:
Mitochondria, Sugarbeet, Microinjection, Cytoplasmic male sterility

Reporting period: April 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

To characterise the mitochondrial genomes of sugarbeet and in doing so identify the lesions within them which result in cytoplasmic male sterility. In addition we wish to develop a technology for transferring mitochondria between different varieties of a given species and to examine the consequences of such a transfer.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

To complete the characterisation of the minicircular DNA molecules present in sugarbeet mitochondria; this involves sequencing them and establishing the nature of any transcripts. A study has also been initiated to detect uniquely transcribed genes in CMS and MF mitochondria. A second objective has been to produce protoplasts of sugar beet which can be cultured at very low densities so that they can be used in the microinjection procedures developed in this laboratory.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Mitochondrial characterisation

Preparation of mitochondria, isolation of mitochondrial nucleic acids and Northern/Southern blotting were all as described previously (Thomas, 1986). SI-nuclease protection analysis was performed as described by Berk and Sharp (1977).

Results

The DNA sequence of the three minicircular DNAs present in the mitochondria of most male fertile (MS) lines of sugarbeet have been

determined and their relationship to each other and the main genome determined (Thomas, 1986). Also, sequences common to all three DNAs which may be essential to their maintenance, perhaps functioning as replication origins have been detected by computer analysis. We report here on our recent experiments to define sequences regulating transcription of sugarbeet minicircular mtDNAs. Also, we report preliminary experiments to identify unique gene sequences transcribed in MF and cytoplasmic male sterile (CMS) mitochondria.

Northern blot analysis of mtRNA from CMS and MF sugarbeet showed two minicircle a (Mc.a) transcripts of c.550 and c.450 nucleotides. A single major transcript of minicircle d (Mc.d) of c.480 nucleotides is present exclusively in MF lines as are several transcripts (1120, 920, 720 and 660 nucleotides) of minicircle c (Mc.c). The polarity of these transcripts has been determined previously (Thomas, 1986). Cloned subgenomic restriction fragments terminally labelled with P32 were used in SI-protection experiments to determine the approximate location of transcription start points. In subsequent experiments SI-protected fragments were electrophoresed against Maxam/Gilbert sequence ladders of end-labelled DNAs to enable precise determination of transcript termini. Mc.c transcripts, which appear to be 5' co-terminal, mapped to a sequence showing extensive homology to the Mc.d transcript 5' terminus, and one of the two Mc.a transcripts (see Table 1).

TABLE 1 - Sequences surrounding the 5' terminus of the three sugarbeet minicircle transcripts showing their homology to each other, to the recently determined Oenothera cytochrome oxidase subunits I and III (Hiesel et al, 1987) and a recently proposed consensus for plant mt promoters (Young et al. 1987). The arrow indicates the proposed transcription start points.

Mc.c transcripts	T A A A A T C A T A A G T G A T
Mc.d transcript	A A A T A C C A T A A G T G A C
Mc.a transcript -1	A A A T A T C G T A A G T G A G
Oenothera CoxI/CoxIII	C A A T T G C G T A A G T G A G
Consensus sequence for mt promoters	A A A T Y T C N T A A G $\frac{A}{T}$ G A A
	↑

A second Mc.a transcript was detected in SI-mapping experiments which protects a restriction fragment encompassing 90% of a short open reading frame (ORF). The ORF (111 amino acids) exhibits several features of expressed plant mitochondrial genes (Thomas, 1986). The putative 5' terminus is located 6 nucleotides downstream of the second in-frame AUG codon. However, the nucleotide sequence in this region does not resemble any putative promoter sequence and raises the possibility that this "terminus" is a splice function. This is currently being investigated. As yet, the 3' termini of Mc.a transcripts have not been mapped unambiguously.

Experiments designed to detect uniquely transcribed genes in CMS and MF mitochondria have also been performed. P32-labelled mtRNA

from each line was used sequentially to probe Southern blots of restricted CMS and MF mtDNA. The bulk of the labelled RNA hybridizes to 5S, 18S and 26S rRNA genes (identified by heterologous hybridization using clones from wheat, a gift of D. Falconet). Several differences in hybridization pattern between MF and CMS lines were observed in presumptive protein coding sequences. Further experiments are being performed with an exclusively mRNA fraction. This should facilitate easier identification of restriction fragments harbouring uniquely transcribed sequences which will be isolated from restriction fragment libraries of MF and CMS mtDNA constructed in this laboratory.

Discussion

A thorough understanding of the molecular biology of sugarbeet minicircular mtDNAs in terms of their sequence organization, transcription and replication will be useful in their development as potential transformation vectors for higher plant mitochondria. The sequences of all three DNAs present in MF lines have been determined (Thomas , 1986) and we now have some details of their transcripts. The proposed promoter sequences of all three DNAs show a striking resemblance to the few putative plant mt gene promoters (see Table 1). The use of extrachromosomal DNAs to determine transcription control signals has considerable advantages over the main genome. The DNA sequences can be easily and quickly determined and the structure of mature transcripts more readily defined.

Minicircle DNAs will be useful markers for transferred mitochondria, although for sugarbeet this technique will only be of use in detecting MF mitochondria transferred to a CMS recipient. Main genome markers are required for detecting CMS mitochondria transferred to an MF recipient. It is envisaged that the preliminary investigations described here to define uniquely transcribed genes in CMS or MF mitochondria will provide suitable main genome markers in addition to providing a basis for understanding the molecular mechanism of CMS in sugarbeet.

References

Thomas, C.M. (1986) - Nucl. Acids Res. 14, 9353-9370.
Young, E.G. et al. (1986) - Nucl. Acids Res. 14, 7995-8006.
Berk, A.J. and Sharp, P.A. (1977) - Cell 12, 721.
Hiesel, R. et al. (1987) - EMBO J. 6, 29-34.

Mitochondrial transfer

Methodology and Results

1) Production of protoplasts

Sugar beet seeds are sterilized by soaking in a 0.3mg ml^{-1} solution of ethyl mercury phosphate with a few drops of Tween 20 for 20 minutes. After 5 washes in sterile distilled water they are plated on MS salts (Murashige and Skoog, 1962) solidified with 0.7% agar and incubated at 20°C in the dark. After 2 days they are again washed in sterile distilled water, plated on fresh MS salts agar and

incubated as before. Hypocotyls are removed when they reach 15mm in length, cut into 5mm sections and plated on MS medium with 0.7% agar and 3mgl^{-1} Benzyl amino purine (BAP) to initiate callus. The callus is then plated on MS medium containing half the concentration of macro-nutrients (½ MS) and 1mgl^{-1} BAP. When sufficient callus is available from a single hypocotyl it is transferred to liquid MS medium with 0.1mgl^{-1} BAP. Established suspension cultures are subcultured every three days and protoplasts prepared from them using a modification of the method of Szabados and Gaggero (1983). A solution containing 2% cellulysin (Calbiochem), 0.5% Macerozyme R10 (Yakult Honsha Co. Ltd.), 0.8M mannitol, 0.3mM MES buffer, 0.64mM NaH_2PO_4 and 6.8mM $CaCl_2$ is added to a similar volume of suspension culture and the mixture shaken gently for 16 hours at 25°C. The protoplasts released are filtered through a 100µ nylon mesh and then floated on a cushion of BE salts (Banks and Evans, 1976) with 16% sucrose to remove debris. They are washed twice with WS solution (Menczel et al., 1981) then resuspended in A2 medium which is the A medium of Kao and Michayluk (1981) with 0.03M sucrose, 0.39M mannitol, 0.1mgl^{-1} Naphthalenacetic acid (NAA), 0.5mgl^{-1} BAP but omitting glucose and 2,4-dichloro-phenoxyacetic acid (2,4-D).

2) Low density protoplast culture

The basic method for low density culture is as previously described by Lawrence and Davies (1985) except that A2 medium is used throughout and a modified system for feeding microinjected protoplasts is employed. Agarose microdrops are prepared by the standard method and 1µl of protoplasts in 0.6% agarose is placed in the centre of each. Protoplast density is adjusted to give the required final number per microdrop - usually 5 to 15. Microdrops are then transferred directly to feeder plates by sliding them from coverslips onto the feeder plate surface. Feeder plates consist of a layer of protoplasts at $5x10^4$ ml^{-1} embedded in A2 medium with 0.7% agarose and covered with the same medium to avoid direct contact with the microdrops. Using this method at least 16% of protoplasts plated in microdrops give rise to large calli capable of sustained growth. In certain cases the plating efficiency can be as high as 40%.

3) Transfer of mitochondria between protoplasts by microinjection

Microinjection procedures are those of Lawrence and Davies (1985). Standard microinjection needles with tip diameters of 1µl or less are broken to give a bevelled snape with a larger aperture. Both MF and CMS protoplasts are prepared in microdrops. Cytoplasm is withdrawn in the needle from one protoplast and injected into another. Once all the protoplasts in a microdrop have been injected it is placed directly onto a feeder plate without covering the injected protoplasts.

4) Regeneration of injected protoplasts into calli

After 2-3 weeks on feeder plates calli derived from protoplasts in the microdrops are up to 1mm in diameter. The microdrops are then removed from the feeder plates and placed on ½ MS medium with 1mgl^{-1} BAP. On this medium callus multiplies rapidly.

Discussion

The basic tissue culture procedures are now developed to a satisfactory stage, and thus will enable experiments on the transfer of mitochondria to be attempted, and methods for detecting the persistence of transferred organelles to be tested.

References

1. Banks, M.S., Evans, P.K. (1976) Plant Science Letters 7, 409-416.
2. Kao, K.N., Michayluk, M.R., (1981) In Vitro 17, 645-648.,
3. Lawrence, W., Davies, D.R. (1985) Plant Cell Reports 4, 33-35.
4. Menczel, L., Nagy, F., Kiss, Z.R., Maliga, P. (1981) Theor. Appl. Genet. 59, 191-195.
5. Murashige, T., Skoog, F. (1961) Physiol. Plant. 15, 473-497.
6. Szabados, L., Gaggero, C. (1983) 6th Int. Protoplast Symposium. 38-39.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

I. Thomas, C. M. (1986) Nucleic Acids Research 14, 9353-9370.
Lawrence, W. and Davies, D.R. (1987) Plant Science 50, 125-132.

IV. Thomas, C. M. The enzymology of cauliflower mosaic virus DNA replication. PhD Thesis. 1986.

TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	No
Joint meeting(s)	No

Descriptive information for the above data.

Exchange of Material

Dr. Thomas has received mitochondrial ribosomal RNA clones (26S, 18S and 5S) from the laboratory of Prof. F. Quetier, in Orsay, France.

Exchange of staff

Dr. W. Lawrence visited the laboratory at Orsay, Paris in April 1986 for a period of 3 weeks.

Dr. S. Bazetoux and Dr. I. Sissoeff from Orsay, Paris, visited the John Innes Institute from 15-19th June 1986.

(Centre d'Orsay, Universite de Paris-Sud, Orsay 91405, Paris, France)

Joint Meetings

In view of the fact that there was an annual meeting of all EEC contractors no special meeting was arranged. However one is planned for September 1987.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Vrije Univ., Amsterdam

Contract no.: BAP - 0020 - NL

Project leader: H.J.J. NIJKAMP

Scientific staff: J.M. de Haas, M. Haring, C.E. Jansen, H. Witsenboer, M.L.M.C. Tan, V. Rodeva, M.I. Spassova and J. Hille

Address: University of Amsterdam
Dept. of Genetics
De Boelelaan 1087
NL - 1007 MC AMSTERDAM

Telephone no.: +31.20.548.57.52 or 548.57.53

Telex no.: 11329 DPVVU NL

Other contractual partners in the joint project:

M. Briquet, U. C. L. (Louvain-la-Neuve)
A. Cornu, I. N. R. A. (Dijon)
D.R. Davies, John Innes Institute (Norwich)
F. Quétier, Université de Paris XI

Title of the research activity:
Mitochondrial molecular genetics in relation to crop improvement.

Key words:
Petunia hybrida, Lycopersicon (tomato), Cell organelle replication origin, Selectable cell organelle marker, Cybridization

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The structure and function of the genetic systems of plant mitochondria is only beginning to be elucidated, however, the technologies of molecular biology and of somatic cell genetics are now allowing rapid advances to be made. The aims of the joint project are to characterize the mitochondrial genomes of several cultivated species and to transfer mitochondria, or specific mitochondrial encoded genes, to different plant species. From this will come the ability to manipulate these genetic systems for crop plant improvement.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The most direct approach for introduction of desirable cytoplasmic properties is transfer of specific genes to plant cell organelles. As a first step towards this goal potential replication origins of organelle genomes are characterized and analyzed with respect to *in vitro* functioning. This information is used for the design of vectors which are applied for the development of a cell organelle transformation method. For an efficient transfer of cell organelles using cybridization it is advantageous to have the availability of selectable markers. To this end, a programme has been set up for the isolation of mutants containing selectable markers encoded by the chloroplast and mitochondrial genomes.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Methodology

Autonomous replicating sequences (ARS) in yeast of *Petunia hybrida* chloroplast (cp) and mitochondrial (mt)DNA were isolated as described (1).

The chloroplast DNA synthesizing lysate system was prepared from resuspended purified chloroplasts and assay conditions for *in vitro* DNA synthesis were performed as described (2, 6). Molecular techniques (hybridizations, DNA isolations, digestions gelelectrophoresis, cloning, sequencing) were performed according to Maniatis *et al.* (4).

Nicotiana tabacum SR1 leaf mesophyll protoplasts were transformed with electroporation or the PEG/Mg^{++} method according to Negrutiu *et al.*, (5). Selection for plant cell transformants was performed at 50 mg/l for kanamycin and 20 mg/l for chloramphenicol. Plant cell mutagenesis was essentially performed as described by Hosticka and Hanson (3).

Results

Potential replication origins of cell organelle genomes.

Because replication origins contain potential stem and loop structures, GC-rich blocks, direct and inverted repeats and AT-rich stretches of nucleotides, we isolated *P. hybrida* cp and mt ARS sequences (which originate from AT-rich regions of the DNA containing such structures).

Three cpDNA ARS regions (ARS A, B and C) have been isolated, mapped on the physical map, subcloned and sequenced. The cpDNA region adjacent to ARS B might be part of a potential replication origin because it contains a 100bp AT-rich region which shows extensive sequence homology to a *Euglena gracilis* cpDNA fragment which is part of the replication origin (divided in 5 boxes of nucleotides), and it shows many characteristics that have been found in sequenced replication origins (1).

The cpDNA ARS sequences were tested for functioning as replication origins in the *in vitro* DNA synthesizing system isolated from purified chloroplasts (2). When pPCY20-1 (containing the 1.8 Kbp *Hind*III/*Eco*RI ARS A fragment) is added to the organelle free lysate system the vector and the ARS A insert are both labeled in a non-specific (random) manner (Fig. 1A), probably as a result of repair DNA synthesis. However, when pPCY62 (containing the 5.9 Kbp *Hind*III ARS B/C fragment) is used as a template, part of the cloned ARS B region is more strongly labeled than the vector (the 1.60 Kbp fragment in Fig. 1B.

Moreover, when a mixture of all *Hind*III/*Eco*RI and EcoRI subfragments of pPCY62, cloned in M13, serves as a single stranded DNA template in the lysate system, and when after isolation these labeled template DNA's are used as probe for a blot containing a *Hind*III/*Eco*RI/*Sac*I digest of plasmid pPCY62, the 1150 and 450 bp fragments are labeled more intensively than the other pPCY62 insert fragments. (Fig. 1D). The 100 bp region that forms part of, or is localized closely to, the putative cpDNA replication origin is located in the 1150 bp fragment. This suggests that *in vitro* the DNA synthesis initiates in or close to this 100 bp region (2). The observed DNA synthesis proceeds towards the ribosomal RNA genes (in the inverted repeat), but till now "bidirectional DNA synthesis" cannot be excluded.

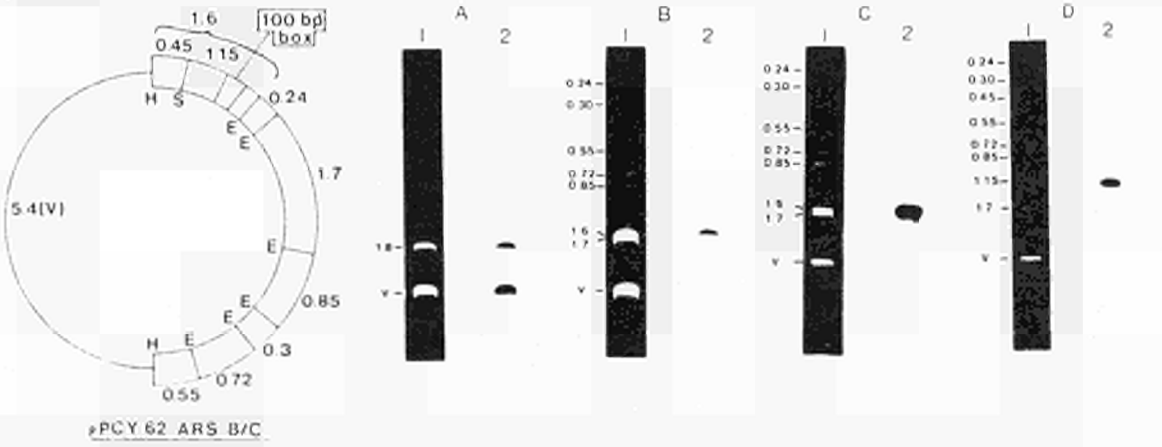


Fig. 1. Labeling of ARS containing plasmids in DNA synthesizing chloroplast lysate system.
1: EtBr stained agarose gel, 2: Autoradiograph of agarose gel.
A/B: Labeling of double stranded pPCY20-1 (lane A; ARS A) and pPCY62 (lane B; ARS B/C. digested with *Eco*RI (E)/*Hind*III (H).
C/D: Labeling of a mixture of pPCY62 single stranded *Eco*RI and *Eco*RI/*Hind*III subfragments (cloned in M13) which was used as a probe for blotted *Eco*RI/*Hind*III digested pPCY62 (C) and *Eco*RI/*Hind*III/*Sac*I (S) digested pPCY62 (D).

Four mtDNA/ARS regions have been isolated, subcloned and sequenced (ARS I, II, III and IV, called pPMY-plasmids). All four ARS's are mtDNA and not cp or nuclear DNA as was determined by Southern blot analysis. The mt ARS's contain, in addition to the

conserved yeast core ARS consensus sequence (5' A/TTTTATPuTTTA/T, which is essential for ARS activity in yeast) two other semi-conserved mt yeast ARS consensi, one upstream (5' TNTPuAA) and one downstream (CTTTTAGCA/TA/TA/T) towards the core consensus. They all contain several origin of replication characteristics. All four mt ARS fragments show, apart from the presence of the yeast ARS consensi, no specific homology with the 100 bp cpDNA region which is part of the potential cpDNA replication origin region. However, in ARS III and IV there is a 150 bp region which shows homology with that part of the replication origin region of the yeast 2 micron plasmid that can replicate autonomously both *in vivo* and *in vitro*. The homologous region consists of 6 blocks of nucleotides (ranging from 4-9 nucleotides). In the centre of this region a stable stem and loop structure can be folded in both ARS IV and in the 2 micron plasmid. In the corresponding stem and loop of the opposite strand both an ARS consensus and a potential yeast gyrase recognition site are present. Furthermore ARS III and IV contain the same structural components as the yeast mitochondrial replication origin (ARS core, GC-rich clusters, stem and loops, yeast primase and gyrase recognition sites). Moreover, ARS IV shows the same orientation and spacing of these structural yeast mtDNA replication origin components.

Attempts to develop a cell-organelle transformation method.

Chimaeric chloroplast marker genes were constructed using chloroplast DNA promoter fragments and the transposon Tn9 encoded chloramphenicol acetyltransferase (Cat) gene. A 190 bp and a synthetic 55 bp *psb*A, a 371 bp *rbc*L and a 437 16S rRNA promoter fragment were fused to the promoterless Cat-gene. The fusion sites were sequenced and it was shown that all chimaeric genes are expressed in *E. coli*.

Potentially replicating vector plasmids were constructed by combining the rbcL-Cat gene with plasmids containing respectively the arsA, B and C fragments. Integrating vectors contain segments of so-called "silent regions" of the chloroplast genome. To this end, the rbcL Cat gene was cloned into cpDNA containing plasmids, outside of structural or regulatory chloroplast DNA sequences. This results in a chimaeric gene being flanked by DNA segments that allow homologous recombination to direct the integration into the chloroplast genome.

Both the potentially replicating and the integrating vectors were applied for transformation of *N. tabacum* SR1 leaf mesophyll protoplasts using electroporation or the PEG/Mg^{++} method. A plasmid, containing the Npt-II gene from Tn5 under transcriptional control of the 35S promoter of CaMV and containing the polyadenylation signal of the nopaline synthase gene, was used as a control for nuclear transformation under the applied conditions. However, with optimal transformation conditions for selection of kanamycin resistant calli, (transformation frequency approximately 1%), we were not yet able to obtain chloramphenicol resistant calli.

Isolation of selectable cell organelle markers.

Since spontaneous plant cell organelle mutations occur only in low frequency, we choose a mutagen to enhance this frequency. To this end, nitroso methyl urea (NMU) is used because this mutagen was shown to be most effective for the induction of cell organelle mutations (3). Effective concentrations of NMU have been determined at the level of tomato-protoplasts, leaf explants and seeds. Following this approach we wish to obtain selectable markers for chloroplasts (e.g. streptomycin resistance) and mitochondria, which will subsequently be used in somatic cybridization experiments.

Discussion

The experiments with the *in vitro* DNA synthesizing lysate system demonstrate that (part of) the cpARS B region functions as origin of replication *in vitro*. Whether it is also functioning *in vivo* will be further investigated by looking for replication in cell organelles. Investigations will focuss on experiments aimed at determination of the direction of DNA synthesis and on EM studies aimed at the detection of replication loops.

The homology with the functional 2μ origin of replication and the presence of structural replication origin components (which are also localized in the 2μ plasmid and in the yeast mitochondrial replication origins) make the ARS III and especially the ARS IV region of the *P. hybrida* mtDNA at the moment the best candidates for being a replication origin. This will further be investigated along the same lines as described for the chloroplast work. At the moment an *in vitro* DNA synthesizing system is developed using purified mitochondria.

The cell organelle transformation experiments have not been successfull yet. This may be partly due to the fact that the selection procedure for chloramphenicol resistance is not yet very reliable. Experiments are now in progress to compare the level of resistance when the Cat-enzyme is present in the cytoplasm and when the enzyme is specifically present in the chloroplasts. This information might enable us to distinguish between nuclear and cell-organelle transformed plant cells.

Procedures have been developed in this lab. for isolation, fusion, culture and regeneration of protoplasts from *Solanum* and *Lycopersicon* species. Now it is important to obtain selectable cell organelle markers in order to perform a positive selection on transfer of cytoplasmic properties in cybridization experiments. Attempts to obtain such markers are in progress using the mutagen NMU.

References

1. de Haas, J.M., Boot, K.J.M., Haring, M., Kool, A.J., and Nijkamp, H.J.J. (1986). Mol. Gen. Genet. 202, pp. 48.
2. de Haas, J.M., Kool, A.J., van Brug, W., Overbeeke, N., and Nijkamp, H.J.J. (1987). Current Genetics (in press).
3. Hosticka, L.P. and Hanson, M.R. (1984). J. Heredity 75, pp. 242.
4. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982). Molecular cloning, a laboratory manual. C.S.H., New York.
5. Negrutiu, I., Shillito, R., Potrykus, I., Biasini, G., and Sala, F. (1987). Plant Molec. Biol. 8, pp. 363.
6. Overbeeke, N., de Waard, J.H. and Kool, A.J. (1984) in: Proteins involved in DNA replication (Hubscher, S.U., Spadari, eds.) Plenum Press, London, pp. 107.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

- Mei-Lie M.C. Tan, Hedwig S. Boerrigter and A.J. Kool. (1987) A rapid procedure for plant regeneration from protoplasts isolated from suspension cultures and leaf mesophyll cells of wild Solanum species and Lycopersicon pennellii. Plant Science 49, 63-72.
- Mei-Lie M.C. Tan, E.M. Rietveld, Gijsbert A.M. van Marrewijk and A.J. Kool. (1987) Regeneration of leaf mesophyll protoplasts of tomato cultivars (L. esculentum): factors important for efficient protoplast culture and plant regeneration. Plant Cell Reports 6: 172-175.
- Mei-Lie M.C. Tan, C.M. Colijn-Hooymans, W.H. Lindhout and A.J. Kool (1987). A comparison of shoot regeneration from protoplasts and leaf discs of different genotypes of the cultivated tomato. Theor. Appl. Genet. (in press).
- J.M. de Haas, A.J. Kool, W. van Brug, N. Overbeeke and H.J.J. Nijkamp (1987). Characterization of DNA synthesis and chloroplast DNA replication initiation in a *Petunia hybrida* chloroplast lysate system. Current Genetics (in press).

IV.2.

- J.M. de Haas, M.A. Haring, H.J.J. Nijkamp and A.J. Kool (1986). Isolation and characterization of a potential replication origin of *Petunia hybrida* chloroplast DNA. Book of abstracts (C37), Strassbourg (July 20-24 '86). "International Symposium on Plant molecular Biology".
- J.M. de Haas, A.J. Kool and H.J.J. Nijkamp. (1986). Searching for replication origins in plant cell organelle DNA. Book of abstracts (22), Airly House, USA (October 19-24, '86). "International Workshop on higher plant mitochondrial DNA".
- A.J. Kool, J.M. de Haas, M.A. Haring and M.Q.J.M. van Grinsven. (1986) Book of abstracts (6) Paris (Nov. 22, '86) "Aspects recents de l'utilisation en Biologie vegetale des methodes de culture de tissues et de cellules de plantes".
- M.L.M.C. Tan, E.M. Rietveld, A.J. Kool and G.A.M. van Marrewijk (1986) Rapid and efficient procedure for plant regeneration from leaf mesophyll protoplasts of tomato cultivars. Int. Assoc. Plant Tissue Culture, Minneapolis, Minnesota, U.S.A., Aug. 4-8, 1986. Book of Abstracts, p. 322, Ed.: Somers, D.A. *et al.*
- M.L.M.C. Tan, E.M. Rietveld, A.J. Kool and G.A.M. van Marrewijk. (1986) Rapid and efficient procedure for plant regeneration from leaf mesophyll protoplasts of tomato cultivars. Tomato Biotechnolgoy Meeting, Univ. of California, Davis, U.S.A. Aug. 20-22, 1986.
- M.A. Haring (1987). Chloroplast gene expression and development of a chloroplast gene transfer method. Seminar at Free University of Brussel (VUB), March 12, 1987.
- J.M. de Haas, F. Kors, A.J. Kool and H.J.J. Nijkamp (1987). Isolation and (sequence) characterization of potential organelle DNA replication origins of *Petunia hybrida*. B.A.P. Meeting, Louvain la Neuve, March 23-26.
- M.A. Haring, M.Q.J.M. van Grinsven, T.J.A. Kneppers and H.J.J. Nijkamp (1987) Progress in chloroplast transformation: development of integrating and replicating vectors. B.A.P. Meeting, Louvain la Neuve, March 23-26.
- C.E. Jansen, W. van Uden, V. Rodeva, and H.J.J. Nijkamp (1987). Improvement of selection of cybrids by introduction of selection markers: future research. B.A.P. meeting Louvain le Neuve, March 23-26.
- M.L.M.C. Tan, E.M. Rietveld, F. van de Mark, and H.J.J. Nijkamp (1987). Regeneration and analysis of somatic hybrid plants of *Lycopersicon peruvianum* and *Lycopersicon penelli*. B.A.P. meeting Louvain la Neuve, March 23-26.
- J.M. de Haas, A.J. Kool and H.J.J. Nijkamp (1987). Isolation of potential replication origins of plant cell organelle DNA. Book of Abstracts (MOP181) Amsterdam (June 10-15, '87) "Fourth European Congress on Biotechnology".

J.M. de Haas, F. Kors, A.J. Kool and H.J.J. Nijkamp (1987) Isolation of putative *Petunia hybrida* chloroplast and mitochondrial replication origins and analysis of the initiation of DNA synthesis. Book of abstracts, Copenhagen (June 10-20, '87) "Nato Advanced Studies on Plant Molecular Biology".

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No *
Joint experiment(s)	Yes	
Joint meeting(s)		No **

Descriptive information for the above data.

Exhange of material
exchange of sequence data with Colwyn Thomas (Davies' Lab., Norwich).

Joint experiment
sequence computer analysis with Colwyn Thomas (Davies' Lab., Norwich).

* Transnational cooperation
with Free University of Brussels (VUB)
Dept. of Plant Genetics
Prof. M. Jacobs and Dr. I. Negrutiu

Subjects:
- Optimalization of direct DNA transfer method with CAT as selection marker. Presently a student from our lab., E. Rietveld, is working on this subject in Brussels.
- Analysis of cytoplasmic DNA from fusion products of Atropa belladonna and N. Plumb.

** A joint meeting is scheduled for September 18, 1987 in Norwich.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: C. N. R. S., Orsay

Contract no.: BAP - 0022 - F

Project leader: F. QUETIER

Scientific staff:

Address: Université de Paris XI
Lab. de Biologie Moléculaire Végétale
U.A. 1128, Bâtiment 430
F - 91405 ORSAY CEDEX

Telephone no.: +33.1.69417135 or +33.1.69417960

Telex no.: 692166 F

Other contractual partners in the joint project:

M. Briquet, U. C. L. (Louvain-la-Neuve)
A. Cornu, I. N. R. A. (Dijon)
D.R. Davies, John Innes Institute (Norwich)
H.J.J. Nijkamp, University of Amsterdam

Title of the research activity:
Mitochondrial molecular genetics in relation to crop improvement.

Key words:
Organization, Expression, Mitochondrial genome, Higher plants

Reporting period: June 1986 - July 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The mitochondrial genome of higher plants exhibits very peculiar characteristics when compared to the mitochondrial genome of other organisms (mammals, fungi...). It is unusually large (200 to 2500 kb according to the plant) and the presence of repeated sequences makes this genome highly recombinogenic : master molecules of 200-600 kb give rise to various subgenomic DNA molecules. A better understanding of the molecular organization, the replication process and the regulation of gene expression is especially important since mitochondria are directly involved in agricultural traits such as cytoplasmic male sterility (CMS) in addition to their key-role in energy metabolism.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The main goal of this laboratory is the understanding of the basic mechanisms which control the molecular organization of the mitochondrial genome. Our main material is wheat and the principal approaches are :

- the study of repeated sequences involved in recombination and of DNA sequences of chloroplastic origin which have been transferred into the mitochondrial genome.
- the identification of enzymatic activities and enzymatic complexes involved in mt DNA recombination.
- the variation of molecular organization induced by *in vitro* culture.
- the attempts to modify the mitochondrial compartment by direct transfer of isolated mitochondria into a recipient protoplast and subsequent plant regeneration.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Methodology

We have used the usual methods of DNA cloning and DNA sequencing (M13 and dideoxy procedure). We had previously constructed the complete library of Sal I restriction fragments of the wheat mtDNA in cosmid pHC 79; two other mtDNA libraries in pHC 79 have been obtained since (sunflower and alfalfa). A peculiar technology lies in the microinjection of isolated mitochondria into recipient protoplasts (see section V).

Results and discussion

Repeated sequences, chloroplastic DNA inserts and recombination.

The mitochondrial genome is highly recombinogenic, especially in wheat. In addition to the several repeated sequences (RS) already studied in previous reports, the area carrying the 26S rRNA gene (D. Falconet) has been completely sequenced (coding sequence, putative promotor region). The organization of the wheat 26S rRNA gene has been compared to the maize and *Oenothera* counterparts (conserved and variable regions). This part of the genome is involved in some restriction pattern variations found in *in vitro* cultures (see below).

On wheat DNA (B. Lejeune, M.F. Jubier) a 1108 bp insert of chloroplastic origin has been found several kb downstream the 18S - 5S genes. This sequence is the internal part of the chloroplast α-ATPase. 5' and 3' ends of the chloroplast gene are not found in the mitochondrial genome, even elsewhere. The complete sequence of the insert has been determined and shows

98% homology to that of the genuine chloroplast gene; it is bordered by a direct 9 bp repeat, likely involved in the transfer process. This insert is not transcribed and has been found in all the wheat ancestors tested so far with the same location in the genome This indicates that the transfer of a chloroplast DNA sequence into the mitochondrial genome has taken place long ago in the phylogeny.

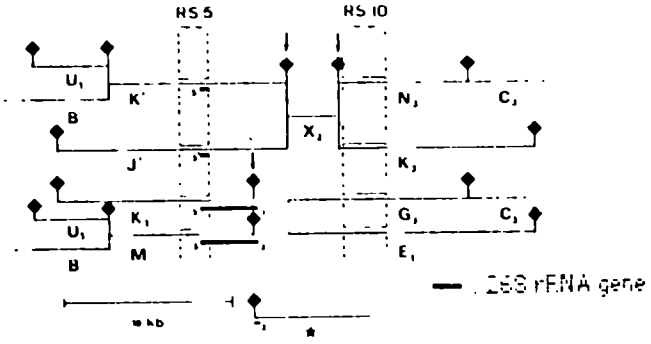
In cauliflower (C. Hartmann, A. Rode) a Pst I chloroplast fragment has been shown to hybridize to two Sal I mitochondrial fragments. Sequencing of the region homologous to these fragments has allowed to detect two putative $tRNA_{His}$ genes which carry 97% homology to the corresponding chloroplast gene. However, the flanking regions are markedly different in both cellular compartments. On the contrary, flanking regions of both mitochondrial genes are identical (20 nucleotides upstream their 5'-end and 50 nucleotides downstream their 3'-end). These results suggest that this gene would have been duplicated by a retroposition phenomenon.

In collaboration with the group of Dr Litvak (Bordeaux), two enzymatic activities involved in DNA replication and recombinaison have been or are investigated (B. Lejeune). A mitochondrial protein fraction prepared by M. Etcheverria in Bordeaux is capable of nucleotide incorporation into mtDNA. Nevertheless, in spite of 10 000 cpm incorporation, experiments achieved on our cloned mtDNA failed to show any EM picture of replication . Thus this activity likely concerns a DNA repair-enzyme. Such protein extracts, prepared in Bordeaux, are used in Orsay by M. Etcheverria (Bordeaux) and B. Lejeune (Orsay) who have constructed plasmids carrying wheat repeated sequences, specific flanking stretches and reporter genes. These plasmids are mixed with the protein extracts and *in vitro* recombined sequences are presently looked for.

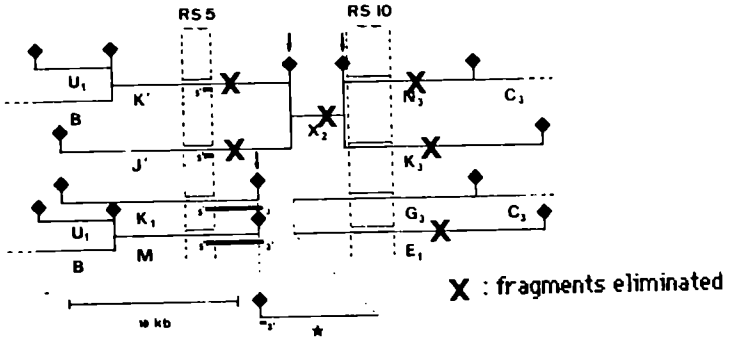
Modifications of the molecular organization of the wheat mitochondrial genome through in vitro culture (somaclonal and gametoclonal variations). (A. Rode, C. Hartmann, A. Benslimane)

The haplo-diploid wheat regenerated through androgenesis by E. Picard (G.I.S. Moulon, Orsay) have been analysed; no variation has been detected in the mtDNA restriction patterns of the regenerant lines tested, indicating thus that androgenesis *per se* does not systematically induces variation.

In collaboration with J. de Buyser and Y. Henry (Orsay), tissue cultures derived from immature wheat embryos have been investigated at the mtDNA level. Their mtDNA restriction patterns undergo marked differences : the stoichiometry of bands is altered, some bands are either underrepresented or overrepresented and several disappear completely. One area of the master molecule mainly concerned with these variations has been thoroughly studied : in the wheat plant, the 26S rRNA gene is carried by the different contexts represented below:



In tissue cultures derived from the cultivar Aquila, several restriction fragments are completely lost (they do not occur in the mitochondrial genome, even elsewhere else, like fragment X_2 for instance). The figure below shows the eliminated and conserved sequences:



It must be underlined that both arrangements still present (K_1-G_3 and M-G_3) give rise to a correct rRNA transcript. The deleted stretch of DNA amounts to about 17 kb, *i.e.* the length of an entire mammal mitochondrial genome! Whereas cv Aquila gives rise to nonembryogenic cultures, cv Chinese Spring is an embryogenic variety whose *in vitro* culture also reveals variation on mtDNA restriction patterns. For instance, the time-course of variation of mtDNA organization has been followed on 12 successive 2-months subcultures: variation occurs during the very early process of callogenesis and is rapidly stabilized. These results suggest a variation of the replication rates of some subgenomic molecules, induced by *in vitro* culture itself. Possible relationship between regeneration properties and these modifications are considered.

Identification of native mtDNA molecules (F. Quetier, S. Bazetoux, I. Sissoeff)

The previous report indicated that the direct lysis of wheat coleoptile protoplasts on a vertical agarose slab gel led to several discrete bands hybridizing to nick-translated wheat mtDNA. This preliminary experiment has been now optimized (physiological state of protoplasts and protoplast concentration are critical). Nine distinct bands appear and several ones migrate very slowly, on a range >> 100 kb (size predicted for master circles). Adequate size markers are under construction by concatening lambda DNA and probing by specific cloned mtDNA sequences is underway. Attempts on protoplasts isolated from tissue cultures displaying mtDNA variation are on course.

Mitochondrial genome of 2 other plants important in agriculture.

The complete Sal I library of sunflower mtDNA has been obtained (H. Récipon) in cosmid pHC79 and fragments carrying the mitochondrial ATPase subunits alpha, 6 and 9 are identified. They show polymorphisms when fertile-cms lines are compared.

The library of alfalfa mtDNA has been recently constructed in cosmid pHC79 (A. d'Hont) in order to study the mtDNA rearrangements in somatic hybrids obtained in Orsay (Dattée, Théoulé).

Transformation of the mitochondrial compartment

See transnational activities, Part V.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

Publications in Scientific Journals

B. LEJEUNE, S. DELORME, E.DELCHER and F. QUETIER. Recombination in wheat mitochondrial DNA: occurence of nine different genomic contexts for the 18-5 S rRNA genes. Plant Physiol. and Biochemistry (1987), 25.

E. PICARD, A. RODE , AA. BENSLIMANE and L. PARISI. Gametoclonal variations in doubled haploids of wheat: biometrical and molecular aspects. in "Somaclonal variations and crop improvement", J. Semal ed. Martinus Nijhoff Publishers (1986) 136.

A. RODE, C. HARTMANN, AA. BENSLIMANE, E. PICARD and F. QUETIER. Gametoclonal variation detected in the nuclear rDNA from doubled haploid lines of a spring wheat. Theor. Appl. Genet. (1987) 74, 31.

A. RODE, C. HARTMANN, D. FALCONET, B. LEJEUNE, F. QUETIER, A. BENSLIMANE, Y. HENRI and J. DeBUYSER. Extensive mitochondrial DNA variation in somatic tissue cultures initiated from wheat immature embryos. Curr. Genet. (1987) under press

C. HARTMANN, J. DeBUYSER, Y. HENRY, D. FALCONET, B. LEJEUNE, AA. BENSLIMAN, F. QUETIER and A. RODE. Time-course of the mitochondrial genome variation in wheat embryogenic somatic tissue cultures. Plant Science, under press.

Submitted Papers

DHONT A., QUETIER F., THEOULE E. and Y. DATTEE. Mitochondrial and chloroplast DNA analysis of interspecific somatic hybrids of a leguminosae, *Medicago*. Submitted to Plant Science.

P. LEBACQ, D. SQUALLI, M. DUCHENNE, P. POULETTY and M. JOANNES. Easy detection of subpicogram quantities of specific DNA sequences on blot-hybridization with sulfonated probes. Journal of Biochemical and Biophysical methods (revised version submitted).

Short Communications, Internal Reports

LEJEUNE B., FALCONET D., RODE A., JUBIER M.-F., DRON M., HARTMANN C. et QUETIER F., A review on recombination events involved in the physical organization in higher plant mitochondrial genome. Recent data on wheat mt DNA.Inter. Symp. Plant Mol. Biol., STRASBOURG, July1986. (**Lecture**).

LEJEUNE B., JUBIER M.-F., DELCHER E., RODE A. et QUETIER F The wheat mitochondrial DNA contains part of the chloroplastic gene coding for the α-ATPase subunit., Inter. Symp. Plant Mol. Biol STRASBOURG, July 1986.(**Poster**).

HARTMANN C, DRON, M. BENSLIMANE AA., BUSCHEN, S., QUETIER,F. and RODE A. The same cauliflower chloroplast DNA sequence may be transferred at 2 different loci into mitochondrial DNA. Inter. Symp. Plant Mol. Biol STRASBOURG, July 1986.(**Poster**).

RODE A., HARTMANN C., LEJEUNE B., BENSLIMANE AA., FALCONET D., QUETIER F., HENRY Y. et De BUYSER J., Unusual mitochondrial DNA variation in a non-regenerating cell culture of *Triticum aestivum*, Inter. Symp. Plant Mol. Biol STRASBOURG, July 1986 (**Poster**) .

RODE A., HARTMANN C., LEJEUNE B., BENSLIMANE, FALCONET D., QUETIER F., HENRY Y et DE BUYSER J., Mitochondrial DNA variation in a non-regenerating cell culture of Triticum aestivum.2nd Workshop on Higher Plant Mitochondrial DNA, AIRLIE (USA),October1986. **(Poster and lecture).**

LEJEUNE B., JUBIER M.-F., DELCHER E., RODE A. et QUETIER F.Recombination in wheat mitochondrial DNA : insertion of the internal part of the chloroplastic gene for alpha-subunit ot ATPase. 2nd Workshop on Higher Plant Mitochondrial DNA, AIRLIE (USA), October 1986.**(Posterand lecture).** .

F. QUETIER, B. LEJEUNE and S. BAZETOUX. Catching native mt DNA molecules of higher plants?2nd Workshop on Higher Plant Mitochondrial DNA, AIRLIE (USA), Octobre 1986 **(Poster and lecture).**

LEJEUNE B., JUBIER M.-F., DELCHER E., RODE A. et QUETIER F...Recombination in wheat mitochondrial DNA : insertion of the internal part of the chloroplastic gene for alpha-subunit ot ATPase. EEC meeting on Genetic Engineering of Plants and Microrganisms important for Agriculture, LOUVAIN-LA-NEUVE (Belgique) March 1987.**(Poster).**

JUBIER M.F., DELCHER E., LEJEUNE B. et QUETIER F., Characterization of a DNA sequence involved in recombination in wheat mitochondrial DNA. EEC meeting on Genetic Engineering of Plants and Microorganisms important for Agriculture, LOUVAIN-LA-NEUVE (Belgique) March 1987 **(Poster).**

RODE A., HARTMANN C., LEJEUNE B., FALCONET D., QUETIER F., BENSLIMANE AA., De BUYSER J. et HENRY Y., Extensive mitochondrial DNA variation in non-embryogenic somatic tissue cultures initiated from wheat immature embryos. EEC meeting on Genetic Engineering of Plants and Microrganisms important for Agriculture, LOUVAIN-LA-NEUVE (Belgique) March 1987 **(Poster and lecture).**

DeBUYSER J, HENRY, Y, HARTMANN C., RODE A. ORY, C and YOLLE N. Morphology, chromosome and mitochondrial DNA variations in wheat somatic tissue culture. EEC meeting on Genetic Engineering of Plants and Microrganisms important for Agriculture, LOUVAIN-LA-NEUVE (Belgique) March 1987 **(Poster).**

HARTMANN C., DRON M., BENSLIMANE AA., BUSCHEN S., QUETIER F. and RODE A. DNA transfert from chloroplastic to mitochondrial genome? EEC meeting on Genetic Engineering of Plants and Microrganisms important for Agriculture, LOUVAIN-LA-NEUVE (Belgique) March 1987 (**Poster).**

FALCONET D., SEVIGNAC M. and QUETIER F. Nucleotide sequence of the wheat mitochondrial 26S rRNA gene. EEC meeting on Genetic Engineering of Plants and Microrganisms important for Agriculture, LOUVAIN LA-NEUVE (Belgique) March 1987 (**Poster).**

FALCONET D.; SEVIGNAC M. and QUETIER F.Identification of a recombination site within the 26S rRNA gene in wheat mt DNA. International Symposium on Plant Molecular Biology, Strasbourg July 1986 (**Poster).**

QUETIER F. Bilan et perspectives en biologie moléculaire végétale. Journée d'Etude Biologie Moleculaire Végétale, Soc. Franç. Bot. Nov. 1986, Orsay.(**Communication).**

LEJEUNE B. Organisation et expression du génome mitochondrial des plantes. Journée d'Etude Biologie Moleculaire Végétale, Soc. Franç. Bot. Nov. 1986, Orsay. **(Communication).**

FALCONET D., LEJEUNE B., SEVIGNAC M. and QUETIER F. Organisation de l'ARN ribosomique dans le génome mitochondrial de blé. Colloque de la Société de Chimie biologique : Le ribosome et ses ligands. Biosynthèse, structure et fonctions. April 1987 Strasbourg. **(Lecture).**

P. LEBACQ, M. JOANNES, D. SQUALLI (1986). Easy detection of sub-picogram quantities of DNA by improvement in the use of non-radioactive DNA probes. Application to the study of plant genome. International Symposium on Plant Molecular Biology. July 1986 Strasbourg. **(Poster)**.

D. SQUALLI. Utilisation de sondes non-radioactives pour détecter des quantités sub-picogrammiques d'ADN. Application à l'étude des ADN végètaux. Colloque Franco-Maghrebin de Biologie et Génétique Moléculaire. October 1986 Paris .**(Poster)**.

P. LEBACQ, Easy detection of sub-picogram quantities of DNA by improvement in the use of non-radioactive DNA probes. Application to the study of plant genome.Genetic and Cellular Engineering of Plants and Microorganisms Importants for Agriculture. March 1987. Louvain-la-neuve BELGIQUE.**(Lecture).**

P. LEBACQ, M. JOANNES and D. SQUALLI. (1987) Possible occurence of Proto-oncogenes in the plant genome . Genetic and Cellular Engineering of Plants and Microorganisms Importants for Agriculture. March 1987 Louvain-la-Neuve BELGIQUE. **(Poster)**.

H. RECIPON, A. RODE and F. QUETIER. Molecular cloning and identification of the genes coding for ATPase subunits 6, 9 and a in *Helianthus annus*. Genetic and Cellular Engineering of Plants and Microorganisms Importants for Agriculture. March 1987, Louvain-la-Neuve, BELGIQUE.**(Poster)**

HARTMANN C., LEJEUNE B., FALCONET D., QUETIER F., BENSLIMANE AA, DeBUYSER J., HENRY Y. et RODE A. Réarrangements du génome mitochondrial du blé consécutifs à la culture *in vitro* de cellules somatiques. Colloque de la Société Française de Physiologie Végétale; Biologie Moléculaire Végétale, Orsay 1987. **(Communication).**

RODE A. and HARTMANN C. Dedifferentiation of wheat immature embryo cells induces a marked variation of the mitochondrial DNA organization. Colloque "Differenziamento e coltura de tessuti", San Miniato Italie.June 1987. **(Lecture introductive).**

B. LEJEUNE, F. QUETIER, D. FALCONET, MF JUBIER, A. RODE, C. HARTMANN. Organisation moléculaire et expression du génome mitochondrial des plantes supérieures. Bull. Soc. Bot. France, 1987,under press

Thesis

A. BENSLIMANE. 1987. Contribution à l'étude des séquences répétées de l'ADN nucléaire des végétaux supérieurs. Structure et variabilité. Ph. D. Thesis, Orsay.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff	Yes	
Joint experiment(s)	Yes	
Joint meeting(s)		No

Descriptive information for the above data.

Exchange of materials

Orsay has sent the complete library of Sal I restriction fragments of wheat mitochondrial DNA to Louvain la Neuve (Pr BRIQUET), various clones to Dijon (Dr BERVILLE), Bordeaux (Dr LITVAK) and Edinburgh (Pr LEAVER). Clones were received from Edinburgh (Pr LEAVER) and Norwich (Pr DAVIES), and protein extracts from Bordeaux (Dr LITVAK).

Exchange of staff

F. QUETIER : one week in Edinburgh (Pr LEAVER)
I. SISSOEF and S. BAZETOUX : several days in Norwich (Pr DAVIES)

M. ECHEVERRIA (Bordeaux) in Orsay for several weeks
W. LAWRENCE (Norwich in Orsay for two weeks

Visits

F. QUETIER to Louvain la Neuve (PR BRIQUET),Bordeaux (Dr LITVAK) and Dijon (Drs CORNU and BERVILLE)
B. LEJEUNE to Bordeaux (Dr LITVAK) and Dijon (Dr BERVILLE)

Joint experiments

We have designed in Orsay a modified procedure to isolate axenic mitochondria from cell suspensions, protoplast or axenic seedlings. W. LAWRENCE (Norwich) has been trained in this technique two weeks in our lab. Complementarily, I. SISSOEF and S. BAZETOUX have been trained one week in Norwich (Pr DAVIES) to shape beveled needles for injection of mitochondria into recipient protoplasts. The joint experiments consist of i)the isolation of axenic mitochondria from a plant A (*Nicotiana, Solanum*) ii)the microinjection of controlled amount of isolated mitochondria into recipient B (other lines of *Nicotiana, Solanum*) which contains a mt DNA different from that of A. The mechanical injection is carried out through an oil-driven microsyringe under a laminar flow hood and using agarose-immobilized protoplasts (LAWRENCE's method). The main technical problems have been now overcome and experiments are under way in Orsay and Norwich. Modification of the cells will be followed by mt DNA analysis of the tissue culture and regenerated plants.

The cytoplasm 447 in *Vicia faba* is CMS and contains virus-like particles. These particles have been purified in Dijon (Drs DUC and CORNU) and aliquots have been mechanically microinjected in ovuls/embryos of fertile *V. faba* in Orsay. The mitochondria of the progeny will be analyzed for the presence of particles (Dijon) and for mt DNA analysis (Louvain la Neuve).

C. THOMAS (Norwich) and M. DUCHENNE (Orsay) have identified several cytoplasms of sugarbeet in respect to the presence of several minicircular DNAs. Exchange of sequence data and of DNA clones between Norwich and Orsay have prompted these determinations.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Univ. degli Studi di Siena

Contract no.: BAP - 0204 - I

Project leader: M. CRESTI

Scientific staff:

Address: Università degli Studi di Siena
Dipt. Biologia Ambientale
Via Mattioli 4
I - 53100 SIENA

Telephone no.: +39.577.281228 or +39.577.281248

Telex no.: 572459 UNIVSI I

Other contractual partners in the joint project:

Ch. Dumas, C. N. R. S. (Villeurbanne)
H.J. Wilms, Agricultural University of Wageningen

Title of the research activity:
Pollen biotechnology in cultivated crops.

Key words:
Pollen biotechnology, Biology of reproduction

Reporting period: January 1987 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

- Development and improvement of technical methods for storage of pollen in order to prolong viability.
- Male Germ Unit (MGU) characterization and isolation; storage while preserving the original viability.
- Charaterization of pollen, pollen tube, generative cell and sperm cytoskeleton.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

- Male Germ Unit characterization and isolation (generative cell formation and division to form two sperm cell).
- Viability and storage of pollen grains.
- Pollen and pollen tube cytoskeleton (freeze-substitution fixation, monoclonal antibodies, against motility protein, preparation).

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1) Male germ unit characterization and organization

Electron microscopy studies were carried out in mature Euphorbia pollen (trinucleate species). The formation and organization of the generative cell and after division, of the sperm cells show the presence, in the wall of structures like "septa" that probably have an important function in comunication between the cells. After freeze-substitution treatment the generative cell in Nicotiana shows a uniform and regular structure.

Three dimentional reconstructions (computer assisted work) on sperm cells in Euphorbia are in progress.

2) Viability and storage of pollen grains.

We have tested several culture medium to better understand the behaviour of the pollen grain "in vitro" in order to determine the viability after storage.

Tests of viability using low temperature have permitted us to maintain, a high percentage of pollen germination in Nicotiana after 3 days. No morphological changes in the structure of the vegetative and generative cell and sperm formation have been observed. Experiments on the generative cell formation "in vitro" are under way.

3) Pollen and Pollen tube cytoskeleton

The pollen and tube cytoskeleton were studied with immunofluorescence, biochemical and electron-microscopical methods. For immunofluorescence, monoclonal antibodies against proteins responsible for the cell motility (tubulin and actin) were prepared in our lab.

In the vegetative cell, microtubules and microfilaments are clearly present; they are arranged longitudinally according to the direction of growth. The generative cell contains only microtubules which are longitudinally arranged and organized in many bundles. In its proximal part, the generative cell has a structure like a "tail" which probably plays an important role in motility.

Biochemical studies on the isolation and characterization of tubulin (i.e. different isoforms) are in progress.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1

CRESTI M., TIEZZI A. and A. MOSCATELLI. Pollen and pollen tube cytoskeleton. In: "Genetic and cellular engineering of plant microorganisms important for agriculture". BAP Meeting (Commission of the European Communities) Louvain-la-Neuve March 23-26, 1987 pp. 86-88.

IV.4

CAI G. Proteine citoscheletriche in cellule animali e vegetali. Tesi di Laurea. Facoltà di Scienze, Matematiche, Fisiche e Naturali. Università di Siena. Giugno 1987.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Exchange of material - Brassica oleracea pollen prepared in Lyon was cut and observed at electronmicroscopy in Wageningen by a Researcher from Siena University (Dr. M. Murgia) in order to characterize the Male Germ Unit.

Exchange of staff - Dr. M. Murgia (Dipartimento di Biologia Ambientale - Università di Siena) have visited several time (for 2-3 months) the Agricultural University at Wageningen in order to collaborate with the staff. Also Prof. M. Cresti, with some students, have visited Wageningen for a short period (10 days). During the visit several conference were organized and also Prof. Cresti have spoken on "Pollen tube cytoskeleton".

Joint experiments - Several experiment concerning Brassica oleracea are prepared in collaboration with Lyon and Wageningen Labs.

Joint meeting - A meeting in Lyon (19-20-21 May 1987) took place.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: C. N. R. S., Villeurbanne

Contract no.: BAP - 0203 - F

Project leader: Ch. DUMAS

Scientific staff: E. Matthys-Rochon, C. Kerhoas, S. Detchepare A.M. Thierry, C. Moulin (technical assistance)

Address: Reconnaissance Cellulaire et Amélioration des Plantes - UM CNRS 380 024 UCB-Lyon 1
Bât. 741 / 5ème
43, Boulevard du 11 Nov. 1918
F - 69622 VILLEURBANNE CEDEX

Telephone no.: +33.78898124 EXT.3302

Telex no.: 310810 BUSLYON F

Other contractual partners in the joint project:

H.J. Wilms, Agricultural University of Wageningen
M. Cresti, Università degli Studi di Siena

Title of the research activity:

Pollen biotechnology in cultivated crops.

Key words:

Pollen quality, Pollen storage, Male Germ Unit, Sperm cell isolation

Reporting period: January 1987 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The final aim of this joint E.C. project is to perform an efficient cellular tool for further genectic manipulations with the male germ cells, i. e., sperm cells (SC) or the generative cell of some crop pollen species

In order to reach this goal we have developed a strategy based on several steps:

a)- to precise some key parameters which control the pollen quality and to store the pollen for a long term

b)- to elucidate the Male Germ Unit (MGU) organization and formation,

c)- to isolate both MGU and viable SC,

d)- to store these SC and to micromanipulate them

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

According to our own background and potential we have planed to provide answers to the step (Ia) with the help of a multidisciplinary approach (biophysical, biochemical and cytological tools) and to the step (Ic) in which we started earlier than our two co-contractants.

For the specific step (Ib) we have developed a strict joint program with exchanges of both scientits and material. In this view, we have provided the full technics available on the non confidential part of this program, i. e., sperm cell isolation to our european partners.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Each step in progress in the frame of this projet is briefly summarized below.

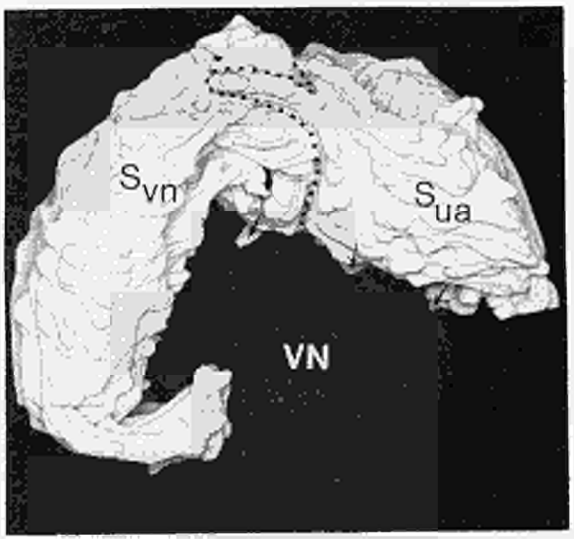
1-METHODOLOGY

1a. Pollen quality aspect

- Plant material: *Cucurbita pepo*, *Zea mays* L. (several lines or hybrids), *Brassica oleracea* and *B. campestris*

- Quantitative cytology of the sperm cells

B. oleracea and *B. campestris* pollen grains were fixed in glutaraldehyde and post-fixed in OsO4, dehydrated in graded ethanol series and embedded in epon (see DUMAS et al., 1985, Protoplasma 124: 168-174). Three-dimensional reconstructions were prepared according to McCONCHIE et al., 1987, Protoplasma 127: 57-63



- Biophysical analysis:

Differential scanning calorimetry (DSC) has been used in order to detect the presence of freezable water and to estimate transition phase of pollen water from a liquid to a solid state by the enthalpy change (see KERHOAS et al., 1987, Planta 171 1-10)

Nuclear Magnetic Resonance Spectrometry (1H-NMR) has been used to evaluate water mobility with the help of the transverse relaxation time (T2) following DUMAS and DUPLAN (1984) (see in KERHOAS et al., 1987, Planta 171: 1-10)

- Plasma membrane analysis:

The fluorochromatic reaction (FCR according to HESLOP-HARRISON and HESLOP-HARRISON, 1970, Stain Technol. 45. 115-120) has been performed for each pollen sample used around 400 counted grains.

Freeze fracture replicas and esterase activity allow pollen membrane structure during each controlled step of pollen dehydration according to ESAIG and NICOLAS, 1976, C.R.Acad.Sci. 283. 1245-1248.

- Biochemical analysis. Esterase activity has been tested following a technique that we previously performed (GAY et al., 1986, Electrophoresis 7: 148-149)

1b. MGU and SC isolations

Each methodology is in progress and partly published (see MATTHYS-ROCHON et al., 1987, Plant Physiol. 83: 464-466). For SC isolation, part of the methodology is summarized on the diagramme included in the section discussion

2- RESULTS

2a. Pollen quality

Water content and water mobility form together a quite good parameter to estimate pollen quality A multidisciplinary approach with NMR, DSC, freeze-fracture, esterase activity (FCR and isoelectric focusing analysis) and, seed setting allow to conclude that the main modification that occurs during normal pollen ageing or experimentally controlled pollen dehydration is the formation of gel-phase microdomains in the plasma membrane. And, the combination of wall deformation and water replacement would permit pollen survival until oxidative damage occuring in the dehydrated grain (KERHOAS et al., 1987).

The quality of pollen is affected by dehydration and, we think that some specific molecular signals may trigger parthenocarpy at a distance This particularly new and exciting area could be the study of defective pollen in inducing fruit and haploid embryo development. Through the possibility by obtaining these haploid embryos is uncertain, it should not be neglected

2b Male Germ Unit (MGU)

Three dimensional reconstructions associated with quantitative cytology have precised the significant differences which exist between the two sperm cells (Sua and Svn) associated to the vegetative nucleus (Svn) or not (Sua) in terms of potential cytoplasmic DNA (mitochondria), from 11 to 15 in Svn at 6 to 14 in Sua in *Brassica oleracea*, for example (McCONCHIE et al., 1987).

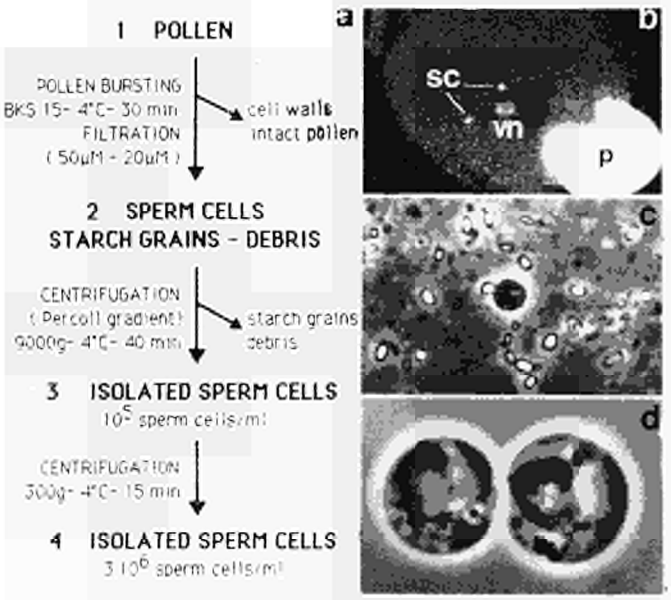
In vitro MGU isolation in several species allows us to confirm the validity of our previous concept of MGU (DUMAS et al., 1984, What's New in Plant Physiol. 15: 17-20).

3- DISCUSSION

At this stage of the programme we have performed several analysis which are progressing in our laboratory on the pollen quality. Part of these basic research constitutes the theoretical support to perform a pollen bank especially for pollen grain with high water content and short time life (corn, for example).

We have continued to analyze MGU with the help of quantitative cytology and demonstrated the validity of the MGU concept.

Finally, the *in vitro* MGU isolation constituted the first pre-requisied for SC isolation. And, the first evidence for viable SC was successfully obtained in corn (see below for methodology).



Conclusion. This multidisciplinary approach is necessary to expect to use the reproductive route for further genetic manipulations.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV-1 PUBLICATIONS IN SCIENTIFIC JOURNALS

- KERHOAS C., GAY G., DUMAS C., 1987. A multidisciplinary approach to the study of the plasma membrane of *Zea mays* pollen during controlled dehydration. **Planta** 171. 1-10
- GAY G., KERHOAS C., DUMAS C., 1987 Quality of a stress-sensitive *Cucurbita pepo* L pollen **Planta** 171. 82-87
- McCONCHIE C., RUSSELL S.C., DUMAS C., TUHOY M., KNOX R.B., 1987. Quantitative cytology of the sperm cells of *Brassica campestris* and *B. oleracea*. **Planta** 170: 446-452
- MATTHYS-ROCHON E., VERGNE P., DETCHEPARE S., DUMAS C., 1987. Male germ unit isolation from three tricellular pollen species *Brassica oleracea*, *Zea mays* and *Triticum aestivum*. **Plant Physiol**. 83. 464-466.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Exchanges of material(s)

Descriptive information for the above data.

- From *Brassica oleracea* anthers fixed and included in epon at very precise stages (male programme) controlled with a DNA probe (Lyon's group) a cytological study is performing in Wageningen by an italian researcher (details in joint experiment section).

- We provided the full technic developed in Lyon on SC isolation to both partners during the first joint meeting last may.

Joint experiment(s)

- To increase the efficiency of our collaborative project all cocontractants have decided to focuse their main interest on *Brassica* as a model with controlled genotypes

- The aim of our first experiment is to understand on a tricelled pollen species the origin of this unique association between two sperm cells and the vegetative nucleus with a sophisticated cytological analysis combined with computer aid for three dimensional reeconstruction.

- The use of micro-manipulator on isolated SC might constituted the next step of joint experiment.

Joint meeting

Held in Lyon, 19-20 may 1987, the first workshop was devoted to exchange technics and new data and to plane staff exchanges and joint programme.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: De Landbouwhogeschool Wageningen

Contract no.: BAP - 0202 - NL

Project leader: H.J. WILMS

Scientific staff: C.H. Theunis, M. Murgia

Address: Agricultural University
Dept. of Plant Cytology and Morphology
Arboretumlaan 4
NL - 6703 BD WAGENINGEN

Telephone no.: +31.8370.82658 or +31.8370.82155

Telex no.: 45015 BLHWG NL or 45917 BURLH NL

Other contractual partners in the joint project:

M. Cresti, Università degli Studi di Siena
Ch. Dumas, C. N. R. S. (Villeurbanne)

Title of the research activity:
Pollen biotechnology in cultivated crops.

Key words:
Pollen, Sperm cells, Male germ unit, Gametes

Reporting period: January 1987 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

1. Male Germ Unit (MGU) characterization in the in vivo condition (1987,1988, and partly 1989).
2. In vitro MGU characterization (starting end 1987).
3. Germ plast and its potential for biotechnology (partly 1988, 1989).

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Research programme 1987 for WAGENINGEN (W) and in collaboration with partly Siena (S) and Lyon (L).

1. MGU characterization in the in vivo conditions:

- Tricelled pollen species (spinach, Brassica sp., sugar beet, Euphorbia) (S;W).
  Three dimensional reconstruction using transmission electron microscopy and monitored with computer assistance for Euphorbia.
  The cytoskeleton analysis of spinach pollen to understand the origin of the sperm cells.
- Origin of sperm cells (sc): Kinetic and control of microgametogenesis, i.e. sc formation using cytological approaches in Euphorbia (W;S).

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

I. In March C.H.THEUNIS started with research of the immunolabeling of tubuline in order to characterize the cytoskeleton of the sperm cells in Spinacia pollen grains.
The preliminary results are promissing.

2. The ultrastructural development of Euphorbia dulcis pollen from microspore at the tetrad stage to the bicellular pollen grain was investigated. For the summerizing scheme see figure 1. The pollen grain at anthesis consists of 3 cells, two sperm cells and a vegetative cell.
The ultrastructure and the three dimensional reconstruction of the sperm cells are in progress. The results are being analized.

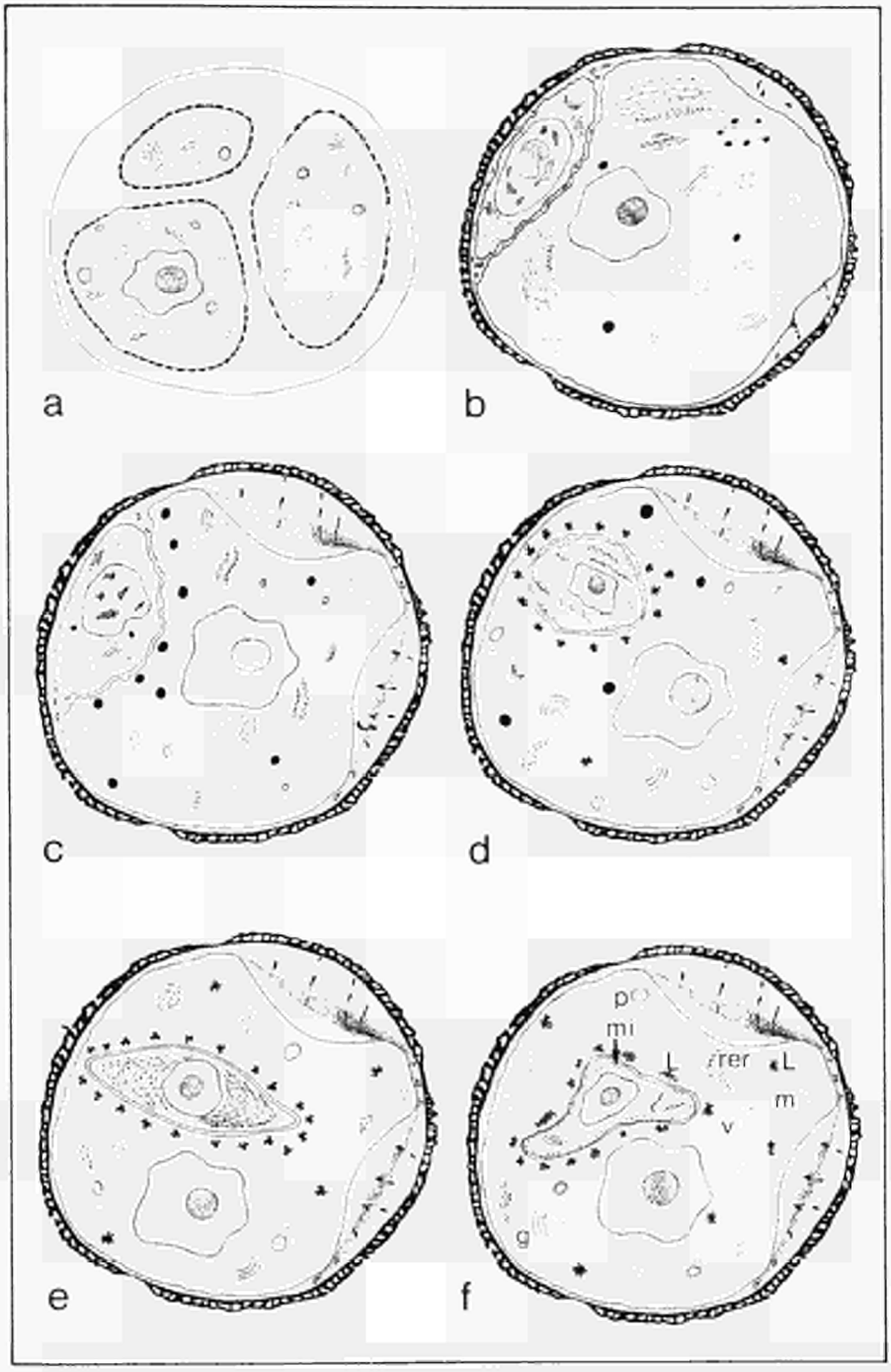


Figure 1. Schematic development of Euphorbia dulcis pollen from microspore to bicellular pollen grain. a=microspore and f=bicellular pollen grain. L=lipid; g=Golgi body; m=mitochondrium; mi=microtubule; v=vesicle; rer=rough endoplasmatic reticulum.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

1.---

2. Internal reports:

a. C.H.THEUNIS, H.J.WILMS 1987. Report workshop "Pollen Biotechnology" 19-20 May 1987 in Lyon. Department Plant Cytology and Morphology, Agricultural University Wageningen. 8 pp.

b. C.H.THEUNIS 1987. Report workvisit to "DE DANSKE SUKKERFABRIKKER", 15-17 June 1987 in Copenhagen, Denmark. Department Plant Cytology and Morphology, Agricultural University Wageningen. 5 pp.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

In March all contractants were together in Louvain-la-Neuve for the first introduction of the research proposals.

In May 1987 the contractants of this BAP programme were together for the first workshop in Lyon. They have decided to focus their main activities on one plant species, namely Brassica, as a model to reach the first step of our programme, i.e.: SC formation and the origin of this unique association between the vegetative nucleus and the pair of SC termed "MALE GERM UNIT (MGU). See for further details the note from C.DUMAS.

Exchange of material	L --- W
Exchange of staff	S-----W : M.Murgia 6 months 8 students 1 week
Joint experiment	L --- W
Joint meetings	"BAP meeting in Louvain-la-Neuve",23-26 March "Workshop Lyon (W,S and L)",19-20 May "Visit to DDS Copenhagen", P.Olesen,15-17 June

BIOTECHNOLOGY OF ANIMALS

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Trinity College, Dublin
Contract no.: BAP - 0131 - IRL

Project leader: T.J. FOSTER
Scientific staff: M. O'Reilly

Address:
Trinity College
Moyne Institute
Microbiology Dept.
IRL - DUBLIN 2

Telephone no.: +353.1.722941 EXT.1814 or 1345

Telex no.: 25442 TCD EI

Other contractual partners in the joint project:

A.J. Bramley, Inst. for Research on Animal Diseases (Newbury)

Title of the research activity:

Genetic analysis and biologic assessment of virulence determinants of Staphylococcus aureus.

Key words:

Staphylococcus aureus, Mastitis, -toxin, coagulase, Protein A

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The objectives of this project are to identify, using molecular cloning and allele-replacement mutagenesis, the major determinants of virulence of Staphylococcus aureus strains that cause bovine mastitis. Mutants of S. aureus lacking virulence factors (either singly or in combinations) will be tested for virulence using the mouse mastitis model. This research will identify components that could be included in a subunit vaccine to protect against mastitis caused by S. aureus.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The specific objectives for the first period of the project were as follows: **(i)** Inactivation of the α-toxin gene of a bovine strain of S. aureus **(ii)** Construction of gene libraries of S. aureus DNA in a λ replacement vector. **(iii)** Identification of recombinants expressing β-toxin, coagulase and protein A. **(iv)** Subcloning of protein A, coagulase and β-toxin genes on plasmids in E. coli. Detailed mapping of the coagulase determinant. **(v)** Inactivation of the protein A gene of S. aureus 8325-4 by allele-replacement mutagenesis.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY

Gene libraries of S. aureus genomic DNA were prepared in λL47.1. Plaques were screened for haemolysis to detect expression of β-toxin and by filter-immunoblotting with anti-coagulase serum and pre-immune rabbit serum to detect protein A.

Proteins expressed in extracts of E. coli strains were examined by SDS-polyacrylamide gel electrophoresis and Western immunoblotting.

Genes were subcloned into plasmid vectors and mapped using standard methods. Allele-replacement mutagenesis of the S. aureus α-toxin gene was performed by a procedure we have previously described (O'Reilly et al. 1986, Microbial Pathogenesis **1**, 125-138). This technique was modified for inactivating the protein A gene as described below.

The β-toxin gene of strain 8325-4 was inactivated by lysogenic conversion. This cannot be used for the bovine strain M60 because it is insensitive to the converting phages. Allele-replacement will be employed in this case.

2. RESULTS AND DISCUSSION

The genes coding for β-toxin (hlb), protein A (spa) and coagulase (coa) of S. aureus have been cloned in E. coli phage vectors and subcloned in plasmids. A detailed molecular analysis of the cloned coagulase gene has been performed. The protein A and β-toxin genes have been cloned and analyzed by others so a minimal analysis sufficient of facilitate construction of mutants by allele replacement mutagenesis was performed.

(i) Inactivation of the α-toxin gene (hly) of a bovine strain

We have previously isolated hly::Em^r substitution mutations in the laboratory strain 8325-4. The same procedure was used to inactivate the hly gene of the bovine strain M60.

(ii) Inactivation of the β-toxin gene of strain 8325-4

β-toxin can be inactivated in some strains by lysogenization with a converting bacteriophage which inserts in the hlb gene. Hlb$^-$ mutants of S. aureus 8325-4 were isolated by lysogenization with phage 42E. Strain M60 is resistant to converting phages so allele-replacement methods will be employed to isolate an Hlb$^-$ mutant.

(iii) Inactivation of the protein A gene of strain 8325-4

The cloned spa gene was inactivated by substituting a 1.2 kb BclI fragment (which encodes the 5 IgG binding domains) with a 2 kb BglII fragment expressing resistance to ethidium bromide. This in vitro-constructed mutation was transferred into S. aureus 8325-4 on a shuttle plasmid and introduced into the chromosome in place of the wild-type spa$^+$ gene by recombinational allele-replacement. Plasmid incompatibility was used to eliminate the shuttle plasmid and enrich recombinants. Western blotting showed that no wall-bound or extracellular protein A was expressed by the mutant and Southern blotting revealed that the chromosomal mutation had the predicted physical structure. Extensive phenotypic characterization ensured that the mutation was not pleiotropic and only affected Spa production.

(iv) Construction of mutants defective in more than one virulence factor

A set of strains derived from 8325-4 was constructed with all possible combinations of wild type and mutant hly, hlb and spa alleles. A double mutant of 8325-4 defective in both protein A and α-toxin was constructed by transducing the hly::Em^r allele into an Hly$^+$ strain carrying spa::Eb^r. β-toxin deficient derivatives were constructed by lysogenization with phage 42E. These strains will be tested for virulence in the mouse mastitis model by Dr Bramley.

(v) Molecular cloning and expression of the coagulase gene

A λcoa phage was identified by screening the gene library with antibodies. λcoa lysates of E. coli had plasma clotting activity and an immunoreactive protein of 60 kDa which comigrated with the major protein in S. aureus culture supernatants was detected. The coa gene was subcloned in pUC plasmid vectors. One recombinant expressed plasma clotting activity and a 60 kDa immunoreactive peptide. A hybrid β-galactosidase-coagulase protein and truncated derivatives were formed by subcloning. Their properties are

consistent with published biochemical data that the prothrombin binding domain of coagulase is located in the N-terminus of the protein. Mutants defective in the expression of coagulase are currently being isolated.

The cloned coa gene was transferred into S. aureus on a shuttle plasmid for expression studies. The level of coagulase was higher in a strain with a mutation in the agr locus which controls the level of several exoproteins. This suggests that coagulase is a member of a class of proteins that are negatively controlled by agr.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1. Publications in scientific journals.

Foster, T.J. (1986). A new genetic approach to defining the virulence determinants of Staphylococcus aureus strains that cause bovine mastitis. Irish Veterinary Journal 40; 110-115.

IV.2. Short communications.

O'Reilly, M. and T.J. Foster. (1986) Genetic analysis of Staphylococcus aureus virulence. Proceedings of the 14th World Congress of Diseases of Cattle, Dublin. (Abstract).

Foster, T.J., O'Reilly, M. and J. Anderson. (1986). Site directed mutagenesis in the study of bacterial toxins. Poster P17.2. Microbe 86.

Patel, A., Phonimdaeng, P., O'Reilly, M, and T.J. Foster. (1986). Molecular cloning, expression and inactivation of the protein A and coagulase genes of Staphylococcus aureus. Poster P19.9. Microbe 86.

Patel. A., Nowlan, P. and O'Reilly, M. and T.J. Foster. (1987). Inactivation of the protein A gene of Staphylococcus aureus by allele-replacement. Studies on the role of α-toxin and protein A in virulence. 108th Meeting of the Society for General Microbiology. St. Andrews.

TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	No
Exchange of staff	Yes	No
Joint experiment(s)	Yes	No
Joint meeting(s)	Yes	No

Descriptive information for the above data.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: I. A. D. R., Newbury

Contract no.: BAP - 0146 - UK

Project leader: A.J. BRAMLEY

Scientific staff: R.J. Foster

Address: Institute for Animal Disease Research
Compton Laboratory
Compton
UK - NEWBURY, BERKS RG16 ONN

Telephone no.: +44.635.578411 EXT.322

Telex no.:

Other contractual partners in the joint project:

T.J. Foster, Trinity College (Dublin)

Title of the research activity:

Genetic analysis and biologic assessment of virulence determinants of Staphylococcus aureus.

Key words:

Mastitis, Staphylococci, Virulence, Toxins, Neutrophils

Reporting period: November 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Staphylococcus aureus is a common cause of mastitis in cattle and sheep and various haemolysins, extracellular enzymes and surface components may contribute to virulence. The project investigates the role of α and β haemolysins, protein A and other components in virulence using single site deletion mutants produced by the University of Dublin and an infection model in lactating mice developed at Compton. By understanding which components play important roles in the pathogenesis of staphylococcal mastitis it is hoped to aid the development of vaccines to reduce the major economic loss associated with this disease.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Set up a breeding group of mice for the experimental model. Train a new staff member in the various techniques involved in the model. Perform an initial screening of the virulence of strains of Staphylococcus aureus elaborating α and β toxins in various combinations using various doses. Determine the strategy to be applied for the future comparisons and obtain preliminary data on the pathological changes developing during infection.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY. Lactating Compton White mice (BSVS) are inoculated into the right and left fourth abdominal mammary glands with washed suspensions of Staphylococcus aureus via the teat under halothane anaesthesia. After various intervals the mice are sacrificed and the mammary glands removed aseptically. The dissected glands can then be used either for assessment of bacterial growth or fixed appropriately for light or electron microscopy.

2. RESULTS. The majority of bovine mastitis isolates of Staphylococcus aureus elaborate both α and β haemolysins. A wild type bovine strain M60 intramammarily inoculated in doses between 1000 and 100 million colony forming units (cfu) lead to death within 48 hours in 8 of 15 mice challenged. A mutant of M60 in which the α haemolysin gene had been inactivated by site-directed mutagenesis, but still produced β toxin produced no deaths over the same period. A similar result was obtained with an additional strain (8325-4). The numbers of bacteria recovered from the mammary gland at either 24 or 48 h. were similar for both wild types and mutants (Table 1). Comparison of strains DU 5719 and DU 5720, derived from 8325-4, but with inactivated β and α & β genes respectively did show a tendency for more rapid intramammary growth for the α

Table 1. Effect of inactivating the α toxin locus of Staphyloocccus aureus on intramammary growth and deaths in mice over 48 hours.

Strain	Inoculum $\log_{10}$	$\log_{10}$ mean cfu recovered (SD)		No. deaths	
		α+β+	α−β+	α+β+	α−β+
M60	8.4-8.7	10.06 (0.03)	10.27 (0.05)	5	0
M60	6.2-6.3	8.44 (1.38)	9.01 (0.29	1	0
M60	3.4-3.6	8.09 (2.1)	8.35 (2.0)	2	0
8325	8.4	9.47 (.02)	10.25 (.12)	4	0

n = 5 in each case

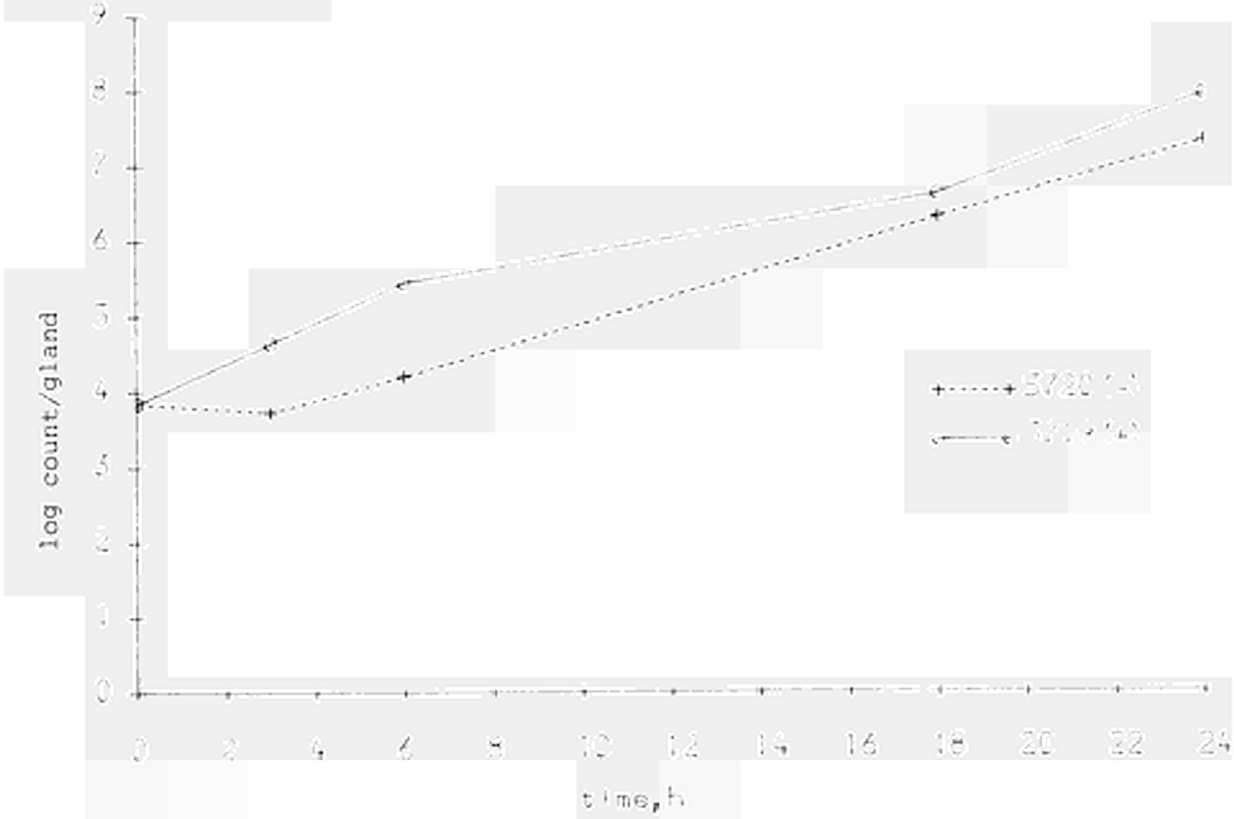


Figure 1. Growth of alpha toxigenic and non-toxigenic Staphylococcus aureus in the mammary gland.

toxigenic strain in the initial stages following inoculation (Figure 1). Data also suggest that the elaboration of β toxin may promote more rapid growth or protect from intramammary defences since recoveries of β toxigenic strains from the mammary gland were generally higher than for the inactivated strain although these differences were not statistically

Table 2. Effect of inactivating of the β toxin locus of Staphylococcus aureus 8325-4 on intramammary growth in the mouse over 48 hours.

Inoculum	Mean cfu recovered/gland	
	α-β+	α-β-
8.4	10.25 (0.12)	10.01 (0.14)
8.0-8.2	9.02 (0.26)	8.12 (0.65)
4.1-4.3	7.62 (1.77)	6.03 (2.4)

n = 5 in each case

significant (Table 2). Differences have been observed in the degree of neutrophil infiltration of the alveoli following inoculation with the various strains and this will be further investigated.

3. DISCUSSION. The preliminary findings indicate that the mouse mastitis model can be used to study virulence differences of staphylococci which have been altered by site-directed mutagenesis. This technique allows the precise study of virulence because only a single gene or a known combination of genes are disabled. The combination of α and β haemolysin produces highly virulent organisms which even when inoculated in doses of 1000 cfu may be fatal for a high proportion of mice in 24-48 h. The inactivation of the α haemolysin gene prevented lethality. Strains which produced α or β haemolysin were recovered in greater numbers following inoculation than deletion mutants devoid of haemolysin. This suggests that the α and β haemolysins may confer more rapid growth in vivo or protect from host defence mechanisms. Future work will examine these preliminary findings carefully and examine also the expression of the genes in vivo relative to the stage of bacterial growth.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

Foster, T.J., Reilly, M.O., Patel, A.H., Nowwlan, P. & Bramley, A.J. (1987) Genetic analysis of the role of toxins and protein A in the pathogenesis of Staphylococcus aureus infections. Properties of an unexpressed α-toxin locus from a clinical isolate. Proc. 3rd European Workshop on Bacterial Protein Toxins.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

Strains of Staphylococcus aureus have been exchanged.

Several meetings have taken place between Dr. Foster and Dr. Bramley and telephone and letter communication is frequent.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: I.N.R.A., Thiverval-Grignon

Contract no.: BAP - 0156 - F

Project leader: J. COHEN

Scientific staff:

Address: INRA
Station de recherches de
Virologie de Thiverval
Route de Thiverval
F - 78850 THIVERVAL-GRIGNON

Telephone no.: +33.1.30 54 45 45

Telex no.:

Other contractual partners in the joint project:

M.A. McCrae, University of Warwick (Coventry)

Title of the research activity:

Non-group A rotaviruses : characterization, contribution to disease and vaccine construction.

Key words:

C. Rotavirus, Purification, cDNA, Library, Structural proteins

Reporting period: September 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Are to use recombinant DNA technology in a detailed molecular characterization of non group A rotaviruses.

The first goal being to evaluate the importance of these viruses in the overall rotaviruses problem and to prepare and disseminate diagnostic reagents for the various atypical groups.

In the case of demonstration of the prevalent of atypical rotaviruses in epidemic situation, the long term goal of this project will be also the production of viral vaccine to provide protection against non group A rotaviruses.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

For the beginning of this project the laboratory has focussed his interest on group C porcine rotaviruses with 3 objectives :

- Transpose to atypical rotaviruses the purification protocole established for group A rotaviruses. This step requires special attention because atypical rotaviruses are known to be much more labile than group A rotaviruses.

- Obtain cDNA clones from semi-purified RNA and identify a set of cDNA clone corresponding to each genome segments.

- Characterize structural proteins in comparision with those of group A rotaviruses.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY

- Purification of virus and viral RNA :

Virus was obtained from faeces of infected gnotobiotic piglet (Pedley and al.). Fecal material is diluted in 50 mM PIPES pH 6.8 and extensively extracted with "Freon 113". The virus is semi-purified by centrifugation on a sucrose column, or on a cesium chlorid gradient. The purification steps were controlled by electron microscopy. Viral RNA was phenol extracted from semi-purified virions. In some experiments individual viral ds RNA segment was gel purified from direct phenol extract of clarified faeces (J.C. Nicolas and al.).

- Obtention and screening of genomic library :

Genomic cDNA library was constructed in pBR 322 from 2 ug of ds RNA using a strategy as previously described (Cohen and al.). Briefly, both strands were polyadenylated in vitro and reverse transcribed

using oligo dT as primer. Plus and minus strand of cDNA were reannealed, tailed with oligo dC and inserted in the dG tailed Pst 1 site of pBR 322. Screening of recombinant clones was achieved using Northern blot hybridization of ds RNA separated on 10% SDS-PAGE and electrotransferred on activated DPT paper.

- cDNA sequencing and proteins analysis :

a) Sequencing :

Sequencing of cDNA clones was done by using the dideoxy chain terminator method of Sanger and the shotgun strategy after subcloning random fragments in M 13 phage.

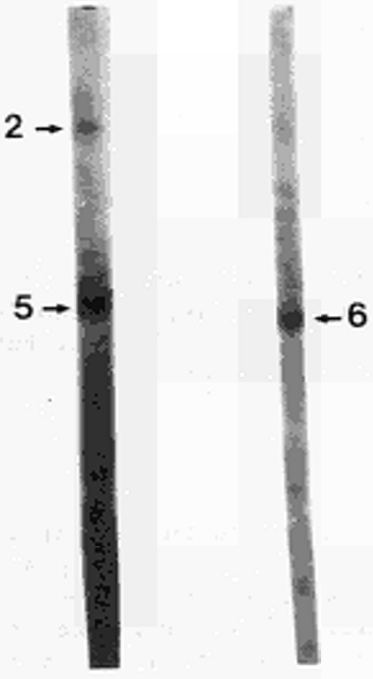
b) Proteins analysis :

Polypeptides pattern analysis of cesium chlorid purified particles was done on 12,5% SDS PAGE. Gels were stained with silvernitrate according to Oakley and al.

2. RESULTS

Characterization of cDNA clones :

Using the same strategy employed for cloning group A rotavirus, we have obtained about 0.5 ug of ds cDNA from 2 ug of viral DNA. Once tailed, insertion of one tenth of this material led to 800 recombinant clones. In a first step we selected clones having an insert longer than 500 bp and used them for making nick translated probes. Due to the fact that the virus was semi-purified, only 40% of the probes hybridize with the genomic RNA in Northen blot. An exemple is shown in Figure 1.

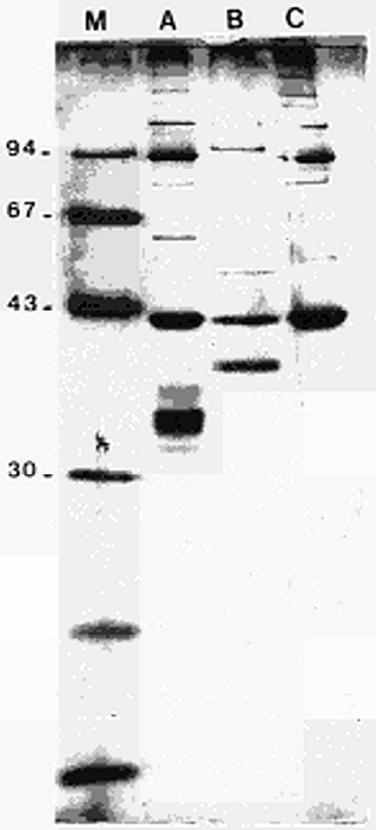


We have already selected several clones that hybridize with 5 different genomic segments. The size analysis indicates that these clones are partial.

First sequence data obtained from cDNA corresponding to gene 6, indicate that there is no homology for this segment with all the available group A rotavirus sequences. This result corralate the lack of hybridization of RF bovine rotavirus (group A) probes with the cDNA library.

Structural proteins of group C rotavirus

Comparison of polypeptides pattern of group C and group A rotavirus is shown in Figure 2.



Legend : (M) = Molecular wweight markers.
(A) = RF bovine group A rotavirus L particles.
(B) = Porcine group C rotavirus.
(C) = RF bovine group A rotavirus D particles.

Gel was 12° : 5% SDS-PAGE and silver stained.

We can notice several similarities between these two viruses. Particularly on both patterns, one can identify polypeptides having molecular weight of 125, 89 and 43 kdt. But the patern of the group C virus exhibit significant differences.

3. DISCUSSION

We and many research groups in the rotavirus field have focussed on developping new vaccine against group A rotavirus. But the evidence of non group A rotavirus in an severe gastroenteritis epidemy in humans (Hung and al. 1984) and the relatively high occurence of positive sera against non group A viruses in livestock animals

implies better molecular and antigenic characterization of these new viruses. During this period, we have achevied molecular cloning of a group C isolate. Screening and sequencing of the recombinant clones is in progress and will give us informations on the primary structure of structural proteins. Moreover the already available cDNA clones could be used as diagnostic probes. Characterization of structural proteins could be helpful in the gene assignment and in the identification of the gene(s) coding for the group and the type antigens.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

None

TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)		No
Joint meeting(s)	Yes	No

Descriptive information for the above data.

Dr. Mac Crae visited us recently to discuss the progress of the project and the experiments, both of us, could perform during the next period. We received from him anti-group C rotavirus antiserum for identification of viral proteins.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Univ. of Warwick Contract no.: BAP - 0114 - UK

Project leader: M.A. McCRAE
Scientific staff:

Address: University of Warwick
Dept. of Biological Sciences
UK - COVENTRY CV4 7AL

Telephone no.: +44.203.52.35.24

Telex no.: 31406 UNILIB COVENTRY G

Other contractual partners in the joint project:

J. Cohen, I. N. R. A. (Thiverval-Grignon)

Title of the research activity:
Non-group A rotaviruses : characterization, contribution to disease and vaccine construction.

Key words:
Rotaviruses, Non-group A rotaviruses, Viral vaccines, Viral gastroenteritis

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The non-group A rotaviruses are a recently defined complex of viruses falling into at least four serologically distinct groups. They appear to be widespread throughout Europe in a range of domestic livestock, however assessment of their contribution to the problem of acute viral gastroenteritis has been severly hampered by the absence of suitable reagents and assays for epidemiological surveying of the different non-A groups. The major objective of the project is therefore to exploit recombinant DNA techniques to provide both reagents for diagnosis of these infections and starting materials for the development of virus vaccines should this prove necessary.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The major objective has been the isolation of c-DNA clones from the different genomic RNA segments of the group B prototype virus. A secondary objective was the in vitro translation of the RNA segments into protein to facilitate the characterisation of protein encoded by each of the viral genes.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

(i) Methodology:- c-DNA cloning was undertaken using a strategy developed in this laboratory for use on group A rotaviruses (1). The fragility of the group B virions meant that it was not possible to purify the intact virus particles from the infected faeces to obtained purified genomic RNA as a starting material for c-DNA synthesis. Therefore c-DNA cloning was undertaken on the crude nucleic acids extracted directly from faeces. In vitro translation of denatured ds RNA was performed using the method that we originally developed for work on the mammalian reoviruses (2).

(ii) Results:- The use of a crude nucleic acid fraction extracted from virus infected faeces as starting material for c-DNA synthesis produced an obvious screening problem when trying to identify virus specific c-DNA clones. Despite these difficulties by using a combination of Grunstein-Hogness and Northern-blot hybridisation against paper strips carrying electrophoretically separated genomic RNA segments a number of viral specific c-DNA clones have been isolated. To-date c-DNA's have been obtained from four of the eleven genomic RNA's, segments 1, 2, 3 or 4 and 8. In the case of the larger segments the viral inserts range in size from 400-600 bp's and are therefore only partial copies of the genes in question which are 2-2.5 kbp's in length. The largest c-DNA isolated from gene 8 is large enough

(approx. 1 kb) to be full length or near full length, a restriction map of it has been made and sequencing of it by the di-deoxy chain termination method following sub-cloning into M13 has been started. A section of the gene 8 insert has also been sub-cloned into the pEX expression vectors (3) and expressed into protein as a carboxy-terminal fusion onto E.coli β-galactosidase. This fusion protein is currently being used to immunise rabbits to ascertain if the expressed viral sequences are immunogenic.

In vitro translation studies on the mixed population of genomic RNA's have revealed a number of putative viral gene products. Work is in progress to isolate large enough quantities of individual genomic RNA segments to allow their individual in vitro translation and hence identification of the particular protein product of each viral gene.

(iii) Discussion:-

The comparative analyses performed on the different groups of rotaviruses (4) prior to this project being started suggested that each group would represent a distinct 'gene pool' and could therefore be considered as a completely separate virus from a vaccine point of view. The limited amount of information that has been obtained in this project to-date has confirmed that original suggestion at least in the case of groups A-C. The DNA sequencing of the gene 8 clone that has been carried out has failed to reveal any significant level of homology with its group A homologue, in fact there was no homology with any of the other group A RNAs for which the sequence is known.

The E.coli expression work that has been undertaken is orientated towards providing a ready source of viral antigen for use in serologically based diagnostic assays, since the inability to grow these non-group A viruses routinely in tissue culture remains as a major hurdle to producing reagents for epidemiological survey work. Gene 8 was chosen as the initial target as the largest c-DNA in our initial bank of clones came from this gene. It is of course possible that the protein product of that particular gene is a non-structural protein precluding its use in the diagnostic assays and it is for this reason amongst others that the in vitro translation work has been commenced. It is hope that the results of these translation studies will provide information both on the major structural proteins of the virus, and also on the RNA coding assignments for them.

References

1. McCrae, M. A. and McCorquodale, J. G. (1982) J. Virol. 44, 1076-1079.

2. McCrae, M. A. and Joklik, W. K. (1978) Virology 89, 578-593.

3. Stanley, K. K. and Luzio, J. P. (1984) E.M.B.O. J. 3, 1429-1434.

4. Pedley, S., Bridger, J. C., Chasey, D. and McCrae, M. A. (1986) J. Gen. Virol. 67, 131-137.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)	Yes	
Joint meeting(s)	Yes	

Descriptive information for the above data.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: N. E. R. C., Oxford
Contract no.: BAP - 0120 - UK

Project leader: D.H.L. BISHOP
Scientific staff: P. Roy, S. Inumaru

Address: Natural Environment Research Council (NERC)
Institute of Virology
Mansfield Road
UK - OXFORD OX1 3SR

Telephone no.: +44.865.512361

Telex no.: 83147 VIROX G

Other contractual partners in the joint project:

A. Flamand, C. N. R. S. (Gif-sur-Yvette)

Title of the research activity:
Baculoviruses expression vectors for animal virus vaccines.

Key words:
Baculovirus expression vectors, Bluetongue virus vaccines

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT: The aims of the project are to use available (single) and new (multiple) *Autographa californica* (AcNPV) baculovirus expression vectors for the production of low-cost proteins that can be used as diagnostic reagents or subunit vaccines, and to test the latter for immunogenicity and protection of model animal species against infection. Multiple vaccines are required for many diseases to guard against the different viral serotypes that are operative in nature. They are also of value for the protection of an animal against different agents. In the UK laboratory subunit vaccines will be produced for five serotypes of bluetongue virus (BTV), in the French laboratory they will be prepared for both virulent and avirulent strains of rabies.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD: The aim of the UK laboratory in this reporting period was to obtain BTV DNA clones that were representative of the type-specific (L2) neutralization gene and group-specific (L3) gene of five BTV serotypes (BTV-2, -10, -11, -13, and -17) and to sequence and express them into proteins in high yields. The latter is to be accomplished by inserting cloned DNA copies of the genes into single and multiple AcNPV expression vectors, followed by recovery of the antigens, their characterization and, for the L2 genes, demonstrating their efficacy in eliciting neutralizing antibodies.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY.

A. Cloning and sequencing of bluetongue genes: The procedure used to obtain the complete nucleotide sequence of specific BTV RNA species involved the synthesis of cDNA followed by cloning and sequencing the derived viral specific plasmid DNA. In brief, the viral RNA species were polyadenylated at their 3' ends and cDNA copies of both strands synthesized using reverse transcriptase, deoxyribonucleoside triphosphates and an oligo(dT) primer. After removal of the RNA templates by hydrolysis with KOH, the cDNA products were self-annealed and, to ensure that the products were full-length, their 3' ends repaired with the Klenow fragment of *E.coli* DNA polymerase. The DNA species were then tailed with dC, and cloned into the *Pst* 1 site of pBR322. The clones representing the viral genes were identified by colony hybridization. Plasmid DNA prepared from positive colonies were digested with *Hinf* 1 and the sizes of the inserted DNA determined by electrophoresis of the products in 4% polyacrylamide gels. The recombinants with restriction patterns showing the largest viral DNA inserts were selected and grown in large scale. In order to confirm that they represented BTV, viral RNA was resolved by agarose gel electrophoresis, blotted on Genescreen paper and hybridized to nick-translated plasmid DNA. All the clones annealed specifically to particular BTV RNA species representing the original virus serotype used for cloning. The sequence of the BTV genes was determined by the Maxam and Gilbert method (1980) using strand-separated, end-labelled, restriction DNA fragments.

B. Expression of single BTV genes in baculovirus expression vectors. *Autographa californica* nuclear polyhedrosis virus (AcNPV) has been shown to be an efficient expression system for foreign genes. DNA representing BTV-17 RNA segment 3 was inserted into a baculovirus transfer vector in lieu of the 5' coding region of the polyhedrin gene of AcNPV. After verification of the construction, the plasmid DNA was purified and used to transfect *Spodoptera frugiperda* insect cells together with infectious AcNPV DNA. Recombinant viruses lacking a functional polyhedrin gene were recovered by selecting plaque-cloned progeny which did not produce visible occlusions in the infected cells. The derived plaques were used to grow polyhedrin-free virus. These were

screened for BTV DNA and mRNA by standard "Southern" and "Northern" hybridization procedures. Virus infected cell extracts were then screened for virus protein production. As expected, BTV protein was produced in high yield.

2. RESULTS:

A. Cloning and complete sequence analyses of the BTV L2 gene representing four US serotypes of BTV.

To determine the primary structures of the outer capsid VP2 proteins of 4 BTV serotypes (BTV-10, BTV-11, BTV-13, BTV-17), the L2 genes were cloned and sequenced (Maxam & Gilbert, 1980). From the results, the L2 RNA species of BTV-10 and BTV-11 were deduced to be 2,926 base pairs long (1.87×10^6 Da). Each has, in one strand, an open reading frame capable of coding for a protein with a calculated size of 111,122 or 110,051 Da (956 amino acids), respectively. Segment L2 RNA of BTV-17, on the other hand, was 3 base pairs shorter and encoded a protein of 953 amino acids with a calculated size of 110,574 Da. The predicted amino acid sequences of the VP2 proteins of the three BTV serotypes, although the viruses are serologically distinct, were similar (70% conserved sequences) indicating a common ancestor. Based on the predicted secondary structures, hydrophilicity, and the amino acid sequences, the overall VP2 structures of these viruses were evidently highly conserved (Ghiasi *et al.*, 1987). By comparison with these data, the sequence of the L2 gene of BTV-13 gave a different result (Fukusho *et al.*, 1987). Although the BTV-13 L2 RNA was only slightly longer than that of the other three viruses (2933 base pairs), its VP2 protein only exhibited 40% homology with the VP2 species of the other three BTV viruses. Despite this, the comparative sequence analyses of all four proteins indicated that they belonged to one protein family.

B. Expression of the group specific antigen, VP3: In order to obtain a high yield of the group-specific VP3 antigen, an essentially complete DNA copy of the L3 RNA segment of BTV-17 has been expressed. The derived protein corresponded in size to the BTV-induced VP3 protein, and, in mice and rabbits, induced antibodies that reacted with authentic BTV-10 or BTV-17 VP3 antigens (Inumaru *et al.*, 1987).

3. DISCUSSION:

A study of the genetic and antigenic variation of BTV at the molecular level has been instigated by comparing the complete nucleotide sequences of the VP2 genes representing 4 of the 5 different US bluetongue virus serotypes.

The comparative sequence data indicated that the L2 genes of BTV-10. BTV-11 and BTV-17 were more closely related to each other than that of BTV-13. Within the VP2 proteins of the respective viruses there were regions of the proteins with greater variability than other regions, as expected for proteins that vary antigenically but are structurally similar.

Recombinant baculoviruses containing the L3 gene of BTV-17 have been constructed and used to express the group-specific VP3 antigen of BTV in *S.frugiperda* cells. Immunoprecipitation of the VP3 protein produced in the insect cells showed that the product was similar to the BTV VP3 both antigenically and in size. The expressed VP3 antigen was also shown to elicit antibodies in mice and rabbits that recognized the BTV protein. Since VP3 is a group-specific antigen, this genetically engineered BTV antigen is being developed as a reagent for the diagnosis of BTV.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1 PUBLICATIONS IN SCIENTIFIC JOURNALS:

1. Ghiasi, H., Fukusho, A., Eshita, Y. and Roy, P. 1987. Identification and characterization of conserved and variable regions in the neutralization gene of bluetongue virus. Virology, *in press*.

2. Inumaru, S., Ghiasi, H. and Roy, P. 1987. Expression of bluetongue virus group specific antigen VP3 in insect cells by a baculovirus expression vector: Its use for detection of bluetongue virus antibodies. J. Gen. Virol., **68**: 1627-1635.

3. Fukusho, A., Ritter, G.D. and Roy, P. 1987. Structure and variation in the bluetongue virus neutralization protein VP2. J. Gen. Virology, *in press*.

IV.2 SHORT COMMUNICATIONS, INTERNAL REPORTS:

None.

IV.3 PATENTS DEPOSITED IN CONNECTION WITH THE RESEARCH PROGRAMME:

None.

IV.4 DOCTORATE THESIS (Ph.D) AND DEGREE THESIS AWARDED DURING THE PERIOD OF CONTRACT:

None.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

1. The UK laboratory supplied the baculovirus transfer vector, pAcYM1 to the French laboratory.
2. C. Prehaud (French laboratory) spent 3 weeks in the UK laboratory to undertake joint experiments.
3. See No. 2.
4. Professor Bishop spent 3 days in the French laboratory in a joint meeting.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: C. N. R. S., Gif-sur-Yvette

Contract no.: BAP - 0151 - F

Project leader: A. FLAMAND

Scientific staff: Ch. Préhaud

Address:
CNRS
Laboratoire de Génétique des Virus
F - 91190 GIF-SUR-YVETTE

Telephone no.: +33.1.69077828 EXT.384/320

Telex no.: 691137 CNRS GIF F

Other contractual partners in the joint project:

D.H.L. Bishop, N. E. R. C. (Oxford)

Title of the research activity:

Baculoviruses expression vectors for animal virus vaccines.

Key words:

Rabies virus, Subunit vaccine, Glycoprotein gene, Baculovirus expression vector

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Among various alternatives to classical rabies vaccination, subunit vaccines prepared from glycoproteins extracted from virions are safe and efficient although too expensive to be used on a large scale. We therefore investigated the possibility of obtaining large quantities of viral glycoprotein (G) through the expression of a cloned G gene in a new expression vector -a baculovirus- constructed at the NERC Institute of Virology (Oxford, U.K.). The gene will be placed under the control of the efficient promotor of the polyhedrin gene which is not necessary for the viral cycle in cell culture.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

This year has been devoted to the obtention of a complete and faithfull DNA copy of the rabies glycoprotein message, starting from total polyadenylated mRNA extracted from CVS-infected BHK cells. Such mRNA preparation has been translated in a rabbit reticulocyte lysate and promotes the synthesis of the rabies glycoprotein.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

cDNA copies of messengers have been obtained with reverse transcriptase primed with oligo dT molecules. After C tailing, they have been cloned in the Pst1 site of PBR 322 G tailed. We have obtained a bank of 4300 clones. After hybridization with viral probes (either rabies genome or cDNA of the G gene both labelled with ^{32}P), we have isolated 72 viral clones of which 21 are specific of the glycoprotein gene. None of them represents the totality of the gene. One clone possesses the 3' end of the mRNA with its AUG codon, another one the 5' end and a third one the intermediate region. The reconstruction of a complete gene is in progress.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

NONE

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Exchange of material : From D.H.L. BISHOP
a baculovirus transfert vector for expression of cDNA (pAcYM1).

Joint experiment :
Ch. Préhaud spent 3 weeks in Oxford

Joint meeting :
D. Bishop spent 3 days in Gif sur Yvette.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Institut Pasteur, Paris

Contract no.: BAP - 0117 - F

Project leader: C. BABINET

Scientific staff: M. Weiss, M. Yaniv, S. Cereghini, T. Chouard, L. Corcos, P. Herbomel, A. Mottura-Rollier, M. O. Ott, M. Raymondjean, J.P. Rousset, F. Tronche

Address:
Pasteur Institute
Dept. of Immunology
25, rue du Dr. Roux
F - 75724 PARIS CEDEX 15

Telephone no.: +33.1.45 68 80 00

Telex no.: 250609 PASTEUR F

Other contractual partners in the joint project:

G. Ciliberto, Università Napoli
R. Cortese, E. M. B. L. (Heidelberg)
M.C. Weiss, Institut Pasteur (Paris)
M. Yaniv, Institut Pasteur (Paris)

Title of the research activity:

Molecular mechanisms of liver specific gene expression and production of proteins of medical interest

Key words:

Hepatoma cells, Transfection, Albumin, Transcription factors, Transgenic mice.

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES

Our project is directed towards the progress of our knowledge of the molecular mechanisms responsible for the differential control of gene expression in liver cells, and aims at the exploitation of this information for the production of abundant and active proteins of medical interest. Our aim is to define the sequences and the transacting factors involved in the regulation of expression of serum protein genes of human and rodent origin, using both cells in culture of defined hepatic phenotype and transgenic mice.

II. SPECIFIC OBJECTIVES

The albumin gene has been chosen as a model for definition of molecular mechanisms involved in liver specific gene expression. Specifically, experiments will be undertaken to define the sequences bordering or within the albumin gene which are involved in its expression, and to determine which types of regulatory factors interact with the sequences thereby defined; to obtain evidence for specific binding of factors to these sequences; definition of the roles during development of these sequences by their introduction into transgenic animals; elaboration of selective methods for isolating hepatoma cells of defined phenotype.

III. SUMMARY OF RESULTS

During the first year of this contract, we have made significant progress in four of the proposed directions.

1) We have undertaken an extensive study of the rat albumin promotor in an attempt to understand the mechanisms and principles that restrict the expression of a certain group of genes to hepatic cells. Deletion analysis of the rat albumin promoter has revealed that roughly 180 bp upstream of the cap site are necessary and sufficient to direct tissue specific expression of reporter genes. In addition, RNAse protection experiments have demonstrated that the transcripts are initiated correctly. Linker scan mutagenesis carried out on these constructions, followed by transfection into albumin producing hepatoma cells and into albumin negative hepatoma cells variants, have permitted us to define sequence elements required for full promotor activity. These elements are defined as DEII, DEI, CCAAT homology and the PE element. Substitutions in the more proximal CCAAT and PE elements are more deleterious than those in DEI and DEII distal elements. In addition, deletions in PE destroy partially the usual strict tissue specificity of expression. It is certainly relevant that these sequence elements are conserved among the albumin genes of mouse, rat and man, and show good homology even with the avian gene.

2) The functional tests described above have been complemented by a biochemical search for transacting transcription factors, using DNAseI footprinting, gel retardation assays and methylation interference. At least four different nuclear proteins interact with the elements defined above. DEII is recognized by NF1/CTF transcription-replication factor.

The CCAAT element is recognized by a novel factor. DEI and PE bind two other factors. The pattern of protein-DNA recognition is conserved between rodents and man. Analysis of dedifferentiated rat hepatoma cells suggest that they contain a negative or repressing dominant factor that interacts with the proximal element.

3) To elucidate the nature and role of elements that control liver gene expression during development, we have prepared transgenic mice that contain different albumin-CAT constructions; the promotor domains under test extend to -180 bp upstream of the cap site, and to -1880 bp, linked to the CAT gene. Preliminary experiments have revealed by dot blot analysis that the introduced material is present in some of the mice. These anaimals are currently being analyzed by Southern blots, and for expression of CAT from the albumin promoter.

4) In view of obtaining new types of hepatoma cells variants that show specific modifications in the types of liver-specific genes expressed, we have developed a new selective system that eliminates cells producing some enzymes of the cytochrome P450 multigene family.

Differentiated rat hepatoma cells lines derived from the Reuber tumor express the microsomal mixed function oxidase system. This enzymatic system includes these products of the cytochrome P450 multigene family which are specific to the hepatocyte and mainly responsible for both detoxification and activation of xenobiotics. Earlier work by others has shown that the sensitivity of rat hepatoma cells to Aflatoxin B1 (AFB1), a potent hepatocarcinogen, is a function of their state of differentiation. Differentiated cells are more sensitive to AFB1 than dedifferentiated variant cells. We used the properties to devise a slection procedure for cells devoid of some cytochrome P450 species.

Cells were treated for 24 hours with 10 μM of AFB1; resistant cells were recovered in single step selection experiments at a frequency of 10^{-5}. Different morphological classes of AFB1 resistant cells can be obtained which are stably resistant to the selective agent. Four clones were characterized for accumulation of P450 mRNA specific to the P450b/e, P450 PB1 and P450 c genes

Three of the four clones produce more P450 c mRNA than the parental hepatoma cells; one produced little or none of this form. Cells of three of the four lines fail to accumulate P450 b/e and P450 PB1 mRNA. In contrast one line presents nearly undiminished P450 PB1 mRNA, but no detectable P450 b/e mRNA. These results implicate the liver specific cytochome P450 b and/or e enzymes in the metabolic activation of AFB1. The selective system described here represents a tool to identify the molecular forms of cytochrome P450 specifically responsible for the metabolism of various hepatotoxic compounds. The AFB1 resistant variants we have isolated will now be characterized for their expression of serum protein genes.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

NONE

For this first year of our contract, results have been obtained but not yet published; one manuscript is submitted and two others are "in preparation", all for scientific journals.

TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	cells lines and plasmids
Exchange of staff		No
Joint experiment(s)		No
Joint meeting(s)	Yes	see below

Descriptive information for the above data.

Joint meeting - At Cold Spring Harbor, New York, during a scientific meeting on liver-specific gene expression (May 1987) involving three project leaders and four collaborators.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: University of Naples Contract no.: BAP - 0116 - I

Project leader: G. CILIBERTO
Scientific staff: M.G. Pizza, R. Arcone, A. Pistone, B. Majello

Address: Università Napoli
Istituto di Scienze Biochimiche
II Facoltà di Medicina
Via S. Pansini 5
I - 80131 NAPOLI

Telephone no.: +39.81.253026

Telex no.:

Other contractual partners in the joint project:

C. Babinet, Institut Pasteur (Paris)
R. Cortese, E. M. B. L. (Heidelberg)
M. C. Weiss, Institut Pasteur (Paris)
M. Yaniv, Institut Pasteur (Paris)

Title of the research activity:
Molecular mechanisms of liver specific gene expression and production of proteins of medical interest

Key words:
Acute phase, C-reactive protein, Transgenic animals, Inducible promoter, Hepatocyte stimulating factors

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The main goals of the project are: a) the clarification of the molecular mechanisms responsible for the selective and differential expression of human genes in liver and other cell types; b) the exploitation of this basic knowledge for applicative purpose such as i) development of eukaryotic vectors for the construction of cell lines producing high levels of liver-specific proteins of medical interest, ii) construction of transgenic animals (initially mice but also sheeps and pigs) harbouring human genes expressing liver-specific proteins; c) identification of inducible promoters for the control of gene expression in transgenic animals.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The main interest of our laboratory is directed to the characterization of the molecular mechanisms responsible for the "acute phase response" in liver. During the last year we have cloned and characterized the structure of the major acute phase reactant in man: C-reactive protein (CRP). The expression of this protein is induced up to 1,000 fold as a consequence of several pathologies (reumatic disease, heart attacks, inflammations, etc.). We have started a detailed analysis of the mechanisms responsible for the gene activation during inflammation both by introducing the human CRP gene into transgenic animals and by transfecting fusions between the CRP promoter and a reporter gene into human hepatoma cell lines.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Methodology

Cloning the human gene for C-reactive protein.

We screened 5 x 10^5 clones from a human genomic library in vector pcos2EMBL using as probe the full-length cDNA for CRP (1). The map of the positive clone pcos-CRP1 was determined according to Rackwitz et al. (2). The coding region of the CRP gene, the 5′ flanking region and the pseudogene were subcloned into M13 vectors TG130 and 131 and pEMBL130 and 131 (3) and sequenced according to Sanger et al. (4). The 5′ flanking region of the CRP gene was fused to the bacterial gene coding for chloramphenicolacethyl-transferase (CAT) (5).Progressive deletions of the 5′ fl. sequence were obtained by digestion with Bal31 enzyme.

Transgenic mice.

F1 (CS7/BL6 x SJL) mice were bred in the mouse colony at the EMBL and the eggs for microinjection were isolated from the F1 crosses. All the manipulations for the microinjections were performed as described in Hogan et al. (6). Total RNA has been isolated according to standard protocols. CRP concentration in the mouse transgenics sera has been determined with a SOPAZYME-CRP test (IBL, Hamburg).

Cell culture and CAT assays.

CRP-CAT fusions were transfected into the human hepatoma cell line Hep3BB with the CaP precipitation technique (7). CAT assays were performed according to Gorman et al. (5). Hep3B cells were stimulated with 10% addition of conditionned medium from peripheral monocytes treated with LPS for 48 hrs (8). S1 analysis was performed as decribed (7).

2. Results

The first obligatory step in our project has been the cloning of the human gene for C-reactive protein. To this aim we have used the full-length cDNA clone (from Dr. Whitehead, Univ. of Boston) to screen a human genomic library in vector pcos2EMBL and obtained a positive clone pcos-CRP1 which was subjected to extensive restriction mapping and sequencing of the coding region and of 1 kb of the 5′ flanking region of the CRP gene. During this analysis we also mapped and sequenced a nontranscribed pseudogene for CRP located approx. 7 kb downstream from the normal gene.

We have then started to study the expression of the CRP gene using two different strategies: a) transgenic animals

b) transfections into human hepatoma cells

a) A 31 kb genomic fragment obtained by digestion with ClaI and containing about 20 kb of 5′ flanking sequences, the 2 kb coding region and approximately 8 kb of 3′ flanking region has been isolated and microinjected into fertilized mouse eggs.

Four founder mice were obtained carrying the human CRP gene integrated in the mouse genome. Immunoprecipitation in agar using mouse serum before and after induction with various agents (LPS, croton oil, conditioned medium from LPS treated human monocytes), revealed the presence of increased plasmatic human CRP levels in all 4 animals and their progeny. The F1 and following generations from two of the original founders were analyzed for the synthesis of human CRP mRNA in all the organs before and after intraperitoneal injection of LPS.

Northern analysis shows that the human gene behaves as a tissue-specific inducible gene also in the mouse context. In fact, we could not detect CRP transcripts in any organ before induction. The CRP mRNA produced after LPS injection is detectable only in liver and has the same M.W. as the human CRP mRNA.

We also performed a time course analysis of CRP expression both for the RNA in the liver and for the protein in the serum. CRP mRNA is already detectable 2 h after LPS injection; the peak of expression is reached at 9 h with a rapid decrease of the mRNA levels after 36 hours. The protein in the serum is appearing at later times, with the peak of protein expression 14 to 18 hrs. after LPS injection. A precise quantitation of the CRP concentration in the serum of the transgenic animals gave maximal values of 500 to 800 mg/lt which is in the same order of magnitude and sometimes even higher than that observed during acute phase conditions in humans. More recent RUN ON experiments allowed us to conclude that the control of CRP gene expression takes place exclusively at the transcriptional level (The work on mouse transgenic was done in collaboration with U. Rüther and E. Wagner at the EMBL).

b) It is possible to mimic on cultured hepatoma cells the change in the pattern of plasmaprotein production characteristic of the acute phase response. It is well known that the acute phase response is mediated by a series of molecules IL-1, TNF, hepatocyte stimulating factors (HSFs) released from activated monocytes. It has been shown that the human cell line Hep3B (9), when cultured in the presence of 10% conditionned medium from LPS induced

monocytes, has the property of undergoing an increased production of acute phase reactants (including CRP) and a decreased production of albumin and lipoproteins (8).
We have exploited this system in order to study the regulation of CRP expression. We have assumed that some if not all the information for the inducibility of this gene must be located in the 5′ flanking region of the gene. We have therefore constructed a fusion between 2.7 kb of 5′ fl. region and a reporter gene (the bacterial gene for chloramphenicolacethyltransferase) and used it for transient expression assays in Hep3B cells. The experiment was designed so as to transfect in duplicate: one plate is incubated with the normal medium, the other with the addition of 10% of monocyte conditionned medium. This initial analysis showed that the CRP promoter is silent in unstimulated Hep3B cells but becomes extremely active upon stimulation.
We constructed a series of progressive 5′ deletions which allowed to define the minimal segment still capable of showing the maximal stimulation to the 90 bp flanking on the 5′ side the CAP site. Furthermore, we showed by S1 analysis that the correct cap site is used in the conditions that give stimulation of CRP expression. The promoter is strictly tissue-specific because stimulation does not take place in other cell lines of non hepatic origin and follows the same rapid kinetic of activation observed in the transgenic mice.

3. Discussion

In our study of the molecular biology of the acute phase response we have focussed our analysis to the expression of the major acute phase reactant in humans, namely the CRP gene. We have shown that CRP gene expression during inflammation is controlled at the transcriptional level, both in the whole animal and in *in vitro* cultured hepatomas. The CRP promoter is strictly liver-specific, is silent in normal conditions, but is rapidly turned on when hepatocytes are reached by appropriate stimuli from macrophages. In these cases the promoter shows a high degree of inducibility and high transcription rates. The levels of CRP protein in the sera of transgenic mice reach sometimes levels even higher than those observed in humans. It is our interest to introduce the gene in animals of bigger size. This will open the chance of producing this protein in large amounts and therefore lower the costs of production (the human CRP purified from human ascitic or pleuric exudates costs approximately 300 US $/mg). In addition, we plan to construct fusions between the CRP promoter and the coding sequence of other genes and introduce these in transgenic animals or in cultured hepatoma cells. The rationale for this approach is to have the expression of several genes under the control of a strong, liver-specific and inducible promoter.

Bibliography

1. Woo, P., Korenberg, J.R. and Whitehead, A.S. (1985). J. Biol. Chem. **260**, 13384-13388. 2) Rackwitz, H.R., Zehetner, G., Murialdo, H., Delius, H., Chai, J.H., Poutska, A., Frischauf, A. and Lehrach, H. (1985). Gene **40**, 258-266.
3) Kieny, M.P., Lathz, R. and Lecocq, J.P. (1983). Gene **26**, 71- . 4) Sanger, F., Nicklen, S. and Coulson, A.R. (1977). PNAS USA **74**, 5463-5467. 5) Gorman, C.M., Moffat, L.F. and Howard, B.H. (1982). Mol. Cell. Biol. **2**, 1044-1051. 6)Hogan, B.L.M., Costantini, F. and Lacy, E. (1986). Manipulation of the Mouse Embryo: a Laboratory Manual. CSH, New York. 7) Ciliberto, G., Dente, L. and Cortse, R. 81985). Cell **41**, 531-540. 8) Darlington, G.J., Wilson, D.R. and Lachman, B.L. (1986). J. Cell. Biol. **103**, 787-793. 9) Knowles, B.B., Howe, C.C. and Aden, D.P. (1987). Science **203**, 497-489.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

1.1) DNA sequence of pseudogene for human C-reactive protein.
G. Ciliberto, M.G. Pizza, R. Arcone and L. Dente.
N.A.R. 1987. In press (July).

2) The human α_1-Antitrypsin gene is transcribed from two different promoters in macrophages and in hepatocytes.
E. Perlino, R. Cortese and G. Ciliberto.
EMBO J. 1987. In press (September).

3) Cis and trans-activity elements responsible for cell-specific expression of the human α1-antitrypsin gene.
V. De Simone, G. Ciliberto, E. Hardon, G. Paonessa, F. Palla, L. Lundberg and R. Cortese.
EMBO J. 1987. In press (September).

2.1) Acute Phase Induction of the human C-reactive protein gene in transgenic mice.
G. Ciliberto, U. Rüther, R. Arcone and E. Wagner.
Meeting on "Regulation of liver gene expression", Cold Spring Harbor Laboratory, April 27-May 3, 487, p. 187.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)	Yes	
Joint meeting(s)	Yes	

Descriptive information for the above data.

During this year we have been collaborating with Prof. Cortese's laboratory at the EMBL on several aspects of plasmaproteins expression both in hepatocytes and in macrophages.

a) We have characterized in detail the structure of the promoter of the alpha1-antitrypsin gene in hepatocytes making use of a detailed site-directed mutagenesis of the 5' flanking region of the gene; in parallel we identified at least two DNA binding factors capable of specifically interacting with the promoter region.

b) We have been able to show that the alpha1-antitrypsin gene is efficiently transcribed in macrophages and that transcription in these cells is under the control of a different macrophage-specific promoter. Its identification is of great interest for the possible use in the future of monocyte and macrophage specific promoters in gene therapy.

c) Our interest in macrophages stems from the observation that these cells actively produce the factors responsible for the changes in plasmaprotein production typical of the acute phase response (HSFs). We have therefore started a collaborative project aiming to the identification, cloning and expression of the hepatocyte stimulating factors from monocytes-macrophages.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: E. M. B. L., Heidelberg Contract no.: BAP - 0115 - D

Project leader: R. CORTESE
Scientific staff:

Address: European Molecular Biology Laboratory
Meyerhofstrasse 1
D - 6900 HEIDELBERG 1

Telephone no.: +49.6221.387200

Telex no.: 461613

Other contractual partners in the joint project:

M. C. Weiss, Institut Pasteur (Paris)
M. Yaniv, Institut Pasteur (Paris)
G. Ciliberto, Università Napoli
C. Babinet, Institut Pasteur (Paris)

Title of the research activity:
Molecular mechanisms of liver specific gene expression and production of proteins of medical interest

Key words:
Plasmaproteins, Gene expression, Transgenic animals, Protein overproduction, Liver

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The main goals of the project are: a) the clarification of the molecular mechanisms responsible for the selective and differential expression of human genes in liver and other cell types; b) the exploitation of this basic knowledge for applicative purpose such as 1) development of eukaryotic vectors for the construction of cell lines producing high lelvels of liver-specific proteins of medical interest, 2) construction of transgenic animals (initially mice but also sheep and pigs) harbouring human genes expressing liver specific proteins; 3) identification of inducible promoters for the control of gene expression in transgenic animals.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

We have developed a research project aimed at the characterization of the mechanisms responsible for liver specific gene expression. In addition to the potentially interesting information for the understanding of the development and differentiation of this organ, we believe that our results will provide useful information for the purpose of expressing large amounts of human proteins in convenient animal or plant systems. In the direction of the transgenic animals we are developing new techniques for the generation of animal mutants mimicking human inherited disease.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Introduction: During the last 12 months we have proceeded to implement our research proposal in three directions:

1) Characterization of the fine structure of the enhancer-promoter region of the alpha-1-antitrypsin gene.

2) Identification and purification of proteins that are responsible for the liver specific expression of several genes.

3) Construction and characterization of transgenic mice carrying the human alpha-1-antitrypsin gene and the human alpha-1-acid glycoprotein gene.

During this period we have collaborated with Dr. Ciliberto, of the University of Naples and co-participant in this EEC program, to characterize in vitro and in vivo the acute-phase response.

We have also established collaborative projects:

a) with Dr. A.J. Clark of the AFRC Institute of Animal Physiology and Genetics Research in Edinburgh, for the construction of transgenic sheep carrying the human alpha-1-antitrypsin gene.

b) with Dr. P. Schreier, at the Bayer Plant Protection Institute, Leverkusen, West-Germany, for the construction of transgenic tobacco plants producing human proteins of medical interest.

c) with Dr. Stunnenberg, at EMBL, for the use of insect cells and recombinant vaccinia virus to produce human proteins of medical interest.

Methodology: cloning and sequencing DNA segments. Northern and Southern blots. S1 mapping and primer elongation. In vitro transfection of human cell lines. Site-directed mutagenesis. Microinjection of cloned DNA into the mouse fertilized egg. Protein immunoprecipitation and Western Blots.

Results and Discussion

1) The enhancer-promoter region of the human alpha-1-antitrypsin gene

Alpha-1-antitrypsin (a_1AT) is one of the main protease inhibitors in human serum. It is believed that AT plays an important role in the control of the inflammatory response by inhibiting the excess of elastase and collagenase released from leucocytes. Single point mutations in the gene, leading to the synthesis of variant forms, are frequently associated with severe lung or liver diseases. a_1AT is synthesized mainly in the liver and to a minor extent in macrophages. It has been shown that the same gene is responsible for a_1AT production in both cell types. The a_1AT gene is therefore expressed in a cell specific manner. However, the fact that expression is found in cell types which originate from two different embryonal layers (hepatocytes from endoderm and macrophages from mesoderm) raises the question of whether a common mechanism for cell-specific expression in macrophages and hepatocytes exists, or whether the same coding sequence is transcribed in response to different regulators in the two cell types. In order to investigate the mechanism of expression of the a_1AT gene in macrophages we have characterized the a_1AT transcriptional units in these cells and discovered that there is a macrophage specific promoter located about 2,000 bp upstream of the hepatocyte specific promoter. Transcription from the two a_1AT promoters is mutually exclusive: the macrophage promoter is silent in hepatocytes and the hepatocyte promoter is silent in macrophages. These results suggest that the a_1AT gene transcription responds to two different cell specific regulatory mechanisms.

We have concentrated our effort on the liver specific control region and shown that the 5' flanking region of the gene contains cis-acting signals for liver specific expression. The functional dissection of the 5' flanking region by deletions and site-directed mutagenesis leads to the following conclusions: i) There are multiple regulatory elements; ii) A minimal tissue specific element has been identified between the nucleotides -137 and -37 (from the transcriptional start site); iii) This element contains at least two regions referred to as the A (-125/-100) and the B (-85/-70) domains, both essential for transcription; iiii) there are at least two other regulatory domains located upstream to this minimal element; the most important of these is located between positions -261 and -210.

2) Purification of nuclear proteins involved in the transcription of liver specific genes.

A considerable effort of our group has been directed towards the identification of those components of the transcriptional apparatus that are responsible for the expression of liver specific genes. On the basis of the genetic information we have set up several assays for nuclear proteins extracted from nuclei of rat liver cells including DNase footprint and gel retardation. The purification of these proteins has been done mainly with conventional chromatographic techniques using an FPLC machine. At the moment we are purifying to homogeneity a protein that binds to the control region of the retinol-binding protein gene and are in the process of obtaining information on its amino-acid sequence. On the basis of this information, oligonucleotides will be synthesized and used as probes for the cloning of the corresponding gene. Slightly less advanced are the projects aimed at the purification of several proteins that bind to the alpha-1-antitrypsin control region. Also in this case the ultimate goal is to purify the corresponding gene.

3) Transgenic mice

The possibility to generate transgenic mice by microinjection of cloned DNA into the fertilized mouse egg has prompted many studies of gene expression in the living organism. Transgenic animals also offer the possibility to be used as model systems for

human genetic diseases. With this possibility in mind we have started a project to study the expression of the human alpha-1-antitrypsin and the human alpha-1-acid glycoprotein in mice. We have shown that in both cases the cloned human DNA segment contains sufficient information for expression in the correct tissues. Furthermore, high serum concentration of the human proteins was found in the transgenic animals. In addition the human alpha-1-acid glycoprotein gene can be induced several-fold if the mouse is experimentally subjected to acute infection. This phenomenon offers an excellent animal model system for the study of the human genetic response to acute phase stimuli.

4) Collaborative projects.

With Dr. Ciliberto's group we have started a collaboration aimed at the identification of the molecular mechanisms responsible for the induction of the rate of transcription of several hepatic genes during the acute phase response. For this purpose we have set up an in vitro system based on the properties of a human hepatoma cell line, Hep3B (made available to us by Dr. B. Knowles), in which several acute phase genes are transcribed only in the presence of acute phase stimuli. This in vitro model complements the in vivo animal model and offers additional possibilities for the understanding of this important biological phenomenon.

Our contribution to the transgenic sheep project has simply been to provide for the human alpha-1-antitrypsin cDNA clone. All the work has been done in Edinburgh. Dr. Clark and colleagues have fused the human cDNA to the promoter of the ovine beta-lactoglobulin gene which is expressed specifically in the mammary gland during lactation. It is hoped that the human alpha-1-antitrypsin will be abundantly secreted in the milk. This should be a convenient source for the production of large amount of functional alpha-1- antitrypsin.

With Dr. Schreier we have constructed new plasmids carrying the neomycin resistance gene for selection of the transgenic tobacco and, in addition, the coding sequence of the human antithrombin gene fused downstream of a plant specific promoter. The transgenic plants were constructed in the Bayer laboratories. We have ascertained the presence of the human DNA segment integrated in the plant genome and are in the process of testing the production of human antithrombin III in the leaves and roots.

With Dr. Stunnenberg we have constructed a vaccinia recombinant carrying the human antithrombin gene. We have shown that the infected cells produce about 1mg per 10^6 cells. The advantage of using insect cell lines is that they grow easily at room temperature, reaching high density in the culture medium.

IV PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT

IV.1. Structure and expression of the genes coding for human α_1 acid glycoprotein. Dente, L., Pizza, M., Metspalu, A. and Cortese, R. EMBO J. (1987) Vol. 6, no. 8.

The human α_1-antitrypsin gene is transcribed from two different promoters in macrophages and in hepatocytes. Perlino, E., Cortese, R. and Ciliberto, G. EMBO J. (1987). In press (September).

Cis and trans-activity elements responsible for cell-specific expression of the human α_1- antitrypsin gene. De Simone, V., Ciliberto, G., Hardon, E., Paonessa, G., Palla, F., Lundberg, L. and Cortese, R. EMBO J. (1987). In press (September).

IV.2. Cell-specific expression of genes coding for human plasmaproteins. Cortese, R., De Simone, E., Frain, M., Hardon, E., Morrone, G., Oliviero, S. and Paonessa, G. Meeting on "Regulation of liver gene expression", Cold Spring Harbor Laboratory, April 27 - May 31, 1987, p. 3.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Exchange of materials: with the French groups of Dr. Weiss and Yaniv we have exchanged plasmids carrying liver specific genes from rat (Paris to Heidelberg) or humans (from Heidelberg to Paris). Likewise we have exchanged human (HepG2 and Hep3B) and rat (Fao) hepatoma cell lines.
Exchange of Staff: Dr. Rosaria Arcone and Luciana Dente from the University of Naples have spent six and nine months respectively at EMBL, working on the joint project on the acute phase signals.
Joint experiments: with Dr. Yaniv, analysis of the down regulation of albumin during the acute phase response. The lab in Paris provides the mutant rat albumin clones and the lab in Heidelberg performs the functional assays. With Dr. Ciliberto, a concerted effort to identify the signals and the target of the acute phase response.
Joint Meetings: Drs. Weiss, Yaniv, Cortese and Ciliberto have held a meeting on the occasion of the Cold Spring Harbor Symposium on "Liver specific gene expression".

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: C.N.R., Roma Contract no.: BAP - 0122 - I

Project leader: A. FALASCHI
Scientific staff: S. Riva, C. Tribioli, G. Biamonti, F. Cobianchi

Address: Istituto di Genetica Biochimica
ed Evoluzionistica del
Consiglio delle Ricerche
Via Abbiategrasso 207
I - 27100 PAVIA

Telephone no.: +39.382.422384 or +39.382.422411

Telex no.: 322883 IGBEPV I

Other contractual partners in the joint project:

J. Rommelaere, U. L. B. (Bruxelles)
P. d'Oultremont, Solvay & Cie (Bruxelles)

Title of the research activity:
Parvovirus-based linear animal vectors for production of vaccines and other useful proteins.

Key words:
Linear vector, DNA replication origins

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The project is aimed at the construction of a linear DNA vector capable of autonomous replication in mammalian cells. In principle such a vector could avoid some of the intrinsic disadvantages of the vectors based on viral replication origins developed so far while maintaining the possibility of cloning and correctly expressing human DNA sequences in human cells. The two constitutive elements of the linear vector are: 1) telomeric structures on both ends; 2) an active origin of replication in the middle of the molecule.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Isolation of human DNA sequences corresponding to origins of replication from synchronized human HL60 cells and cloning into a suitable vector. Characterization and sequencing of selected fragments. Test of replication *in vivo* and assay for binding of specific nuclear factors.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

In an attempt of isolating sequences corresponding to origins of DNA replication in human cells (1) we synchronized a HL60 cell culture at the G1/S border by means of two successive blocks of DNA synthesis with Aphidicolin followed by ^{3}H-BrdU labelling of DNA replicated at the onset of the S-phase. Newly synthesized DNA was purified by several CsCl density gradients and cloned into plasmid pAT153. Structural analysis on newly synthesized DNA revealed that it is significantly enriched in "snap-back" sequences but not in highly reiterated DNA sequences. Moreover our selected sample is not contaminated with mitochondrial DNA. 250 independent recombinant clones were obtained. Two of the largest fragments: pB48(1500bp) and pLC46(700bp) were confirmed by density shift analysis to be replicated at the beginning of the S-phase (see Fig.1).

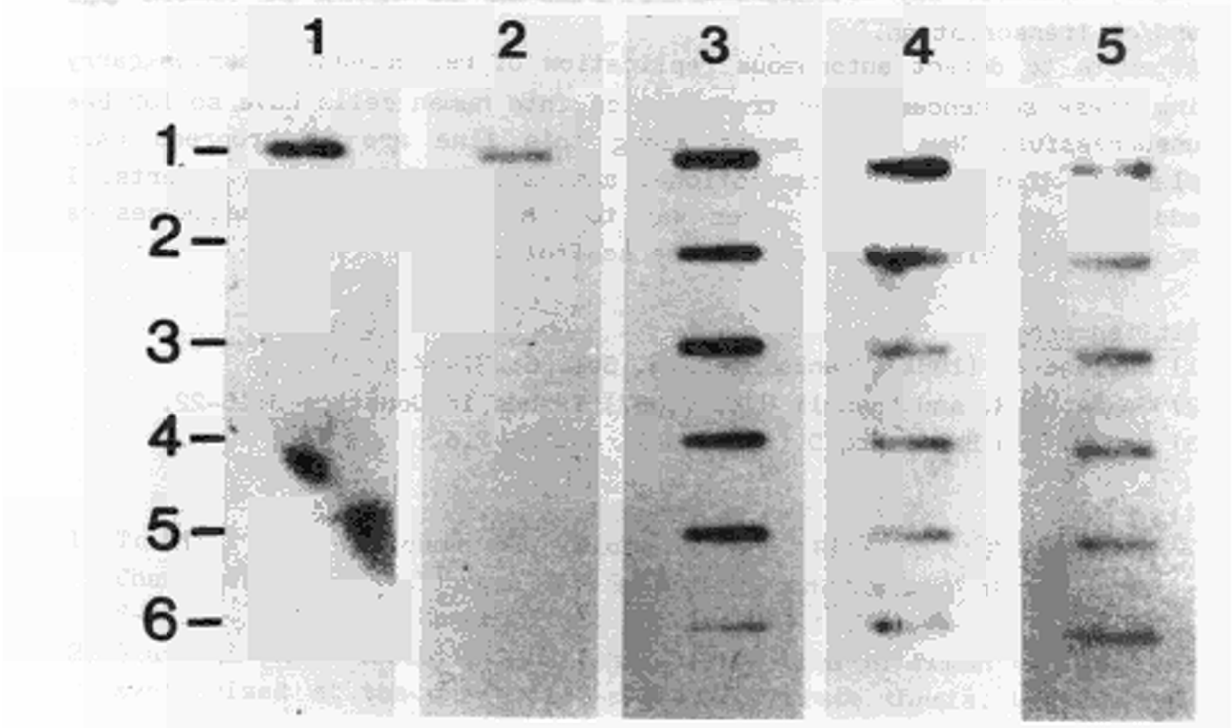


Fig.1: Time of replication of human DNA sequences as revealed by slot-blot hybridization to HL60 cell DNA replicated in successive S-phase intervals (1 to 6). Lanes 1):pB48 DNA; 2) pLC46 DNA; 3) pD8 DNA; 4) Human DHFR gene DNA; 5) Human ß-globin DNA.

As it can be seen both pB48 and pLC46 were highly enriched in the DNA replicated in the first hour of the S-phase. The time of replication of a third fragment (pD8) could not be determined due to the presence of a highly repeated sequence dispersed in the genome (see Fig.1). The nucleotide sequences of pB48 showed a number of features pointing to a possible regulatory function: a) numerous homologies with origin-specific regulatory sites for replication of Papova viruses; b) binding sites for known nuclear factors often associated with potential stem-loop structures on the DNA and c) AT-rich regions with many resemblances to the Scaffold Attachment Regions (SAR) described by Laemmli (2). By means of band-shift experiments we have found in pB48 a region that strongly binds an hiterto undescribed nuclear factor. Footprinting analysis with DNase I showed a corresponding protection of an 18-nucleotides region with the following sequence: 5'CTT CGTCAC GTGATG CGA 3' where the central 12 nucleotides constitute an almost perfect palindrome.

Like pB48, also pLC46 was shown to contain binding sites for nuclear factors. Furthermore the analysis of pLC46 sequence evidenced a number of possible SAR sequences in close proximity to a sequence identical to the enhancer octamer of the human Ig (k-chain) gene (3) and to a TATA box. Thus in both cases many relevant signals can be seen concentrated in a few hundred nucleotides. This observation suggests that in the nucleus

these fragments could interact with several factors (and structures) in a highly specific way with possible functional significance in replication and/or transcription.
Attempts to detect autonomous replication of recombinant plasmids carrying these sequences after transfection into human cells have so far been unsuccessful. New experiments along this line are in progress using plasmids that allow transcriptional activation of the human inserts. In addition experiments are under way to confirm that these sequences can specifically bind to the chromosome scaffold.

Bibliography

1) Harland R. (1981) Trends Biochem. Sci. 6: 71-74.
2) Gasser S.M. and Laemmli U.K. (1987) Trends in Genetics 3:16-22.
3) Sen R. and Baltimore D.(1986) Cell 46:705-716.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

1) Tribioli C., Colonna M., Biamonti G., Riva S., Falaschi A. - Characterization of human DNA sequences synthesized at the onset of S-phase. Atti Ass. Genet. Ital. 32: 211-213 (1986).
2) Biamonti G. - Isolation and characterization of human DNA sequences synthesized at the onset of S-phase. Doctorate thesis, University f Pavia (1987).

TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)		No
Exchange of staff	Yes	
Joint experiment(s)		No
Joint meeting(s)		No

Descriptive information for the above data.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: U.L.B., Rhode St. Genèse
Contract no.: BAP - 0121 - B

Project leader: J. ROMMELAERE
Scientific staff: B. Avalosse, A. Brandenburger, F. Dupont

Address:
Université Libre de Bruxelles
Laboratoire de Biophysique et Radiologie
67, rue des Chevaux
B - 1640 RHODE-ST-GENESE

Telephone no.: +32.2.35.83530 EXT.223

Telex no.: 23069 UNILIB B

Other contractual partners in the joint project:

A. Falaschi, Istituto di Genetica Biochimica (Pavia)
P. d'Oultremont, Solvay & Cie (Bruxelles)

Title of the research activity:
Parvovirus-based linear animal vectors for production of vaccines and other useful proteins.

Key words:
Parvoviruses, Oncolysis, Linear eukaryotic vector

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

1) Identification of the viral factor(s) that are responsible for the toxic effect of parvoviruses on transformed cells.

2) Construction of a linear eukaryotic vector, capable of autonomous replication as an episome.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

1a) Introduction of defined deletions into the cloned genome of parvovirus MVM

1b) Setting-up of cell-transfections with the cloned MVM DNA and its defective derivatives

2a) Construction of a linear plasmid with parvoviral termini and the origin of replication of SV40, capable of replication at a high copy-number under the control of the large T antigen

2b) Preliminary assays to test the functioning of the terminal palindromes of the vector as well as their stability in a cellular system.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

The presentation of the detailed results arising from the research carried out in the laboratory of Biophysics and Radiology (ULB, Brussels) has been deferred to next year, by request of the associated Company Solvay, to allow patent application in due time.

IV PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT

- Avalosse, B.L., Chen, Y.Q., Cornelis, J.J., Duponchel, N., Becquart, P., Namba, M. and Rommelaere, J. (1987)
Amplification of parvoviral DNA as a function of host cell transformation. In : "The role of DNA amplification in tumor initiati n and promotion". Eds. J.R. Schlehofer and H. zur Hausen, J.B. Lippincott Publ., pp. 140-152.

- Rommelaere, J. and Tattersall, P. (1987)
Oncosuppression by parvoviruses.
In : "The Parvoviruses". Ed. P. Tijssen, CRC Press, Boca Raton, in press.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	No
Exchange of staff	No
Joint experiment(s)	No
Joint meeting(s)	Yes

Descriptive information for the above data.

Cooperation with the laboratory of Dr. Falaschi in Pavia :

Once the linear vector with the origin of replication of SV40 will be validated (this will be done in Brussels), it will be transmitted to the laboratory in Pavia where human replication origins will be inserted. These final constructions will be tested in joined experiments.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Univ. College, Galway Contract no.: BAP - 0125 - IRL

Project leader: F. GANNON
Scientific staff: T. Barry, B. Keane, N. MacCarthy, R. Powell, M. Stack, J. Wolff, S. Power

Address:
University College
Dept. of Microbiology
IRL - GALWAY

Telephone no.: +353.91.24411 EXT.284

Telex no.: 28823 UGG EI

Other contractual partners in the joint project:

J.M. Sreenan, Agricultural Institute (Galway)
L.M. Houdebine, C. N. R. S. - I. N. R. A. (Jouy-en-Josas)
V.M. Nigon, Université de Lyon I (Villeurbanne)

Title of the research activity:
The development of transgenic animals (including fish) with novel characteristics.

Key words:
Transgenic, Cattle, Salmonids, Promoters, Growth hormone

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The aim of this joint project is to alter the genetic composition of cattle, rabbits, salmonids and chickens in a finely targeted manner. Two different methodological approaches are being used to achieve these goals: microinjection into 1 cell embryos or, for the chicken work, the use of retroviruses. The genes which are being introduced to the fertilized ova are those for growth hormone (which is the ultimate goal of the project) or readily monitored reporter genes, such as galactosidase or neomycin resistance. To achieve expression of these genes, a variety of homologous and heterologous promoters are being prepared and used in conjunction with other elements, such as enhancers. Ultimately, it is hoped that animals or fish of commercial importance with improved growth characteristics will be produced.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

In this reporting period, this laboratory had four specific objectives: (i) the provision of appropriate available DNA constructions to the laboratory of J.M. Sreenan (Laboratory B) and L-M. Houdebine (Laboratory C) to allow initiation of cattle and fish microinjection experiments and extension of these experiments with rabbits; (ii) the molecular cloning of bovine and salmonid sequences encoded by genes with presumptive strong promoters; (iii) the initiation of the genomic cloning of the corresponding structural genes and control elements and (iv) the analysis of potentially transgenic samples obtained from Laboratory B.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

METHODS

Generation of material for use in microinjection: DNA constructs which are described below were prepared by standard recombinant DNA methods. Plasmids which were required were purified by cesium chloride centrifugation and digested with two different restriction enzymes to separate the plasmid DNA from the gene construct which was of interest. After digestion and phenol extraction the DNA was ethanol-precipitated, ethanol-washed and resuspended at an appropriate concentration in Tris-HCl 10 mM, pH 8, EDTA 1 mM.

Preparation of cDNA banks: PolyA$^+$ RNA was prepared from bovine or salmon liver by the lithium chloride-urea method. cDNA banks were generated by the RNAse H procedure and integrated into pBR-Pst-dG by homopolymer tailing of the double-stranded cDNA. A larger bovine cDNA bank has also been prepared in λ GT10.

Screening of the cDNA banks: The cDNA banks were screened either by a radioactively labelled homologous cDNA probe or by a nick-translated human serum albumin probe (gift from B. Hughes and D. Headon, Galway).

DNA sequencing: DNA fragments required for sequencing were subcloned into an M.13 vector and the dideoxy method used for determination of sequence. The data obtained were analysed by use of the computer programmes Microgenie or PC Gene.

Genomic libraries: A genomic library for salmonids was prepared in λ EMBL3 using partially digested, size-selected Sau3A fragments of Sal gairdneri (rainbow trout) DNA. Screening of the libraries was by nick-translated or oligoprimed (for salmonid library only) DNA fragments.

Analysis of DNA in potential transgenics: DNA from blood obtained from potentially transgenic calves was prepared by leucocyte selection using plasmagel followed by phenol extraction and ethanol precipitations. After digestion by EcoR1, the DNA was transferred to nitrocellulose and probed with a DNA fragment homologous to the microinjected DNA. When the source of material was salmonid embryos, the DNA was prepared as described by the laboratory of L-M. Houdebine (Laboratory C) and used in dot blots. Assays for B-galactosidase involved a fluorometric method using methyllumbiferryl galactoside as substrate.

RESULTS

Provision of DNA constructs for microinjection: To allow immediate initiation of microinjection experiments, DNA samples were prepared in which DNA constructions which were obtained from R. Palmiter, Seattle and which contained either the mouse metallothionein (mMT) promoter fused to the human Growth Hormone (hGH) gene or mMT fused to the E. coli B-galactosidase gene were released from their plasmid vectors. Two novel constructions based on these were prepared by introducing into them a BamH1-BglII fragment from the bovine papilloma virus (BPV). This fragment encompasses the BPV enhancer region (Lusky et al., 1983, Molec. Cell. Biol., 3, 1108-1122). More recently, a new series of constructs have been generated based on the bovine Growth Hormone (bGH) gene (a gift from R. Maurer, Iowa) and either the MT promoter, the BPV-MT combination or the IEI gene promoter region of Cytomegalovirus (CMV) which is reputed to be an ubiquitous promoter/enhancer region (Boshart et al., 1985, Cell 41, 521-530; Foeching and Hofstetter, 1986, Gene 45, 101-105).

Search for a sequence encoded by a gene with a strong promoter: cDNA banks were generated for both salmon and cattle. Using an approach previously described for cattle (see F. Gannon B.E.P. Final Report 1986, p. 355-367), the salmon cDNA bank was probed with a homologous total cDNA probe. Strongly hybridizing colonies were presumed to correspond to most frequently occurring sequences. Two which were selected at random (SL37 and SL94) cross-hybridized in a back hybridization experiment and corresponded to 7% of the sequences. A third sequence (SL85) represents a different sequence which is present in the bank at a level of 2%. SL94 is 890 base pairs long and encompasses SL37 (size 740 bp) and extends beyond it at the 3' end. The orientation of the sequences was demonstrated by a novel probe for polyA regions. The DNA sequence of most of SL94 and portions of SL37 has been established. At the 5' end, both terminate at the same nucleotide and are thus possibly full length at that end. Despite the frequency of occurrence of this sequence in salmon liver, SL94 did not show homology with nucleotide sequences in the available DNA data banks. Northern blot analysis shows it to detect a single RNA band of size 1,200 bases.

The bovine cDNA bank was probed with a hSA probe and colonies containing putative bovine serum albumin (bSA) sequences were isolated. By back-hybridization, this sequence was shown to be present in approximately 8% of the clones. This is 8 times more frequent than the previously isolated CL50 sequence (see F. Gannon B.E.P. Final Report 1986, p. 355-364). The bSA clones were confirmed as such by DNA sequencing where high (frequently 75%) homology with rat or human SA sequences was found. Northern blot analysis showed that the bSA mRNA has a size 2200 bases. From this information and DNA sequence data, it appears that the 5' end of the bSA sequence is missing. With a view to obtaining that, a larger bovine liver cDNA bank has been prepared in λ GT10 and positive clones are being analysed.

Genomic studies: A trout genomic library has been prepared in λ EMBL3 by standard methods. The complexity of the bank is approximately 5×10^5 pfus. The bank is now ready to be screened with a probe from the 5' end of SL94. The bovine genomic bank prepared in a similar manner has been screened with both bSA and CL50 clones. Analysis of the results was initially confused by the presence in the bank (and in all preparations of bovine DNA in this laboratory) of a pBR-like sequence. This sequence is also apparent in Northern blots and independently prepared cDNA banks (A. Cooney and D. Headon, pers. comm.). Correctly hybridizing bSA sequences have now been obtained and await fine analysis when the 5' end of the cDNA is cloned.

Analysis of potentially transgenic cattle and fish: Blood obtained from control and test calves from Laboratory B was digested by EcoR1 (which cuts internally in the injected DNA), Southern blotted and probed with an MT hGH DNA. To date, no positive signals which would indicate transgenicity were obtained. Salmon embryos have also been analysed both for B-galactosidase activity and for the persistence of the injected DNA in the developing ova. DNA homologous to injected sequences was shown to be present in approximately 50% of samples after 14 weeks in a dot blot analysis. Southern blots were not successful because of partial degradation of the DNA and a low yield of DNA ($<$ 1 ug per sample). The galactosidase assays (see Table 1) showed occasional positive samples in our assays and which responded positively to criteria of significance in statistical analysis carried out in Laboratory B.

TABLE 1	Time:	9 weeks	12 weeks	14 weeks
	Positives per test:	2/4	2/8	0/5

DISCUSSION

In the first year, the work in this laboratory has progressed as projected in the timetable submitted with the initial project: (i) a wide range of DNA constructions have been made available to laboratories B and C; (ii) the analysis of cDNAs transcribed by potentially strong bovine and salmon promoters has been achieved; (iii) genomic banks for both salmonid and cattle have been generated and (iv) enzymatic and DNA analysis on potential transgenic calves and fish has been carried out. As by-products, these studies represent the first analysis at the DNA level of the bSA gene and of a major anonymous salmon liver sequence. The promoters corresponding to these sequences are now being actively sought. Finally, the most positive indication that progress towards the goal of transgenic domestic animals and fish is being obtained comes from the finding of a low level of expression of B-galactosidase with the mMT promoter in salmon embryos.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1 McEvoy, T., Stack, M., Keane, D., Barry, T., Sreenan, J. and Gannon, F. (1987). The expression of a foreign gene in salmon embryos. (Submitted to Aquaculture).

Barry, T., Stack, M. and Gannon, F. (1987). A method to locate 3' cDNA clones. (Submitted to Gene Analysis Techniques).

IV.4 Stack, M.C. (1987). M.Sc. Thesis, National University of Ireland. "The Cloning of Frequently Occurring mRNA Sequences from Salmon (Salmo salar) Liver.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Exchange of materials: It is in this section that our cooperation with other BAP contractors has been most active. We have provided DNA constructions to both laboratories B (Sreenan) and C (Houdebine) for use in microinjection experiments in their laboratories. We have also received from laboratory C some DNA which they had sourced and which we used in our constructions. Another major area of interaction has been with laboratory B in the analysis by enzymatic reactions and DNA probing of the resultant animals and fish from their microinjection experiments.

Exchange of staff: Tom McEvoy from laboratory B has worked with this group to learn the enzyme assays involved in the analysis of the fish embryos. This will allow the transferral of this analysis to laboratory B as appropriate.

Joint experiments: As the DNA constructions which we provide to laboratories B and C are for use in microinjection assays, they are correctly defined as joint experiments. As the constructs which are used often involve viral and retroviral promoters and enhancers (CMV, RSV, BPV and Moloney Murine Sarcoma Virus), discussions with members of laboratory C and laboratory D (Nigon) have been useful in directing our choice.

Joint meetings: Because of the interdependent nature of our research and laboratory B, there have been many meetings between all members of both groups. In addition, the project leader in this laboratory has visited and discussed in depth strategies, methods and results, including comparison of raw data, with both laboratory C and laboratory D. These personal contacts are supplemented by periodic telephone calls and letters.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: An Foras Taluntais (The Agricultural Institute) Contract no.: BAP - 0147 - IRL

Project leader: J.M. SREENAN
Scientific staff: T.G. McEvoy, C.B. Ware

Address:
Agricultural Institute
Belclare Tuam
IRL - CO. GALWAY

Telephone no.: +353.93.55455

Telex no.: 28962 AFT EI

Other contractual partners in the joint project:

L.M. Houdebine, C. N. R. S. - I. N. R. A. (Jouy-en-Josas)
V.M. Nigon, Université de Lyon 1 (Villeurbanne)
F. Gannon, University College (Galway)

Title of the research activity:
The development of transgenic animals (including fish) with novel characteristics.

Key words:
Pronucleus, Microinjection, Transgenic, Bovine, Fish

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The production of transgenic animals (bovine) and fish (Salmo salar) by the introduction of heterologous DNA into recently fertilised ova.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The development of fertilised ovum supply, pronuclear and nuclear visualisation and microinjection, in vitro culture and ovum transfer methodologies to allow the direct transfer of various DNA constructs into recently fertilised ova ard to monitor gene expression in any offspring.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

PROJECT 1. THE PRODUCTION OF TRANSGENIC CALVES

Methodology. The approach adopted was to microinject heterologous DNA into one of the pronuclei of single cell ova or one of the nuclei of two-cell ova. To provide a supply of recently fertilised ova, donor heifers were superovulated (PMSG, 2,000 i.u., and prostaglandin, 500 ug) during midluteal phase. Following insemination at oestrus, ovum recovery was carried out under general anaesthesia. As a maximum number of 1-cell ova are required the interval from insemination to ovum recovery was examined (Table 1).

Results. The ovum recovery data are summarised in Table 1. First insemination was carried out at an interval (M±S.D.) of 3±2.1 hours after observed oestrus onset and embryo recovery was then timed from this insemination.

TABLE 1. Effect of interval to recovery on the yield of 1- and 2-cell fertilised ova

	Hours from insemination to recovery	
	41 - 47	47 - 53
Mean (±SD) interval	44.4 ± 1.7	48.2 ± 1.2
No. donor heifers	21	21
No. ova recovered	184	249
No. 1-cell ova (%)	125 (68)	93 (37)
No. 2-cell ova (%)	28 (15)	112 (45)

Ovum recovery rate was lower (x^2 = 6.4; $p < 0.05$) at 47 - 53 hours from A.I. but because of the longer interval from oestrous onset, ovulation rate was higher. The proportion of 1-cell ova recovered was higher (x^2 = 39; $p < 0.001$) for the 41-47 hour interval from A.I. The proportion of 1- and 2- cell ova combined was similar ($p > 0.10$) for both intervals.

Following ovum recovery the next step was pronuclear visualisation. The most effective procedure evolved was ovum centrifugation at 13,000 x g for five minutes allowing visualisation of one pronucleus or nucleus in 60% of ova.

The third major step was the introduction of the DNA and the final procedure adopted was the microinjection of about 2 picolitres of solution containing about 500 copies of the recombinant gene. The ova were then cultured *in vitro* and later transferred (2, 3 or 4 per recipient) to heifers. Echography was used (ca Day 40) to determine pregnancy and foetal survival rate. A number of the recipients have produced calves at term (see Table 2).

TABLE 2. In vivo survival of microinjected bovine embryos

No. of recipients	26
No. ova transferred	66
No. recipients pregnant (%)	14 (54)
Foetal survival in pregnant recipients (%)	21 (55)
Foetal survival in all recipients (%)	21 (32)

The pregnancy (54%) and embryo survival (55%) rates are close to those recorded in studies with non-injected embryos.

The recombinant genes used were pMt-rGH, pMT-rGH-BPV and pMt-B-gal., and their consturction has been described (Gannon *et al.*, 1987) this vol. .

A total of 16 calves have been produced so far and blood and tissue samples are being subjected to dot-blot and Southern-blot analysis (Gannon et al., 1987, this vol.).

Discussion. Successful techniques for the recovery, pronuclear and nuclear visualisation, microinjection and transfer of cattle ova have been developed. The survival rate of microinjected ova (32% overall) is particularly encouraging. These techniques will now be used for the microinjection of various bGH recombinant genes.

PROJECT 2. THE PRODUCTION OF TRANSGENIC FISH

Methodology. Ova from Atlantic salmon (Salmo salar) were microinjected with recombinant genes (Gannon et al., 1987 this vol.) by the method of Chourrout et al., (1986). Ova were monitored for survival and for the presence of the pMt-B-gal gene.

Results. Survival rate of ova is summarised in Table 3.

TABLE 3. Ovum survival (%) at 50, 100 and 150 days

	No. ova	50	100	150
Controls	3,215	98.8	95.5	91.7
Microinjected	3,734	64.8	51.6	40.5

Two of 15 (13%) embryos (14 week old) that had received the pMt-B gal gene were positive (dot blot analysis). A fluorimetric assay was used to measure B-galactosidase enzyme to detect gene expression. Two of 8 injected ova (25%) had an increased level ($p < 0.01$) and 2 of 9 (22%) in a further sample also had an increased ($p < 0.005$) enzyme level (Table 4).

TABLE 4. B-galactosidase enzyme levels (ng/embryo)

Experiment No.	1		2	
	No. Embryos	ng/embryo B-gal	No. Ebmbryos	ng/embryo B-gal
Control	9	1.33	7	0.61
Injected negative	7	1.29	6	0.72
Injected positive	2	3.91	2	3.19

Oiscussion. The survival rate of microinjected ova was about half that of controls at 150 days. There is evidence of persistence and expression of the pMt-B gal gene.

The techniques developed for the bovine and for fish can be used to test various recombinant genes as they become available.

(Chourrout, O., Guyomard, R. and Houdebine, L-M., (1986) Aquaculture 51:143 - 150.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT.

SCIENTIFIC JOURNAL PUBLICATIONS (Short Communication)

McEvoy, T.G., Stack, M., Barry, T., Keane, B., Gannon, F. & Sreenan, J.M. (1987). Direct gene transfer by microinjection . Theriogenology, 27, (1), 258.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Exchange of materials. All DNA constructs for microinjection were provided by Dr. F. Gannon (Lab. A). Bovine and fish tissue samples from progeny have been provided by this laboratory (Lab. B).

Exchange of staff. Mr. T. McEvoy from this laboratory carries out some of the analysis at Dr. F. Gannon's laboratory.

Joint experiments. All work to date has been in full collaboration with Dr. F. Gannon's laboratory.

Joint meeting. Regular meeting of all relevant staff take place between laboratories A and B.

Dr. J.M. Sreenan has undertaken two visits to Dr. L.M. Houdebine, I.N.R.A., France (Lab. C) to discuss the overall project and progress. Mr. T. McEvoy has undertaken one visit to Dr. L.M. Houdebine's laborarory where the fish microinjection methodology was described and demonstrated to him.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: I.N.R.A., JOUY-en-JOSAS

Contract no.: BAP - 0179 - F

Project leader: L.-M. HOUDEBINE

Scientific staff: D. Chourrout, E. Devinoy, M.L. Fontaine, R. Guyomard, L.M. Houdebine, G. Jolivet, C. Puissant

Address: Laboratoire de Physiologie de la Lactation
CNRS - INRA
F - 78350 JOUY-EN-JOSAS

Telephone no.: +33.34.65.23.46

Telex no.: 695431 INRA CRZ F

Other contractual partners in the joint project:

F. Gannon, University College (Galway)
J.M. Sreenan, Agricultural Institute (Galway)
V.M. Nigon, Université de Lyon 1 (Villeurbanne)

Title of the research activity:

The development of transgenic animals (including fish) with novel characteristics.

Key words:

Transgenic fishes and rabbits, Rabbit milk protein genes

Reporting period: July 1986 - July 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

- to obtain transgenic fishes and farm animals with novel characteristics.
- to isolate promoters of rabbit milk protein genes and to fuse them to foreign gene in order to produce large amounts of the corresponding proteins in milk of transgenic animals.
- to study the molecular and cellular mechanism of action of lactogenic hormones on milk protein genes.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

- to define method to obtain transgenic domestic animals
- to isolate promoters of milk protein genes

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

A - Obtention of transgenic fishes

Methodology

Plasmids in their linear or circular form have been injected into trout eggs a few hours after fertilization before the first cleavage. These plasmids contained human GH cDNA under the dependency of SV40 early gene promoter (pSV507, pSV518), human GH gene under the dependency of either mouse metallothionein gene (pMT hGH) or mouse H_2 K histocompatibility gene (pH_2K hGH), rat GH gene under the dependency of either mouse metallothionein gene (pMT rGH or SV40 early gene promoter combined with mouse mammary tumor virus (pSV LTR GH). Trout eggs are opaque and pronuclei cannot be visualized with certainty. DNA was therefore injected into cytoplasm in relatively large amounts (20 x 10^6 copies in 20 nl).

DNA was extracted from whole young fishes just before hatching or separately from muscle, liver and blood in older animals. The presence of the foreign genes in fish genomic DNA was evaluated using homologous probes by slot blot or Southern blot techniques.

Results

About 75 % of the embryos routinely survived after injection and 50 % of fishes which received the foreign DNA in the linear form were

transgenic. This percentage was reduced by half when circular plasmids were used. Up to about 50 copies per genome were found in some fishes. The percentage of transgenic fishes remained essentially constant throughout development of the animal up to one year. A comparison of various tissues revealed that foreign DNA was generally present in all tissues examined although at different concentrations, suggesting that the transgenic trouts are mosaic. A examination of DNA by Southern technique indicated that the central part of the injected linear plasmid is unmodified whereas the fragments of the linearization site reassociated to form head to tail and tandem concaterners (Fig.1). Additional bands of large size were also visible suggesting that the injected DNA was profoundly modified or that it is, at least in part, associated with trout genomic DNA. Preliminary experiments indicated that the foreign gene is transmetted to progeny. Experiments are in progress to evaluate quantitatively the efficiency of gene transmission. To the best of our knowledge, it was the first time that the obtention of transgenic fishes was reported.

Conclusion

Injection of foreign DNA into cytoplasm of trout early embryo leads to a high percentage of transgenic animals. The foreign genes seem, at least in part, to be integrated into genomic DNA of the animals. The various plasmids were equally maintained in adult trouts. The method described here seems highly efficient to obtain transgenic fishes. Experiments have been undertaken to define the most efficient promoters in fish cells.

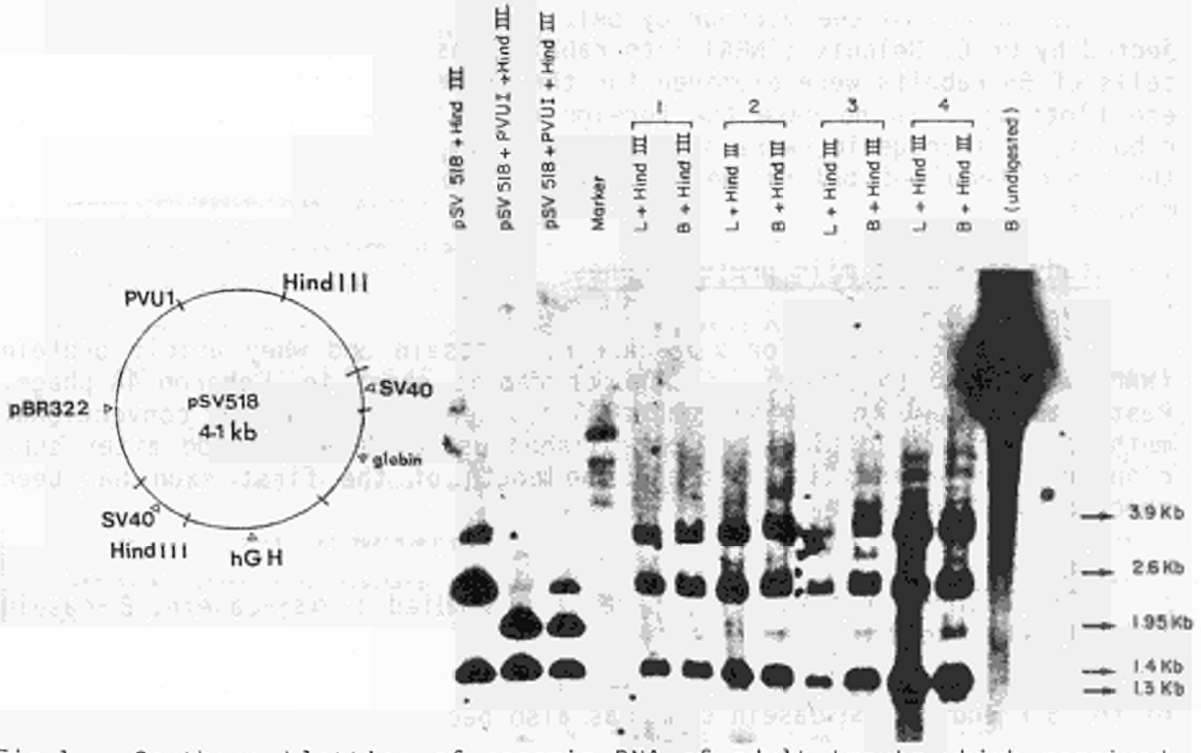


Fig.1 - Southern blotting of genomic DNA of adult trouts which received hGH cDNA in plasmid pSV518 (kindly given by Dr I. Lupker, ELF Biorecherches). The plasmid was open at the pVUI site before being injected. Genomic DNA from liver (L) and muscle (M) of four fishes digested with Hind III and hybridized with labelled pSV518. Bands at 1.3 Kb and 3.9 Kb result from the formation of concaterners. Bands larger than 3.9 Kb may result from an association of plasmid sequence with genomic DNA and they may reflect integration of the foreign DNA with trout genome.

B - Obtention of transgenic mammals

Apart from mouse, direct injection of foreign DNA into early embryo proved to be poorly efficient in most other mammals so far examined. Two alternative methods have been envisaged.

In a recent study, M. Rassoulzadegan et al. demonstrated that plasmids derived from pPy LT1 were very highly efficient to give transgenic mouse (Rassoulzadegan et al., Cell (1986) **46**, 513-519). These plasmids injected in their circular form were stably maintained and they were transmetted to progeny as episomes. These plasmids are therefore good candidate to be used as vectors for other mammals. One of these plasmids kindly provided by Dr M. Rassoulzadegan, pl2 B1 (Leopold et al., Biochim. Biophys. Res. Comm. (1986) **141**, 1162-1169) was injected into pronucleus of early rabbit embryos. About 15 % of the embryos survived and led to new born animals.

DNA of white blood cells of 55 young rabbits was examined for the present of the pl2 B1 plasmid. None of them harboured this foreign DNA. It is concluded that DNA of pl2 B1 cannot replicate efficiently in rabbit cells and cannot be used as vector to obtain transgenic rabbits.

Retroviral vectors have been used successfully by several groups to obtain transgenic mouse. Such a vector derived from Moloney mouse leukemia virus, and harbouring neo ^{r} gene was prepared by Dr J.F. Nicolas (Institut Pasteur). Envelope of the amphotropic virus 4070 A which recognize rabbit cells was given to the vecteur by pA12 cells. Cells of the virus were injected by Dr C. Delouis (INRA) into rabbit blastocysts. DNA of white blood cells of 65 rabbits were examined for the presence of neo ^{r} gene using Southern blotting . In no case the foreign could be detected suggesting that rabbits, if transgenic, were highly mosaic. Improvement of this method is therefore required before being used in rabbit and extended to other mammals.

C - Study of rabbit milk protein genes

Methodology

Full length cDNA for αs_1-casein, β-casein and whey acidic protein (WAP) were used to screen a bank of rabbit genes in λcharon 4A phage. Restriction map of the genes and cDNA has been determined by conventional methods. Sequence of DNA was established using Sanger method after subcloning of fragments in M13 phage. The length of the first exon has been checked by S1 nuclease mapping.

Results

Three milk protein genes have been studied : αs_1-casein, β-casein and WAP (whey acidic protein).

Complete sequence of WAP cDNA has been determined. Partial sequence of the 5'P end of αs_1-casein cDNA has also been established.

Clones harbouring the 5'P region of the 3 genes have been isolated and characterized.

In the clone for αs_1-casein gene, the exon surrounding the site of initiation of translation has been localized, sequenced and compared to the corresponding cDNA. CAP site is being localized.

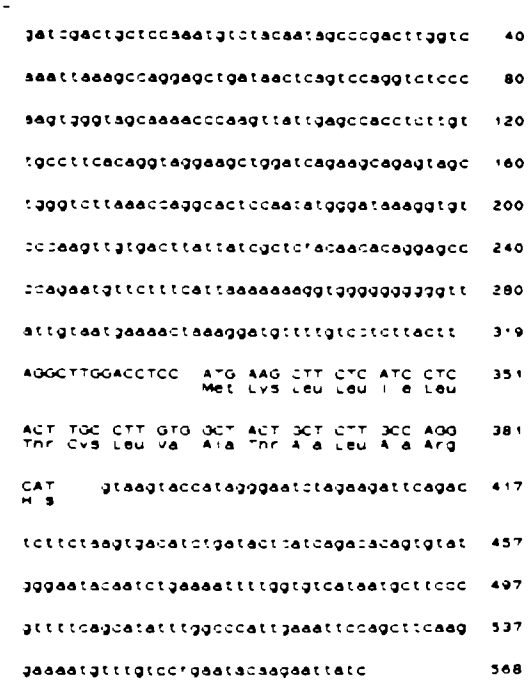
In the clone for β-casein gene, the whole β-casein gene and 380 bp upstream from the CAP site has been characterized. Sequence of the upstream region and of the first exon shows 75 % homology with the analogous sequence in rat β-casein gene. No clone containing further upstream

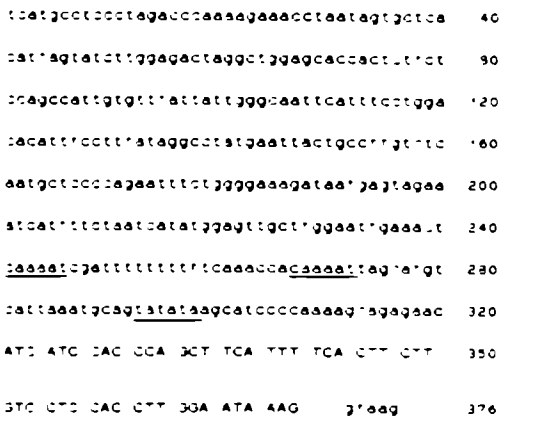
region could be found in the bank.

A clone for WAP gene contains 17 Kb upstream from the CAP site and 255 bp of the coding sequence split into two exons. In 1,8 Kb upstream from the CAP site, 64% homology were found with the analogous rat gene. These data are summarized in Fig. 2.

Conclusion

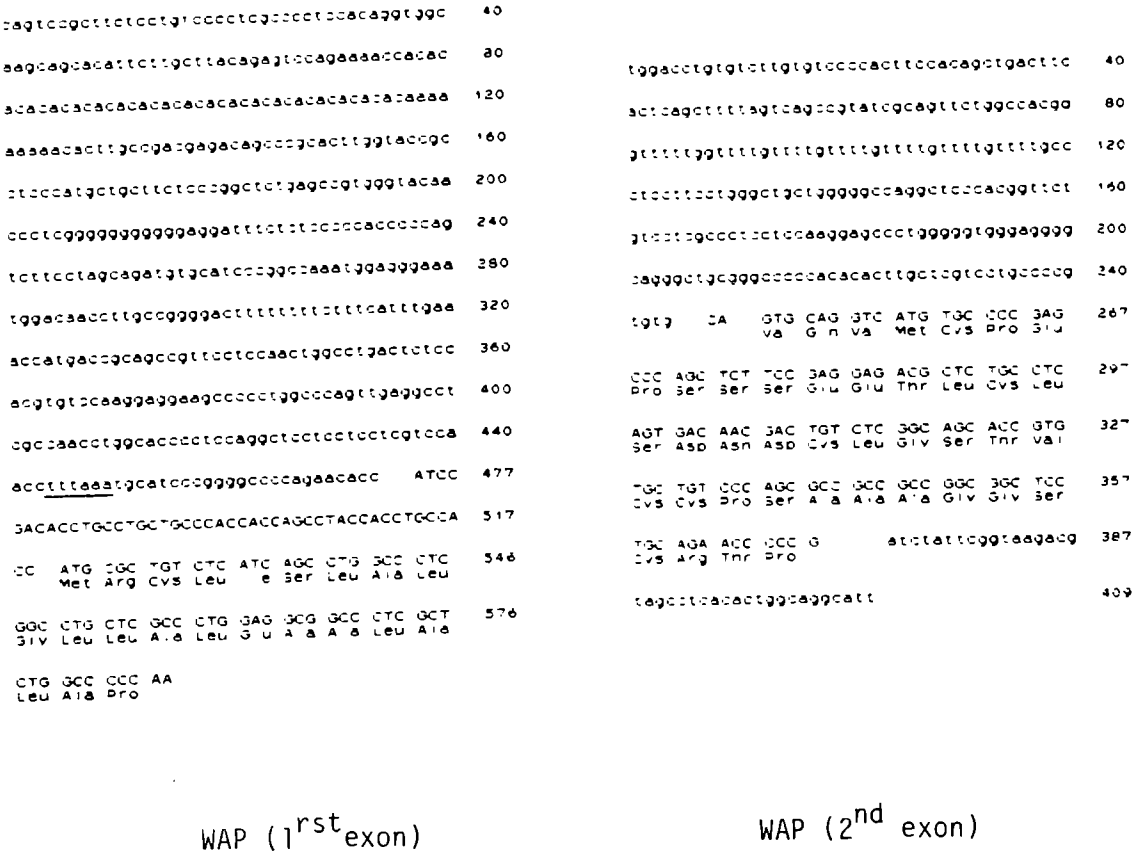
The 5'P end of three rabbit milk protein genes have been characterized and compared to analogous genes in other species. Two of the characterized clones (α_{s1}, casein and WAP) contained long stretches of DNA upstream from the CAP site and β-casein gene is complete in his coding section. Construction of chimaeric genes can therefore be under taken to characterize and to utilize promoters. They will be introduced into rabbit mammary cells in culture and in mouse and rabbit embryos by conventional methods to evaluate their expression.





α_{s1}-casein (2nd exon)

β-casein (1rst exon)



WAP (1^{rst} exon) WAP (2^{nd} exon)

Fig. 2 : Structure of α_{S1}-casein, β-casein and WAP genes

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT

IV.1. Publications in Scientific Journals :

- CHOURROUT D., GUYOMARD R., HOUDEBINE L.M., 1986. High efficiency transfer in rainbow trout by microinjection into egg cytoplasm. Aquaculture, 51, 143-150.

- SERVELY J.L., GEUENS G.M.A., MARTEL P., HOUDEBINE L.M., de BRABANDE M., 1987. Effect of tubulozole, a new synthetic microtubule inhibitor, on the induction of casein gene expression by prolactin. Biol. Cell. 59, 121-128.

IV.3. Review :

- HOUDEBINE L.M., 1987. Les animaux transgeniques. La Recherche, 188, 684-694.

TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	No

Descriptive information for the above data.

- plasmids constructed in Galway (F. Gannon) and containing or not enhancer from bovine papilloma virus to be tested in cell culture.
- plasmids containing various virus promoter sent from Jouy-en-Josas to Galway to be used for construction of vectors.
- plasmids containing GH genes sent from Galway to Jouy-en-Josas to be injected in fish eggs.
- plasmids containing LTR from avian retrovirus (AEV) to be tested into fish cells in culture (from Dr V. Nigon laboratory)
- plasmids p SV518 and p SV507 (from I. Lupker, Elf Biorecherches)
- In 1986, stay of Dr McEvoy (J.Sreenan Laboratory) in Jouy-en-Josas to learn technique of DNA microinjection into fish eggs.
- Utilization of the above mentioned materials
- Multiple exchange of information with other groups to define which vectors should be constructed.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Université de Lyon I

Contract no.: BAP - 0124 - F

Project leader: V.M. NIGON

Scientific staff: G. Verdier, J. Samarut, C. Bagnis, M. Belakebi, M. Benchaibi, F. Chambonnet, Y. Chebloune, F.L. Cosset, C. Faure, O. Gandrillon, P. Jurdic, C. Legras, F. Mallet, A. Reyss-Bryon, P. Savatier, P. Thoraval

Address: Université de Lyon 1
Dept. de Biologie
Générale et Appliquée
43, Bd. du 11 Novembre 1918
F - 69622 VILLEURBANNE CEDEX

Telephone no.: +33.78898124 EXT.3320

Telex no.: UCBL 330208 F (Attn. Prof. Nigon)

Other contractual partners in the joint project:

J. M. Sreenan, Agricultural Institute (Galway)
L. M. Houdebine, C. N. R. S. - I. N. R. A. (Jouy-en-Josas)
F. Gannon, University College (Galway)

Title of the research activity:

The development of transgenic animals (including fish) with novel characteristics.

Key words:

Retrovirus, Vectors, Transgenic, Gallus, Helper

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Methodology for the production of transgenic animals in domestic species.
Fish. Chicken. Rabbitt. Cattle.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOT THE REPORTING PERIOD:

Previous results (SALTER et al., 1986,1987; NIGON et al.,1986) provided arguments that genes can be transfered to germline of chicken through appropriate infection by retroviral vectors. However, from those results, definitive evidence of these interpretations are difficult to assess as all the experiments used virus productive systems. Thus eventual participation of horizontal viral transmission cannot be completely excluded. In order to obtain clear cut results, with eventually practical extensions, appropriate vectors, reproductively defective and as helper free preparations, should be constructed. This require the production of a helper cell line. Obtention of these results have been the main objectives ot this period.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

OPTIMIZED EXPRESSION OF GENES INSERTED INTO RETROVIRAL VECTORS.

Starting from AEV proviral DNA (pAEV), we produced several forms of vectors in which the two oncogenes v-erbA and v-erbB have been deleted and the Neo gene, providing resistance to G418, inserted in place of one of them, with different minor variations. The plasmid DNAs were cotransfected with pRAV1 DNA as helper on chicken embryo fibroblasts (CEFs); then G418 was added to select foci of resistant cells. These produced virions which were used to infect fresh CEFs from which the activity of neomycin phosphotransferase, the enzyme coded by the Neo gene, was assayed. The highest activities were obtained when the Neo gene was inserted in the same position and reading frame as anyone of the original oncogenes. Deletion of the 3' end of the gag gene determines a 50 % reduction of enzymatic activity, suggesting that this gag fragment contains transcriptional control elements as described for RSV. Maintenance of the erbA gene produces a 2.3 times increase of activity. When the inserted gene was in a reading frame different from that of the original oncogenes, a limited translation of active molecules was initiated exclusively at the AUG of the Neo gene; in these cases, very low activities are produced, sufficient to determine foci of resistant CEFs although undetectable by enzymatic assay.

ACTIVITY OF DIFFERENT TRANSCRIPTION CONTROL SEQUENCES IN AVIAN CELLS.

A set of plasmids has been derived from pAG60 (COLBERE-GARAPIN et al.,1981), which contains the Neo gene linked to the thymidine kinase (TK) promoter of Herpes simplex virus; a polyadenylation signal from TK is carried at the 3' end. The TK promoter was removed and replaced either by a tandem of AEV LTR (pAGLTR), or by the transcriptional control sequence of

SV40 early genes (pGAS NEO). In the plasmid pRAST NEO, an enhancer from the RAV2 LTR was associated with the SV40 early promoter. After transfection to CEFs and selection by G418, the highest efficiency, in terms of foci number, was observed with pAGLTR, whereas pAG60 and pRAST NEO did not induce resistant foci. After transfection to QT6, a permanent line of quail cells, pAG60 remains inefficient whereas the other plasmids gave equivalent results. Negative results with pAG60 depend probably from the absence of enhancer element in this construction. The differences observed between CEFs and QT6 might indicate the presence in QT6 of specific factors involved in the activation of enhancers.

ANALYSIS OF RETROVIRAL DNA INTEGRATION STRUCTURES.

Reverse transcription of retroviral RNA produces circular DNA structures, some of them provided with two LTRs in tandem. The junction of two LTRs generate an attachment structure (att) whose normal functioning, in the presence of reverse transcriptase, is required for efficient integration of viral DNA onto cell DNA. We started from a plasmid pX343 which contains a bacterial hygroR gene associated to the SV40 early promoter and polyadenylation signal. A 88 nucleotides sequence including the "att" region, obtained from a two tandem LTR clone, was inserted in the plasmid pX343 in the two possible orientations (pXatt3 and pXatt5). QT6 cells either previously infected, or not, with helper virus RAV1 (carrying the pol gene activity), were transfected with these plasmids, then selected for resistance to hygromycin B (40ug/ml).

DNAs were extracted from 13 clones obtained after transfection with pXatt5 and compared to those from 7 clones issued from transfection with pX343. These DNAs were digested with endonucleases bearing a unique site in the different plasmids; after electophoresis and transfer, the blots were revealed with pX343 as a probe. A major 6 kb band was identified in all cases, associated with two or more faint bands of variable sizes. A main difference between these clones was relative to the very different intensities of the 6 kb bands. From these data we conclude that:

- the 6 kb band corresponds to the predicted size of integrated plasmid linear DNA. The other discrete bands correspond to the junction fragments between cellular and plasmid DNA. The presence, in some clones, of more than two junction bands might result from several independant integrations in the initial cell of these clones.

- the different intensities of the 6 kb band represent integration of multimeric colinear plasmid DNAs of different lengths. To explain the unique length of this band, each linear molecule has to be tandemly associated to another at the same cleavage site and with the same orientation. These multimeric colinear DNAs have been estimated to mean values of 2-7 monomers per cell for pXatt5 used alone in transfection, 2-30 monomers when pXatt5 was associated to RAV infection, but only 2 monomers for transfections with associated to RAV infections.

CONSTRUCTION OF AVIAN PACKAGING CELL LINES PRODUCING HELPER-FREE DEFECTIVE RETROVIRUSES.

Plasmids containing RAV1 proviral DNA were modified by deleting either 54 nucleotides (pHF-13) or 164 nucleotides (pHF-405) between the 5' LTR and the AUG of pr65 gag, removing a structure probably involved in virion packaging. On both plasmids, the 3' LTR has been removed and replaced by the

thymidine kinase polyadenylation signal from the Herpes simplex virus.

pHF-13 and pHF-405 were introduced into QT6 cells by cotransfection using the bacterial hygroR gene as a selectable marker. 90 hygromycin resistant colonies were isolated and screened for production of p27gag both in cell lysates and in culture supernatants. The results suggest that p27gag expression may be governed by two independant stochastic factors occurring during the transfection process: one of them controls the level of intracellular production of p27gag; the other one controls the proportion of this protein released in the culture supernatant. The 22 clones producing the highest level of extracellular p27gag were selected. 15 of them were found to produce infectious viruses. Seven were not, although a few of them produced nearly as much p27gag as RAV-1 infected QT6. Three months later, all of them became virus producers. This result may be interpreted as the occurrence of either fortuitous infections or recombinations of transfected DNA with endogenous viral structures.

pHF-13 and pHF-405 were introduced into QT6 cells by cotransfection using the vector pXJ12 (erbA + NeoR) as a selectable marker. 39 G418 resistant colonies were isolated and screened for their ability to package the pXJ12 vector. Two clones were shown to transmit neomycin resistance via their culture supernatant without producing helper virus. Therefore they have the properties of a helper cell line. These cells accumulate 5 to 10 times less viral RNAs and 50 to 100 times less viral proteins than RAV1 infected QT6. They produce only 50 neo+ virions per ml of culture supernatant which is 10^3 to 10^4 lower than neo+ virion titers obtained usually with XJ12/RAV1 infected cells. These observations suggest a major defect in translation and packaging efficiency.

The results obtained suggest also the hypothesis that continuous production of viral proteins in a cell might adversely affect the cell's physiology; except if viral production discharges a certain amount of these viral proteins. After transfection of helper DNA, viral production may occur either through recombination with endogenous structure or by the properties of a vector like pXJ12.

DISCUSSION AND CONCLUSIONS.

Our results demonstrate that, starting from retrovirus proviral DNA, vectors may be produced transferring simultaneously 2 different genes into chicken cells. Expression of these genes has been optimized by use of appropriate enhancer and translation initiating structures. Properties of some limited parts of the retroviral and not retroviral structures have been analyzed and might lead to the building up of vectors transfering genes submitted to transcription control by non retroviral structures. Avian helper cell lines have been obtained. However, presently, the viral production of these lines is too low for allowing gene transfer to the germline. Further analyses are required to determine mechanisms responsible for limitation of virus production and to show possible ways for disrupting such limitations.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1 PUBLICATIONS IN SCIENTIFIC JOURNALS.

NIGON V.M., SAMARUT J., VERDIER G., FLAMANT F., BENCHAIBI M., PONCET D., SAVATIER P., CHAMBONNET F., THORAVAL P., FAURE C. and LANGLOIS P. ,1986. Use of avian erythroblastosis virus to produce vectors for gene transfer in poultry. 7th European Poultry Conf., Paris, 38-44.

GANDRILLON O., JURDIC P., BENCHAIBI M., XIAO J.-H., GHYSDAEL J. and SAMARUT J. 1987. Expression of the v-erbA oncogene in chicken embryo fibroblasts stimulates their proliferation in vitro and enhances tumor growth in vivo. Cell, 49 (in press).

FLAMANT F., LE GUELLEC D., VERDIER G. and NIGON V.M. 1987. Tissue specificity of retrovirus expression in inoculated avian embryos revealed by in situ hybridization to whole body section. Virology, (in press).

V.M. NIGON, G. VERDIER, J. SAMARUT, C. BAGNIS, M. BENCHAIBI, Y. CHEBLOUNE, F.L. COSSET, C. FAURE, O. GANDRILLON, P. JURDIC, C. LEGRAS, F. MALLET, A. REYSS-BRYON, P. SAVATIER and P. THORAVAL. 1987. Contributions to the construction of retroviral vectors and helper cell lines for gene transfer in chicken. CEE Meeting Salamanca. Ed. E. VINUELA (in press).

IV.3. PATENTS DEPOSITED.

JURDIC P., GANDRILLON D., SAMARUT J., NIGON V.M. Procédé pour la préparation de cultures à long terme de cellules. Brevet 87400771.9 déposé le 7.04.87

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

For the present time, the cooperation remained at the level of meetings with Dr GANNON and Dr HOUDEBINE, mainly for information exchange. Some genetic material has been forwarded to us by Dr HOUDEBINE.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: I.N.R.A., Tours

Contract no.: BAP - 0157 - F

Project leader: G.L. DUBRAY

Scientific staff: G.L. Dubray, M. Zygmunt, N. Bosseray, J.M. Trunde, M. Plommet

Address:
Station de Pathologie de
la Reproduction
INRA - Tours
F - 37380 MONNAIE

Telephone no.: +33.47.42.78.67

Telex no.: 750954 INRATOU F

Other contractual partners in the joint project:

J. Limet, I. I. C. M. P. (Bruxelles)

Title of the research activity:

Gene cloning of Brucella antigens which as vaccines do not interfere with a specific diagnosis.

Key words:

Brucella, Antigens, Genes, Cloning, Vaccine

Reporting period:

July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

To propose a new *Brucella* vaccine and a new serological test that will not suffer neither interference of cross reacting antibodies nor interference of vaccinal antibodies since the vaccine will not contain the antigen used for the diagnosis.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

For the vaccine :
1- production of monoclonal antibodies against the major protective antigens
2- preparation of DNA from *Brucella melitensis* 16M.
3- construction of Brucella genomic libraries in *E. coli*.
4- selection of recombinant *E. coli* clones producing the different protective antigens
For the diagnosis :
1- purification of A2 antigen.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1- METHODOLOGY :

The work done at Nouzilly on A2 antigen has been carried out using standard biochemical methods such as HPLC, ultrafiltration, electrophoresis and western blotting.

Chromosomal DNA was extracted from *Brucella melitensis* 16M By Dr. Verger at Nouzilly.

2- RESULTS :

Protective activity of monoclonal antibodies : Three monoclonal antibodies (IgG1, IgG3 and IgG2a) directed toward the lipopolysaccharide A epitope of *B. abortus*, restricted the infection of spleens on day 7 post challenge in the mouse model (Limet et al. under press).

Screening of the genomic library : the screening of recombinant EMBL phage plates is in progress using a microplate assay.

Purification of A2 antigen : By HPLC on DEAE column, A2 was located in fraction 5 after immunodiffusion analysis, that indicates a pHi< pH 7. This fraction contains 3 major bands of proteins when analysed by SDS-PAGE.

By preparative isoelectric focusing A2 antigen was located in fraction 5 and 6 after immunodiffusion analysis . After immunoblotting, two bands were labelled. The pHi is between PH 5.0 and pH 5.65. The fraction 5 and 6 contains 3 and 5 major bands , respectively, when analysed in SDS-PAGE.

For large scale purification, ultrafiltration was used. A2 antigen is retained by Diaflo membrane XM 300 and YM 10. These results suggest an heterogeneity of A2 antigen.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

J. Limet, A. M. Plommet, G. Dubray and M. Plommet. 1987 Immunity conferred to mice by anti-LPS monoclonal antibodies in murine Brucellosis. Ann. Immunol. Inst. Pasteur, under press.

V TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	No
Joint experiment(s)	No
Joint meeting(s)	No

Descriptive information for the above data.

At INRA Nouzilly, we prepare DNA and all antigens that we send to Dr. Limet, ICP, Brussels. We also, test protective activity of monclonal antibodies.

At ICP Dr. Limet prepare monclonal antibodies and genomic libraries that he send to us.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: U. C. L., Brussels
Contract no.: BAP - 0123 - B

Project leader: J. LIMET
Scientific staff: A. Cloeckaert, P. de Wergifosse, P. Cornélis

Address: International Institute of Cellular and Molecular Pathology
MEXP - ICP 7430
75, Avenue Hippocrate
B - 1200 BRUXELLES

Telephone no.: +32-2-76.47.430

Telex no.: 23722 UCL WOL B

Other contractual partners in the joint project:

G.L. Dubray, Station de Pathologie de la Reproduction (Monnaie)

Title of the research activity:

Gene cloning of Brucella antigens which as vaccines do not interfere with a specific diagnosis.

Key words:

Brucella, Antigens, Genes, Cloning, Vaccine

Reporting period: July 1986 - July 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

To propose a new *Brucella* vaccine and a new serological test that will not suffer neither interference of cross reacting antibodies nor interference of vaccinal antibodies since the vaccine will not contain the antigen used for the diagnosis.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

For the vaccine :

1 - production of monoclonal antibodies against the major protective antigens present in the SDS insoluble fraction of *Brucella* cell walls (SDS-I fraction).

2 - construction of *Brucella* genomic libraries in *E. coli*.

3 - selection of recombinant *E. coli* clones producing the different potential protective antigens, by using polyclonal or monoclonal antibodies.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1 . METHODOLOGY :

For the production of monoclonal antibodies, classical methods were used. Immunisation were done with SDS-I fraction, cell walls or intact bacteria.

For gene cloning : a genomic bank using the cosmid pc2RB was constructed mainly in order to clone the genes corresponding to the enzymes coding for the biosynthesis of O chain of brucella LPS. Brucella DNA was cut by partial restriction with Sau 3AI. Conditions of the restriction were set to produce a maximal amount of fragments between 30 and 45 Kb. The cosmid was clived by Sma I. The clived cosmid was dephosphorylated to avoid its reassociation, then clived again by BamHI, and mixed with brucella DNA fragments. The mixture was submited to a double phenol and chloroform extraction and the DNA was precipitated by isopropanol-acetate. After ligation the cosmids were packaged using the commercial extracts "Gigapack".

For the proteins, banks were constructed in λ EMBL 3 and in λ gt 11 as expression vector For the cloning in λ EMBL 3 the phage DNA was cut with BamHI and the brucella DNA by partial restriction using Sau 3A1. Fragments between 9 to 23 Kb were used. Commercial EcoRI clived and dephosphorylated arms were used. The brucella DNA to be inserted was clived by HaeIII and different linkers will now be compared in order to improve the recombination efficiency. Up to now, DNA fragments between 3 to 7.2 Kb, prepared by electroelution after agarose electrophoresis, were ligated with linkers. These linkers were then clived by EcoRI before ligation with the λ gt 11 DNA. DNA fragments of the convenient size were also produced by moderate ultrasonication.

RESULTS .

Monoclonal antibody :

Several monoclonals antibodies directed against the LPS of *Brucella abortus* were already prepared at the begining of this research programm and passive immunisation experiments have showned that anti-LPS monoclonal antibodies were able to induce good protections (early and late effect as described by Plommet in Infection and Immunity , 1983, 41, 97-104). The efficacity of the protection observed seemed to be linked to the specificity of the antibody towards the A or M dominant antigen and to the abundance of these dominant antigens on the challenge strain. In order to confirm this fact we have produced new monoclonal antibodies directed against one Brucella melitensis strain (H38) in order to obtain anti-M antibodies.

These antibodies were used by the Nouzilly team in passive immunisation experiments. Its is now clear that antibodies directed against the corresponding dominant epitope on the challenge strain are needed in order to get an effective protection. The antibodies used up to now were of the IgG class, we have also prepared antibodies of the IgM and IgA class in order to evaluate their protective efficacity.

In collaboration with Bruno Garin (Maisons Alfort), we have analysed the specificity of these monoclonals towards the A and M antigen, by using LPS from different strains of Brucella prepared according to Dubray by SDS extraction and proteinase K digestion. Preliminary results indicate a good correlation with the classification establish using polyclonals antibodies. A more extended study will indicate if monoclonals could be used instead of polyclonal antibodies for the strain classification.

Balbc/c mice were also immunised with either brucella cell walls, SDS-I fractions or intact cells. On the monoclonals obtained 6 are now characterised. One is directed against a 75 K band in SDS-PAGE, an other recognised a 31-34 K triplet and the 4 others the 25 K band wich is one of the major band of the SDS-I fraction. The later are of the IgG_1 and IgG_{2a} class. Only one react with the 25 K protein coupled to latex particules. At least three of them agglutinate rough bacteria but not smooth one. Therefore, the 25 K protein is probably present at the surface of the bacteria, well accessible to antibody in rough bacteria (45/20) but not in the smouth strain (W99). Twelve other clones are positive in an ELisa test using SDS-I fraction as antigen and are under characterisation, several of them are directed against the 36 K protein, the other major protein of the SDS-I fraction. Protective activity of these anti-"proteins" monoclonal antibodies will be tested at Nouzilly.

Genomic libraries :

Ninety eight percent of the pc2RB recombinants are kanamycin sensitive and could therefore be assumed to contain brucella DNA. The size of the inserted DNA fragments of 10 randomly choosen recombinants was controlled by agarose electrophoresis after restriction of the cosmid DNA by EcoRI. All the recombinants contain brucella DNA fragments from 30 to 45 Kb in size. Screening of this bank with polyclonal and monoclonal antibodies is undertaken.

After cloning in λ EMBL 3, 10^5 pfu/µg brucella DNA were obtain of which 12.2 % lyse the NM 539 strain and could therefore be brucella DNA recombinant.

The genomic bank obtain up to now in λ gt 11 present the same efficacity with 25 % of self recombinant. Different linkers and possibilities of using it will be tested in order to improve the recombination efficiency, mainly in λ gt 11 were problems of expression of the brucella promotors should be less important.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

J.N. Limet, A.M. Plommet, G. Dubray and M. Plommet, 1987. Immunity conferred to mice by anti-LPS monoclonal antibodies in murine brucellosis. Ann. Immunol. Inst. Pasteur, in press.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)	Yes	
Joint meeting(s)	Yes	

Descriptive information for the above data.

Monoclonal antibodies and genomic bank prepared in Brussels were send to Nouzilly and used at Nouzilly.
Docteur B. Garin comes one week in our laboratory, where he learns about PACIA and ELISA. We realysed commun experience using our monoclonal antibodies and his LPS preparations.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Agricult. University Wageningen
Contract no.: BAP - 0118 - NL

Project leader: J.M. VLAK
Scientific staff: J. Roosien

Address:
Agricultural University
Dept. of Virology
NL - 6700 EM WAGENINGEN

Telephone no.: +31.8370.83089

Telex no.: 45015 BLHWG NL

Other contractual partners in the joint project:

D. McCahon, Animal Virus Research Institute (Woking)

Title of the research activity:

High level expression of foot and mouth disease virus antigens using a Baculovirus vector.

Key words:

Foot-and-mouth disease virus, Subunit vaccines, Baculovirus, High-level expression

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

One of the world's most important diseases of domestic animals is caused by foot and mouth disease virus (FMDV). Although the use of inactivated virions as vaccines has been successful in controlling the disease, certain outbreaks of the disease are associated with an inadequate inactivation of the virus particles. Therefore, it would be desirable to use viral capsids as vaccines. The objective of the research is the high level expression of FMDV proteins using the baculovirus expression system. The ultimate aim is to obtain FMDV capsid proteins or complete capsids in such a form that they retain their immunogenicity and can be used as vaccines.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The objectives for the reporting period include the development of a convenient procedure for the detection and analysis of baculovirus recombinants. Furthermore, FMDV cDNA constructs, provided by our partners in the joint project, are to be cloned into a baculovirus transfer vector and to be transferred by homologous recombination into the baculovirus genome. The FMDV cDNA constructs to be tested for expression contained the entire capsid region (four capsid proteins: 1A, 1B, 1C and 1D) along with the proteases (L and 3C), known to play a role in the maturation of the capsid precursor (fig. 1).

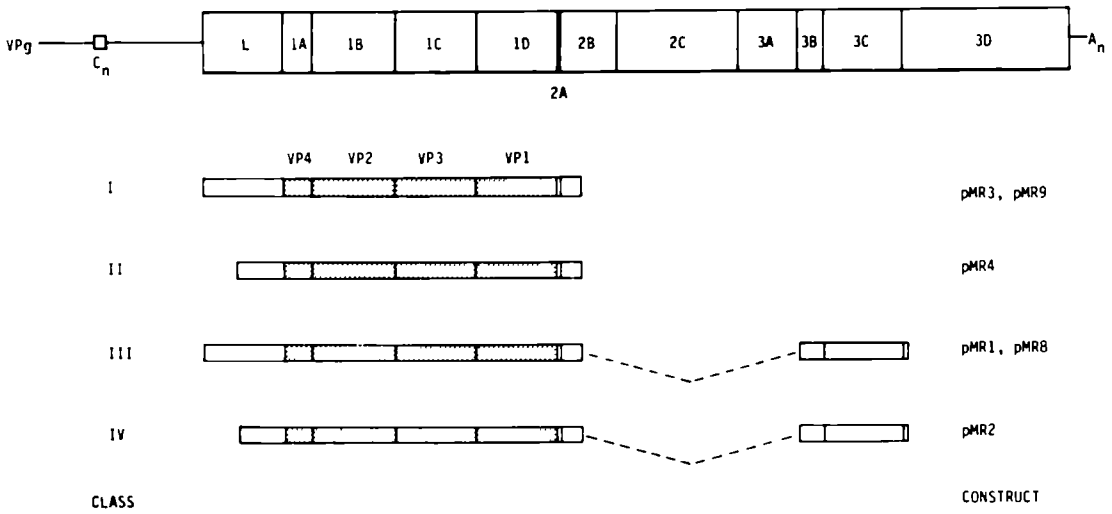
III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY

Wild type (wt) Autographa californica nuclear polyhedrosis virus (AcNPV, strain E2) and recombinants thereof were propagated in a cell culture of Spodoptera frugiperda (Sf) cells (IPLB-Sf21). Viral DNA was isolated from budded virus or from infected cells according to standard techniques.

The various FMDV cDNA constructs diagrammed in Fig. 1 and detailed by our partners in the project, were cloned into the transfer vectors pAc610 and pAc611. These vectors, kindly provided by Drs. M.D. Summers and G.E. Smith (Texas A & M University, College Station, Texas), contain a multiple cloning site in two different orientations in the polyhedrin gene leader region 8 basepairs upstream of the original polyhedrin start codon. The dotted areas in Fig. 1 represent FMDV capsid precursor P1. Class I constructs contain the original FMDV start codon, the L protease and the capsid precursor. In class III, an in-frame fusion of the protease 3C gene after the capsid genes is present. Class II constructs contain the 3'-end of the L protease preceded by an artificial ATG start codon and followed by the capsid precursor P1. In Class IV constructs an in-frame fusion of the 3C protease gene is made with the capsid precursor at the 3'-end. All four classes of constructs contain artificial stop codons.

Fig.1 FOOT-AND-MOUTH DISEASE VIRUS cDNA CONSTRUCTS



Baculovirus-FMDV cDNA recombinants were obtained after cotransfection of Sf cells with wt AcNPV DNA and AcNPV-based transfer vector DNA containing the various FMDV cDNA constructs using routine transfection and plaque-purification techniques. Putative recombinants were recognized as polyhedron-negative plaques.

DNA analysis of the baculovirus-FMDV recombinants was carried out using different restriction endonucleases by comparing the digestion pattern of the recombinants with those of the wt AcNPV and the AcNPV-based transfer vectors containing the FMDV cDNA constructs.

2. RESULTS

For the expression of FMDV capsid proteins in the baculovirus expression system different FMDV cDNA cassettes were prepared (fig. 1). With this set of different constructs it should be possible to determine the expression levels of the capsid precursor and the effect of the presence/absence of the FMDV proteases L and 3C on the processing of the capsid precursor P1. For practical reasons (the availability of cDNA segments of two different FMDV serotypes O_1K and A10) chimeric constructs were used (see Table 1 and the report of the counterpart in this joint project). In Table I the relevant features of the different constructs are summarized.

To obtain recombinants, Sf cells were cotransfected with AcNPV DNA and DNA of the different constructs (except pMR3). At a frequency of 0.1 - 0.3% plaques without polyhedra appeared. For each construct 4-5 individual recombinants were plaque-purified and analyzed by restriction enzyme digestion of the viral DNA. In each of the putative recombinants the DNA segments containing the wildtype polyhedrin gene had disappeared, and had been replaced by the FMDV cDNA constructs in the correct orientation. At present we have obtained a collection of recombinant viruses for all the different classes of constructs.

Preliminary experiments suggested that the expression of FMDV proteins, if any, might be low, since FMDV capsid proteins or its precursor(s) could not be detected on Coomassie Brilliant Blue stained SDS-polyacrylamide gels.

Table I

Construct	Class	Transfer vector	Recombinant	L		P_1		p3C	
				O_1K	A_{10}	O_1K	A_{10}	O_1K	A_{10}
pMR1	III	pAc611	Ac-MR1	+		+			+
pMR2	IV	pAc610	Ac-MR2		+[1]		+		+
pMR3	I	pAc611	Ac-MR3	+		+			
pMR4	II	pAc610	Ac-MR4		+[1]		+		
pMR8	III	pAc610	Ac-MR8	+[2]	+[2]		+		+
pMR9	I	pAc610	Ac-MR9	+[2]	+[2]		+		

[1]the L protein is truncated

[2]the L protein is a fusion product between O_1K and A_{10}

3. DISCUSSION

To study the expression of FMDV capsid genes in the baculovirus expression system, we were able to isolate a number of AcNPV-FMDV cDNA recombinants for each of the four different classes of constructs (Table I and Fig. 1). The FMDV cDNA constructs were properly inserted in the parental AcNPV genomes. Since FMDV proteins were not readily detectable in recombinant virus-infected Sf cells, further studies on these recombinants are required involving immuno blotting with FMDV capsid antisera and testing the plasmid constructs for correct expression in a coupled transcription-translation system (SP6).

The use of wt AcNPV appeared most suitable for the detection of FMDV cDNA-containing baculovirus recombinants by visual inspection. Since high-level expression of FMDV proteins was not readily achieved, it might be important in addition to recognize expression-positive recombinants using immunohistochemical methods.

Initially, transfer vectors pAc610 and pAc611 were used to insert the FMDV cDNA cassettes, since they seemed most appropriate due to the presence of a multiple cloning site. Since the integrity of the polyhedrin gene promoter appears to be essential for high-level expression of passenger genes (Matsuura et al., 1987, J. Gen. Virol. **68**, 1233-1250), it might appear more appropriate to tailor the promoter region according to the original sequence. It is therefore anticipated to make another series of recombinants with the present FMDV cDNA constructs using a transfer vector which contains the complete polyhedrin leader sequence. These recombinants will then be compared with the present collection.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

Roosien, J., Usmany, M., Klinge-Roode, E.C., Meijerink, P.H.S. en Vlak, J.M. Heterologous recombination between Autographa californica MNPV and Mamestra brassicae MNPV. In preparation.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Exchange of materials:

Baculovirus transfer vectors pAc610 and pAc611 as well as Spodoptera frugiperda cells and Autographa californica nuclear polyhedrosis virus have been sent to the Animal Virus Research Institute at Pirbright. Transfer vectors containing FMDV cDNA constructs as well as antisera against FMDV capsid proteins were received.

Exchange of staff:

Dr. Martin Ryan (Pirbright) has visited the Dept. of Virology in Wageningen from May 11 until May 22, 1987 for technical training on the baculovirus system.

Joint experiments:

During Dr. Ryan's visit a joint baculovirus transfection experiment was carried out using various constructs made at the Pirbright end.

Joint meetings:

A joint meeting of the project leaders was held in Pirbright (October 28/29, 1986) and in Wageningen (May 11/12, 1987) to discuss the progress and future strategies. Additional informal meetings between staff were arranged during visits of international meetings in the U.S.A. (June 1986, St. Barbara) and England (April 7-10, 1987, St. Andrews).

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: A. F. R. C., Woking

Contract no.: BAP - 0119 - UK

Project leader: D. McCAHON

Scientific staff: G. Belsham, M. Ryan, J. Brangwyn

Address:
Animal Virus Research Institute
Genetics Dept.
Pirbright
UK - WOKING, SURREY GU24 0NF

Telephone no.: +44.483.232441 EXT.272

Telex no.: 859137 AVRI G

Other contractual partners in the joint project:

J.M. Vlak, Agricultural University of Wageningen

Title of the research activity:

High level expression of foot and mouth disease virus antigens using a Baculovirus vector.

Key words:

Foot-and-mouth disease, Expression, Baculovirus, Capsid, Proteases

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The aim of the project is to achieve high level expression of FMDV capsid proteins in such a form that they retain the immunogenicity associated with these proteins in the intact virion. The chosen strategy is to prepare recombinant baculoviruses containing regions of FMDV cDNA encoding the capsid proteins and other viral functions required for correct processing of the capsid precursors.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The objective of this laboratory was to insert into appropriate baculovirus transfer vectors regions of FMDV cDNA. Such constructs were obtained using cDNA from two different serotypes of FMDV (O1K and A10). Three different regions of the FMDV polyprotein have been implicated in the proteolytic processing of the precursor molecules into mature products. The involvement of each of these regions, individually and in combination, was considered in the construction of the panel of cDNA inserts which were prepared.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

We have introduced into baculovirus transfer vectors (pAC 610 and pAC 611) seven different inserts of FMDV cDNA, their structures shown in Figure 1.

The leader and capsid protein encoding sequences of FMDV O1K were linked to the viral protease 3C via the Xho 1 sites in 2B and 3B (pMR1, see Figure 1). This fusion maintains the open reading frame from the FMDV initiation codon through the capsid region, 2A, the novel 2B/3B fusion protein and 3C. The construct is terminated at the Hind III site within 3D by the addition of a short (-150 bp) poly (A) addition signal and stop codon cassette. The corresponding O1K construct omitting the protease 3C (pMR3) contains the leader and P1 capsid protein region terminated at the Xho 1 site within 2B by the addition of the poly (A) addition and stop codon cassette.

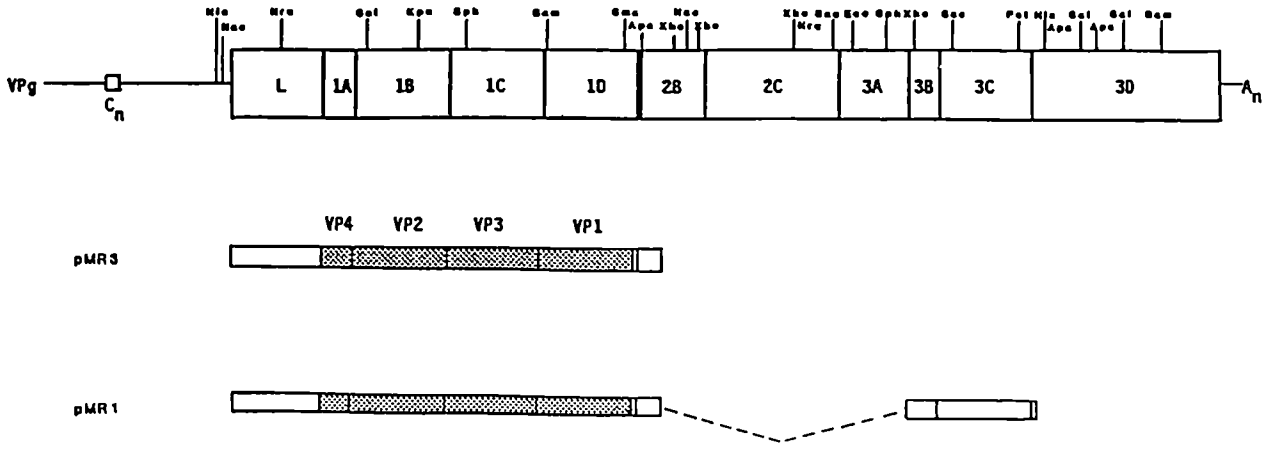
The equivalent FMDV A10 serotype consructs, pMR2 and pMR4, were constructed in an identical manner (see Figure 1). The FMDV A10 cDNA does not contain the entire L coding sequence, nor the FMDV initiation codon. Therefore, a short sequence encoding the N- terminal region of SV40 large T antigen was fused to the 5' terminus of FMDV cDNA, maintaining the correct open reading frame throughout the construct.

The L proteins of FMDV serotypes O1K and A10 show a high degree of homology and contain a homologous Nrul site. This common restriction site was used to construct pMR8 and pMR9 which correspond to pMR2 and pMR4 except that the initiation site and missing L protein sequences are supplied by the FMDV O1K cDNA.

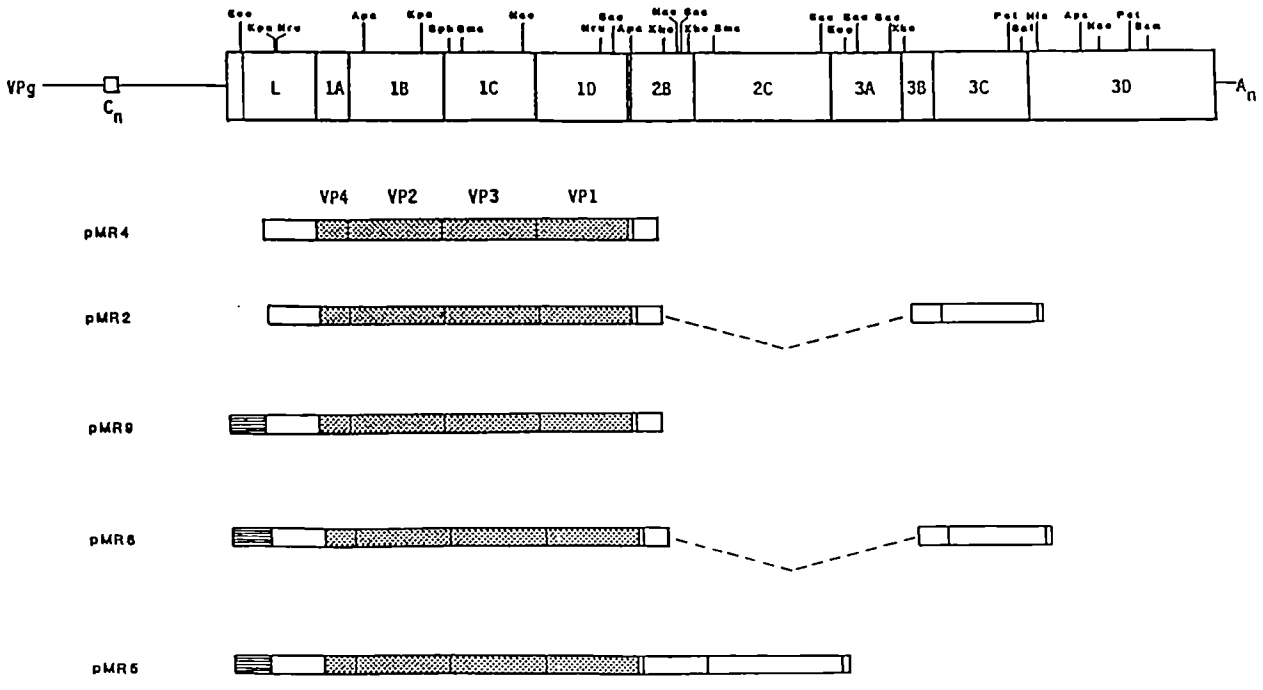
DISCUSSION

Whilst isolation of recombinant baculovirus containing FMDV sequences is in progress in Wageningen (Dr. J. Vlak), experiments to confirm the transcriptional/translational activity of these constructs have been initiated. Preliminary experiments have shown that all the constructs transcribe and translate in agreement with the structures shown in Figure 1, demonstrating that the correct reading frame is maintained in all the constructs. The experiments also indicate that constructs containing sequences encoding the viral protease 3C show processing of the capsid precursor molecules. It should be noted that in all cases the 3C protease has been shown to process O1K capsid precursor molecules. We believe that these results are encouraging with respect to the baculovirus expression system, but are mindful of the possibility that constructs encoding the viral protease 3C may prove to be toxic when expressed in Spodoptera frugiperda cells. Constructs were therefore prepared omitting these sequences. Additionally, we are examining the possibility of using an inducible promoter system to control independently the expression of the 3C protease.

FMDV SEROTYPE O1K cDNA CONSTRUCTS



FMDV SEROTYPE A10 cDNA CONSTRUCTS



IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

NONE

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Exchange of Materials

Six plasmids containing FMDV sequences inserted appropriately into baculovirus transfer vectors have been transferred to Wageningen (Dr. J.M. Vlak) together with appropriate antisera required for analysis of the products made by recombinant baculoviruses when they are isolated. The Spodoptera frugiperda cell line, Autographa california virus and DNA and other materials necessary for baculovirus experimentation has been transferred from Wageningen to Pirbright.

Personal Contact

Dr. J.M. Vlak spent two days at Pirbright in November 1986 discussing ideas on the project.

Dr. M. Ryan visited the laboratory in Wageningen for two weeks in June 1987 to learn the procedures for growing the Spodopteran cells, virus growth, isolation etc. and to perform collaborative experiments on the initial steps in the isolation of baculovirus recombinants carrying FMDV sequences.

Dr. G. Belsham also visited the Wageningen laboratory in June 1987 to discuss progress with Dr. Vlak.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: I.N.R.A., Nouzilly

Contract no.: BAP - 0178 - F

Project leader: A.A. PARAF

Scientific staff: H. Salmon, D. Sage, L. Phan Thanh, B. Ruet, G. Varshney

Address:
INRA
Pathologie Porcine
Laboratoire d'Immunologie
F - 37380 NOUZILLY

Telephone no.: +33.47.42.77.00 EXT.7884

Telex no.: 750954 INRATOU F

Other contractual partners in the joint project:

C. R. Stokes, University of Bristol
D. Sage, Université Claude Bernard (Villeurbanne)

Title of the research activity:

Design of novel techniques to produce pig IgA hybridomas.

Key words:

Mucosal immunology, Pig, Vaccine, IgA, GALT

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Design of novel techniques to create hybridomas producing pig IgA specific for different antigens and possibly to those which are involved in the protection of piglets against transmissible viral gastroenteritis.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Tests for specific adhesion for Ig producing cells on chemically modified plastics by ozone (joint project with Laboratoire des Matériaux Plastiques in Lyon).

Use of some vectors constructed by J.F. Nicolas (Institut Pasteur by micro-injection).

Purification of lymphocytes from duodenal epithelium and lamina propria.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY

Lymphocyte purification, cell culture, molecular biology, biochemistry, immunochemistry and intra nuclear micro-injection.

2. RESULTS

a) two specific techniques for purification of duodenal lymphocytes either from the epithelium or from the lamina propria have been designed. Study of lymphocytic populations in tissue section of the duodenum by different staining and by specific monoclonal antibodies allowed to a quantitative estimation of the lymphocytic populations in the different parts of the organ. Cell purification conformed quantitatively to tissue section studies. In the case of lamina propria, cell preparations were enriched up to 50 % of plasmocytes which were then maintened in culture for 15 days with Ig synthesis. The different Ig classes were identified by monoclonal antibodies kindly given by Dr. Stokes (Bristol, England) which has a joined

project under the present contract.

b) we have been able in a 2-step procedure to purify from either blood or lymph-nodes, SIg^+ cells, T_4 and T_8 cells from pigs of different ages. Characterization of such cells were done by using monoclonal antibodies from Dr. Stokes (Bristol, England) and also from Dr. Lunney (from Beltsville, U.S.A.). The first step of separation on plastics involves the mixture of purified lymphocytes in 40 mg/ml serum albumin medium allowing to SIg^+ sticking on the plastic while T cells stay in suspension. The second step uses the same procedure with a 0 mg 400/ml serum albumin medium.

c) by using a free protein medium for cell suspension all lymphocytes were allowed to stick. After removing lymphocytes from the plastic we could get three major proteins left on the plastic (MW 45 Kd, 32 Kd, 12 Kd respectively) among minor proteins.

d) micro-injection of viral vectors into lymphocytes is not yet at hand. However we were able using blood purified leucocytes to micro-inject different vectors in sticking cells (most probably monocytes) to recover several cell lines after more than three months of culture. These cell lines are under study for complete characterization.

e) three major immunoglobulin classes IgM, IgA, IgG have been chromatographically purified from sow milk.

3. DISCUSSION

Tha aim of the complete project is to able to raise pig monoclonal antibodies of the IgA class without fusion. In order to reach this final gaol, several techniques have to be designed.

1/ we have to purify the different subpopulations of lymphocytes from duodenum. This work has to be completed during the next few months.

2/ we should be able to complete by purified SIg^+ cells : this is already done.

3/ to purify $SIgA^+$ cells : experiments are currently in progress.

4/ to purify $SIgA^+$ cells specific for a given antigen : as a model we shall use ovalbumin and this step will start next spring.

5/ we should be able to design vectors and to be able to introduce them in such a way that IgA antibody producing cells will become permanently transformed. We have already vectors obtained from Dr. Nicolas (Pasteur Institute) which were able to transform blood cells (probably monocytes) obtained from in-bread pigs (kindly given by Dr. Sachs N.I.H., Bethesda U.S.A.).

COLLABORATIVE WORK

CARRIED OUT BY D. SAGE

(Université Claude Bernard, Villeurbanne)

IN ASSOCIATION WITH A. PARAF

B A P - 0 1 7 8 - F

PHYSICOCHEMICAL ANALYSIS OF PLASTIC MATERIALS FOR LYMPHOCYTES SEPARATION

Daniel SAGE and Bernard RUET

Laboratoire d'Etudes des Matériaux Plastiques et des Biomatériaux I
UA 507 - Université "Claude Bernard" LYON I
43, Boulevard du 11 Novembre 1918 - 69622 VILLEURBANNE Cedex, France

* * *

The increase of wettability of polystyrene surfaces by means of various superficial chemical treatments which could be able to graft polar groups is studied.

Surface properties as well as structure and thickness of the modified layer obtained by each treatment were characterized.

MATERIALS AND METHODS.

The surface of 25 µm thick high purity polystyrene films was modified.

The films were dipped into concentrated sulphuric acid for 5 s, 10 s, 30 s, 150 s or 300 s at a temperature of 37°C, 60°C or 80°C.

Ozonolysis with 0.1 % ozone in oxygen mixture during 0.3 h, 1 h, 2 h, 4 h or 8 h at 25°C or 60°C was performed too.

Treated surfaces were chemically characterized with FT-IR and ATR spectrophotometry. The thickness of modified layer was estimated by means of ellipsometry.

Selective binding of various dyes such as methylene blue, eosin and crystal violet would be observed by ultra-violet spectrophotometry.

The contact angles of the prepared surfaces were measured with a specific dynamic Wilhemy surface balance.

RESULTS.

On Figure 1 are represented IR-TF spectra for treated and untreated PS films. A spectral difference is observed only for ozonized film (C) with a strong absorption peak at 1720 cm^{-1} and a wide absorption band between 1250 cm^{-1} and 850 cm^{-1} which are attributed to carboxyl groups.

Colorimetric results are shown on Figure 2. Spectrum A represents ultra-violet absorption of a dye solution composed of methylene blue (650 nm and 600 nm) and eosin (520 nm) in methanol.

Spectrum B represents absorption of an ozonized film after immersion for 10 min in the dye solution and rinsing with methanol. Spectrum C shows the absorbance of an acid-treated film under the same conditions. It appears that the methylen blue cation is adsorbed on the surface of ozonised materials and eosin is not. On acid-treated surfaces nor methylen blue or eosin are fixed. These results clearly indicate the acid character of the chemical groups produced during ozonation.

On Figures 3 and 4 are plotted contact angles of acid-treated and ozonized PS surfaces with pure water (γ_L = 72.4 dynes.cm^{-1}). In both cases, a rapid increase of wettability with temperature is observed as soon as evolution with duration of immersion reached a plateau.

By means of ellipsometry the modified layer thickness can be evaluated as less than 500 Å in the case of sulphuric acid treatment. For ozone-treated materials, the depth of reaction was determined to be set between 1000 Å and 2000 Å.

Then, it appears that the reaction with an acid by wet process leads to a blocked and saturated weak layer of modified polymer while oxidation by gas process with a little molecule permits diffusion at a depth which is similar to cell dimensions.

Preliminary assays shows that particular combinations of time and temperature for ozone treatment may provide substrates with different grafted-sites density of surface and we can then expect with this original gas phase treatment larger capacities in specific cell cultures or separations.

SPECIFIC OBJECTIVES.

Our purpose is to modify the surface of commercial polystyrene products (Petri dishes, microtiters, ...) in order to permit cell attachment and/or isolation on such materials.

In this way, ozonolysis and sulphuric acid treatment were performed on polystyrene films with varying time and temperature to enhance their surface energy.

Surface properties as well as chemical composition, structure and thickness of the modified layer obtained by these treatments will be characterized and connected to cell culture assays. The surface polarity should be studied. Then, physico-chemical parameters implied in the interactions between cell and substrate will be examined. The technics which might be used for this study are : FT-IR/ATR spectrophotometry and ESCA for chemical characterization ; ellipsometry in order to evaluate the depth of reaction ; dynamic contact angles and surface conductivity measurements for surface wettability and polarity.

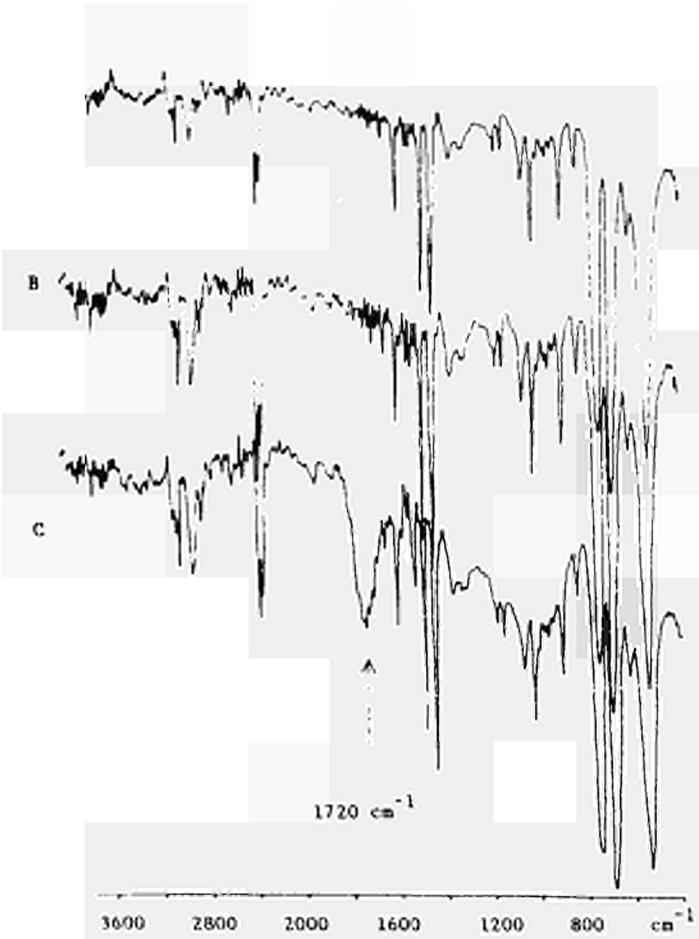


Fig. 1. IR-TF spectra of PS films

A : untreated
B : acid-treated
C : ozonized

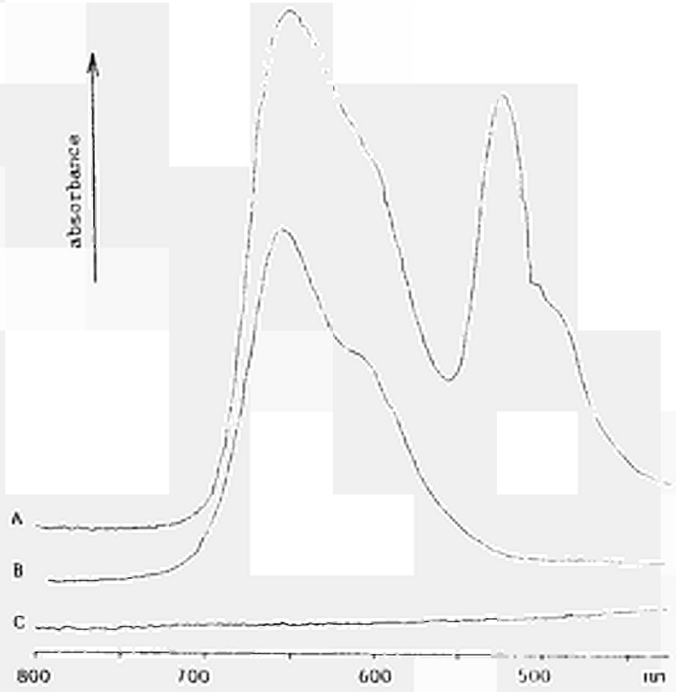


Fig. 2. Ultraviolet spectra of :

A : methylene blue-eosin mixture
B : ozonized film after immersion in A
C : acid treated film after immersion in A

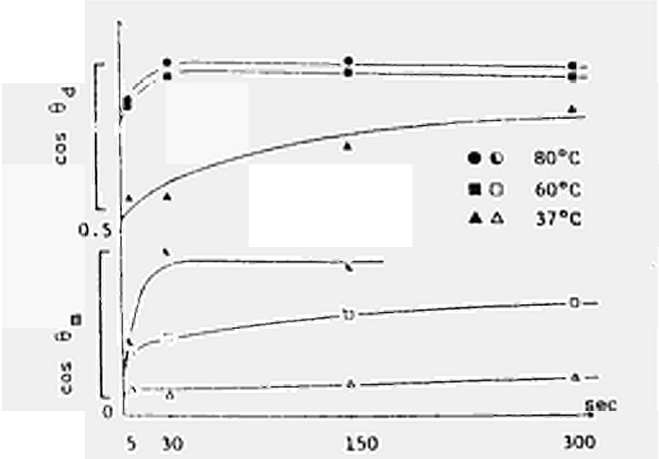


Fig. 3. Advancing (θ_a) and receding (θ_d) contact angles of acid treated films with pure water versus duration and temperature of treatment

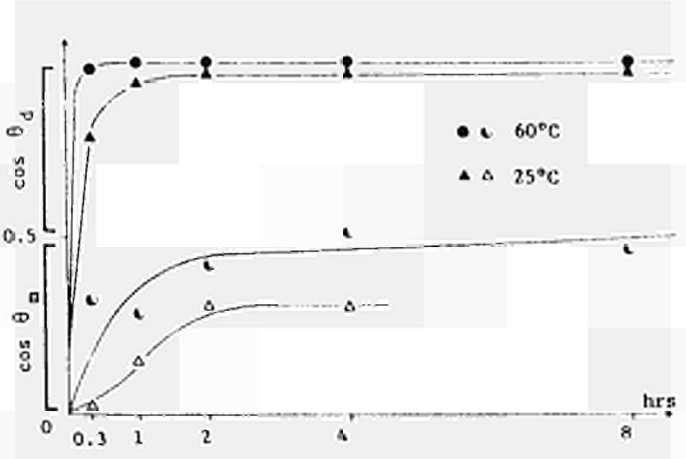


Fig. 4. Advancing (θ_a) and receding (θ_d) contact angles of ozonized films with pure water versus duration and temperature of treatment

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1 - PUBLICATIONS IN SCIENTIFIC JOURNALS, MONOGRAPHS...

L. PHAN THANH, A. PARAF, 1986 - Purification of three porcine immunoglobulin classes from the same biological source. Ann. Rech. Vét. (accepté pour publication).

IV.3 - PATENTS DEPOSITED IN CONNECTION WITH THE RESEARCH PROGRAMME

"Procédé de purification et de séparation de cellules ou de molécules homogènes à partir d'une suspension de cellules ou de molécules hétérogènes, polymère modifié apte à réaliser ladite séparation et procédé de production dudit polymère modifié" - Brevet d'Invention -INRA-

A. Paraf, D. Sage, B. Ruet, H. Salmon, B. Charreau, L. Phan Thanh, M. Olivier, S. Bernard.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:
(with Dr. Stokes, Bristol and with Dr. Sage, Lyon)

Exchange of material(s)	Yes
Exchange of staff	No
Joint experiment(s)	Yes
Joint meeting(s)	No

Descriptive information for the above data.

With Dr. Stokes :

exchange of material was done as following : Dr. Stokes gave me monoclonal antibodies against each class of Pig immunoglobulin. I gave to Dr. Stokes ozonised plastics to purify B, T_4 and T_8 pig lymphocytes.

Joint experiments : Dr. Paraf spent two days in Bristol (9-10 April 1987) with Dr. Stokes staff to make a B cell separations.

Joint meetings : Dr. Stokes came twice to Nouzilly to discuss cooperative experiments.

With Dr. Sage : during the last year Dr. Sage sent 250 plastics Petri dishes treated by different ways with ozone.

Dr. Paraf went 5 times in Lyon to discuss joint experiments.

TABLE

A three steps-procedure for pig lymphocytic subpopulations purification

	Origin	% purified	Yield of recovered lymphocytes
			100
1st step : removal of	Monocytes Polymorphs		75
by carbonyl-irom			
2nd step : removal of SIg^+ cells by panning	Adherent	98	75
	Supernatant	1-3	
3rd step : T_4-T_8 separation by panning	T_4	75-90	75
	T_8	85-90	60

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: University of Bristol

Contract no.: BAP - 0126 - UK

Project leader: C.R. STOKES

Scientific staff: K. Stevens, G. Corfield

Address:
University of Bristol
Dept. of Veterinary Medicine
Langford House
UK - LANGFORD, BRISTOL BS18 7DU

Telephone no.: +44.934.852851

Telex no.: 449174

Other contractual partners in the joint project:

A. A. Paraf, I. N. R. A. (Nouzilly)
D. Sage, Université Claude Bernard (Nouzilly)

Title of the research activity:

Design of novel techniques to produce pig IgA hybridomas.

Key words:

Mucosal immunology, Pig, Vaccine, IgA, GALT

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The aims of the project are twofold. Firstly, to develop techniques for obtaining porcine monoclonal antibodies which will not require conventional cell fusion techniques for their preparation or post fusion screening tests. Secondly to investigate the mechanisms of antigen handling and processing by the gut associated tissue so as to enable the development of a rational strategy for the development of future mucosal vaccines and to provide fusion partners for the production of E. Coli and Transmissible Gastroenteritis virus (TGEV) specific pig IgA monoclonal antibodies.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The specific objectives for the first year of the project were to develop a range of mouse monoclonal antibodies to porcine immunoglobulins with a particular interest in characterising those specific for IgA. To prepare monoclonal antibodies to E. Coli antigens. To develop techniques for the isolation of gut immune cells and enterocytes and to commence studies of immune stimulation and immune potentiation of responses at mucosal surfaces.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Methodology

a) Monoclonal antibodies - mouse monoclonal antibodies to pig immunoglobulins (IgG_1, IgG_2, IgA, IgM & light chains) were prepared from mice immunised with pig immunoglobulins isolated by ion-exchange chromatography, gel filtration and affinity chromatography. Spleen cells were fused with P3 x 63Ag8-653 myeloma cells and resultant hybridomas screened initially by ELISA with immunoglobulin coated plates. Selected monoclonals were then analysed by double diffusion, immunoelectrophoresis and Western Immunoblots. Mice were also immunised with E. Coli (C600 - pFM 205) for the preparation of monoclonal antibodies to K88. Hybridomas were screened by ELISA with plates coated with K88 prepared bacterial shearing to remove pilli, ammonium sulphate precipitation and chromatography on Sepharose-4B.

b) Preparation of Pig Gut Lymphocytes - Techniques for the isolation of pig intraepithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) were developed. Briefly sections of intestine were collected immediately after slaughter, opened lengthwise and washed with ice-cold Hanks' balanced salt solution. The muscularis layer was separated from the mucosal layer by dissection, and epithelial cells removed by shaking vigorously in RPMI containing 2% FCS. IEL's were then purified on a discontinuous Percoll gradient. For isolation of LPL, after removal of the epithelial cells the lamina propria was minced and incubated in RPMI containing collagenase. The lymphocytes were then separated on Percoll gradients.

c) Immune Response Studies In order to stimulate mucosal immune responses a number of approaches to immune potentiation have been investigated. For studies in the pig we have introduced plasmids into laboratory (C600) and pig strains (045) of E. Coli to investigate the influence of LT-B and K88 upon the ability to colonise and induce a mucosal immune response. In mouse we have begun studies to investigate the effect of cholera toxin, Phytohaemaglutinin (PHA) and liposome incorporation upon the cellular (DTH) and humoral response to ovalbumin.

2. Results

a) Pig Monoclonal Antibodies - monoclonal antibodies to pig IgG_1, IgG_2, IgA, IgM & light chains have been prepared and their specificity confirmed by ELISA and immunoelectrophoresis. Two monoclonal antibodies to IgA have been further characterised. The first K61, reacted with 7S, 9S & 11S preparations of IgA in ELISA whilst the second, K60 reacted only with the 11S molecules. Western Immunoblots with the monoclonal antibodies on SDS-PAGE separated IgA showed K61 to bind to a 56 Kd molecule and K60 to a 82 Kd molecule. These molecular weights are compatible with estimates as the size of -chain and secretory component (SC) respectively. From mice parenterally immunised with E. Coli C600. pfM 205 a number of monoclonal antibodies specific for K88 have been prepared.

b) Immune Response Studies Groups of 5 week old pigs were orally immunised for 5 days with E. Coli 045.K with and without the plasmid pUB 3744 which encodes for the B subunit of the heat labile enterotoxin. Serum and secretory antibody levels were measured 5 days after the cessation of dosing. In those pigs dosed with E. Coli 045.k pUB 3744 the serum and secretory IgA antibody response to E. Coli was

significantly enhanced.

In mice we have shown that feeding 10 ug cholera toxin significantly enhanced the serum and secretory antibody response to ovalbumin fed at the same time. A similar dose dependant enhancement of response was obtained when ovalbumin was fed with PHA. Mice fed with ovalbumin (0.75 mg) in liposomes produced a significantly greater delayed type hypersensitivity skin response upon challenge than those mice sensitised with ovalbumin alone.

3. Discussion We have developed a range of monoclonal antibodies to pig immunoglobulins these include reagents specific for IgG_1, IgG_2, IgA, IgM and light chain. In addition we have identified two monoclonal antibodies for IgA one of which binds to an epitope on the -chain whilst the other is specific for secretory component. These reagents will be used to characterise the immune response to a range of fed antigens including those derived from E. Coli and transmissible gastro-eneteritis virus (TGEV). These monoclonal antibodies will be used to identify specific antibody producing cells in the gut associated lymphoid tissue as potential fusion partners for the IgA hybridoma studies. In addition the development of an SC specific reagent will allow us to investigate the route and mechanisms of IgA transport into gut secretions.

The other major part of this year's programme has been to investigate ways of stimulating gut mucosal responses, in order to provide both a convenient source of IgA producing cells as well as a rational strategy for the development of mucosal vaccines. Current evidence would indicate that this is best stimulated by local application of antigen and that whilst the response is highly dose dependant, the use of replicating antigens is by far the most effective. The problem associated with live vaccines (stability, safety etc) necessitates the requirement for effective non-replicating mucosal vaccines.

To this end we have shown the potential of range of immunopotentiators Lt-B, Cholera toxin, PHA and lipsomes. Work during the coming year will focus on developing this approach by investigating their influence upon antigen handling and processing by the gut associated lymphoid tissue.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

None to date

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)	Yes	
Joint meeting(s)	Yes	

Descriptive information for the above data.

All the monoclonal antibodies to pig immunoglobulins prepared at the Bristol laboratory have been passed on to the laboratory at Nouzilly. The techniques of panning on specific plastics for the isolation of pig B cells and T-cell subsets that were developed by Nouzilly have been made available to the Bristol workers. Dr. A. Paraf spent 4 days working in the Bristol laboratory to teach the technique and provide the appropriate plastics. In addition workers from the Bristol laboratory have spent time at Nouzilly on four occasions. These visits have involved the exchange of data and reagents as well as the planning of future experiments. To this end an experiment is planned on mucosal stimulation in France during the autumn.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Università di Torino

Contract no.: BAP - 0113 - I

Project leader: P.M. COMOGLIO

Scientific staff: G. Gaudino, M.F. di Renzo, M. Prat, G. Tarone, D. Cirillo, F. Giancotti, L. Naldini, P. Rossino, R. Ferracini

Address:
Università di Torino
Dipartimento di Scienze Biomediche e Oncologia - Sezione di Istologia
Corso Massimo D'Azeglio 52
I - 10126 TORINO

Telephone no.: +39.11.687516 or +39.11.680416

Telex no.:

Other contractual partners in the joint project:

S. Fisher, I. N. S. E. R. M. (Paris)

Title of the research activity:

New methodology in cultures of human tumour cells.

Key words:

Growth factors, Receptors, Tyrosine-kinases, Cell adhesion

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Human tumor cell cultures are of interest for several practical applications, including the production of biological standards for diagnostic tests. To grow "in vitro" epithelial and mesenchymal cells invariably require specific growth factors. Lack of appropriate growth factors is the main reasons for the failures in establishing cell lines. This report deals with the description of a new methodology suitable for the identification of growth factors and their specific receptors. Moreover, adhesion to specific extracellular macromolecules is a second critical requirement for the replication "in vitro" of most cell types. Studies to elucidate the structure and functions of receptors for extracellular matrix molecules ("Nectins"), critical for cell adhesion "in vitro", will also been accomplished.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

In the first year of work performed under the BAP contract, the main goal attempted has been the identification of the receptor complex for a still largely unKnown growth factor produced by small cell lung carcinomas. The growth factor is a tetradecapeptide named "bombesin".

Studies for the identification of a new growth factor produced by gastric carcinoma have also been undertaken.

Studies on the structure of cell surface receptors for the extracellular matrix protein "fibronectin" have been accomplished.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

(A)Studies on growth factors and growth factor receptors

The known growth factor receptors are associated with a tyrosine-kinase activity which is triggered upon binding to the specific ligand. Moreover these receptors become auto-phosphorylated at tyrosine in response to the specific factor. Previous work from this laboratory has shown that antibodies against Phosphotyrosine (P-Tyr) can be produced (1) and that these can be successfully employed to recognize the autophosphorylated receptors for known growth factors such as EGF and PDGF (2,3). P-Tyr antibodies have been also used to detect the tyrosine kinases coded by viral and cellular oncogenes (2,4). In the past year we exploited this technique to identify new growth factors and their receptors.

The peptide bombesin is known for its potent mitogen activity on murine 3T3 fibroblasts and other cells. Recently it has been implicated in the pathogenesis of small cell lung carcinoma, in which it acts through an autocrine loop of growth stimulation. In Swiss 3T3 fibroblasts, P-Tyr antibodies identified a 115,000 M_r cell surface protein (p115) that became phosphorylated on tyrosine as a specific response to bombesin

stimulation of quiescent cells. The extent of phosphorylation was dose dependent and correlated with the mitogenic effect induced by bombesin, measured by ^{3}H-thymidine incorporation. Tyrosine phosphorylation of p115 was detectable minutes after addition of bombesin and its time course paralleled that described for the binding of bombesin to its receptor. Immunocomplexes of phosphorylated p115 and P-Tyr antibodies bound ^{125}I-Tyr_4-bombesin in a specific and saturable manner and displayed an associated tyrosine kinase activity enhanced by bombesin. Furthermore, the bombesin analog ^{125}I-gastrin-releasing-peptide, bound to intact live cells,was co-precipitated with p115. These data strongly suggest that p115 participates in the structure and function of the surface receptor for bombesin, a new member of the family of growth factor receptors with associated tyrosine kinase activity (5).

It has been hypothesized that bombesin-like peptides produced by small cell lung carcinomas (SCLC) may sustain deregulated proliferation through an autocrine mechanism. We found that phosphotyrosine antibodies recognized p115, phosphorylated at tyrosine, in four human SCLC lines producing bombesin, but not in a non-producer "variant" line. P115 from detergent treated SCLC did bind to bombesin-Sepharose and becames phosphorylated at tyrosine in the presence of radiolabeled ATP and Mn^{++} ions. As in the case of the p115 kinase immunoprecipitated from mouse fibroblast, the SCLC p115 was phosphorylated in an immunocomplex kinase assay. However, the latter did not require the presence of exogenous bombesin. Binding data, obtained using radiolabeled ligand, indicate receptor occupancy in the cell lines producing bombesin. These observations fulfill the hypothesis of autocrine control of human small cell lung carcinoma cell proliferation, via constitutive activation of bombesin receptors.

Antibodies against P-Tyr were also successfully used to identify the receptor for a still unknown peptide growth factor released in the tissue culture medium by human gastric carcinoma lines. Preliminary data on the features of the growth factor and the structure of the receptor (a 190,000 M_r cell surface protein, made of two subunits) strongly suggest that the above molecules, critical for the growth of gastric carcinoma lines "in vitro", have not previously been described.

(B) Studies on surface receptors involved in cell adhesion

Assays to evaluate the specific adhesion requirements of different tumor cell lines have been set up. Cells have been plated on culture dishes coated with purified adhesive factors including: fibronectin, laminin serum spreading factor (vitronectin), entactin, collagens type I, III, IV and glycosaminoglycans. This test allows to detect the presence of cell surface receptors for a given adhesive molecules (6,7) and proved to be useful in

establishing which particular adhesive factor is required for a given cell. Detailed studies on the adhesion properties of hemopoietic cells were performed, using antibodies against cell surface proteins and synthetic peptides derived from the sequence of the adhesive factors. It was found that hemopoietic cells binds to a specific domain of fibronectin via a cell surface receptor of 145,000 M_r.

References:

1. Comoglio, P.M., Di Renzo, M.F., Tarone, G., Giancotti, F.G., Naldini, L. & Marchisio, P.C. 1984. EMBO J. 3: 483-489.

2. Di Renzo, M.F., Ferracini, R., Naldini, L., Giordano, S. & Comoglio P.M. 1986. Eur. J. Biochem 158: 383-391

3. Zippel, R., Toschi, G., Naldini, L., Sturani, E., Alberghina, L. & Comoglio, P.M. 1986. Biochim. Biophys. Acta 881: 54-61.

4. Naldini, L., Stacchini, A., Cirillo, D., Aglietta,M., Gavosto, F. & Comoglio P.M. 1986. Mol. Cell. Biol. 6: 1803-1811.

5. Cirillo, D., Gaudino, G., Naldini, L. & Comoglio P.M. 1986. Mol. Cell. Biol. 6: 4641-4649.

6. Tarone, G., Galetto, G., Prat, M. & Comoglio, P.M, 1982. J. Cell Biol.: 39-179.

7. Giancotti, F., Tarone, G., Damsky, C., Knudsen, K., & Comoglio, P.M., 1985 Exp.Cell Res., 156: 182-189.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

D. Saggioro, R. Ferracini, M.F. Di Renzo, L. Naldini, L. Chieco Bianchi and P.M. Comoglio
PROTEIN PHOSPHORYLATION AT TYROSINE RESIDUES IN v-abl TRANSFORMED MOUSE LYMPHOCYTES AND FIBROBLASTS.
Int. J. Cancer, 37, 623-628, 1986.

L. Naldini, A. Stacchini, D. Cirillo, M. Aglietta, F. Gavosto and P.M. Comoglio
PHOSPHOTYROSINE ANTIBODIES IDENTIFY THE P210 c-ablTYROSINE KINASE AND PROTEINS PHOSPHORYLATED ON TYROSINE IN HUMAN CHRONIC MYELOGENOUS LEUKEMIA CELLS
Mol. Cell. Biol., 6, 1803-1811, 1986

M.F. Di Renzo, R. Ferracini, L. Naldini, S. Giordano and P.M.Comoglio IMMUNOLOGICAL DETECTION OF PROTEINS PHOSPHORYLATED AT TYROSINE IN CELLS STIMULATED BY GROWTH FACTORS OR TRANSFORMED BY V-ONCOGENE CODED TYROSINE KINASES
Europ. J. Biochem., 158, 383-391, 1986

E. Sturani, L.M. Vicentini, R.Zippel, L. Toschi, A. Pandiella-Alonso, P.M. Comoglio and J. Meldolesi.
PDGF-INDUCED RECEPTOR PHOSPHORYLATION AND PHOSPHOINOSITIDE HYDROLYSIS ARE UNAFFECTED BY PROTEIN KINASE C ACTIVATION IN MOUSE SWISS 3T3 AND HUMAN SKIN FIBROBLASTS
Biochim. Biophys. Res. Comm., 137, 345-350, 1986

D. Cirillo, G. Gaudino, L. Naldini and P.M. Comoglio
RECEPTOR FOR BOMBESIN WITH ASSOCIATED TYROSINE KINASE ACTIVITY
Mol. Cell. Biol, 6, 4641-4649, 1986.

A. Palumbo, P. Rossino and P.M. Comoglio
BOMBESIN STIMULATION OF c-fos AND c-myc GENE EXPRESSION IN CULTURES OF SWISS 3T3 CELLS.
Exp. Cell Res. 167, 276, 1986.

E. Sturani, L. Toschi, R. Zippel, P.M. Comoglio and L. Alberghina PHOSPHORYLATION OF THE PDGF RECEPTOR IN MOUSE SWISS 3T3 FIBROBLASTS UNDER DIFFERENT GROWTH CONDITIONS
In "Biological Regulation of Cell Proliferation", (R. Baserga ed.), Raven Press, N.Y., 1987, in press

S. Giordano, M.F. Di Renzo, D. Cirillo, L. Naldini, L. Chiado'Fiat and P.M. Comoglio
PROTEINS PHOSPHORYLATED ON TYROSINE AS MARKERS OF HUMAN MALIGNANCIES
Int. J. Cancer, 39, 482-487, 1987.

P.M. Comoglio, M.F. Di Renzo and G. Gaudino
PROTEIN TYROSINE KINASES ASSOCIATED WITH HUMAN MALIGNANCIES
Annals of the New York Academy of Sciences, 1987, in press

F.G. Giancotti, P.M. Comoglio and G. Tarone
A 135,000 MOLECULAR WEIGHT PLASMA MEMBRANE GLYCOPROTEIN INVOLVED IN FIBRONECTIN-MEDIATED CELL ADHESION. IMMUNOFLUORESCENCE LOCALIZATION IN NORMAL AND RSV TRANSFORMED FIBROBLASTS.
Exp. Cell. Res., 163, 47-62, 1986.

F.G. Giancotti, P.M. Comoglio and G. Tarone
FIBRONECTIN-PLASMA MEMBRANE INTERACTION IN THE ADHESION OF HAEMOPOIETIC CELL LINES
J. Cell Biol. 103, 429-437, 1986.

TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)	Yes	
Joint meeting(s)	Yes	

Descriptive information for the above data.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: INSERM, Paris

Contract no.: BAP - 0112 - F

Project leader: S. FISHER

Scientific staff:

Address:
INSERM
24, rue du Faubourg Saint Jacques
F - 75014 PARIS

Telephone no.: +33.1.43201240 EXT.392 or +33.1.40460411

Telex no.:

Other contractual partners in the joint project:

P.M. Comoglio, Universitàà Torino

Title of the research activity:

New methodology in cultures of human tumour cells.

Key words:

Transformed cells, Tyrosine kinase, Growths factors, Cellular onc

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

A - Tyrosine phosphorylation of specific cellular proteins in response to growth factors and other metabolites required for cell survival

B - Changes in the level of some c.onc (C.src and lck) in transformed and normal cells cultured in various conditions

C - Establishment of procedures for the maintenance in culture of tumor cells from both human and murine origin

D - Role of growth factors and other metabolites (i.e. Vanadate) in the establishment of these cultures

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

- Purification and modulation of the activity of $p56^{lck}$ from lymphoma cells of both human and murine origin

- $p60^{src}$ levels in several megakaryocytes cell lines at various stages of differentiation

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY : Cell lines and culture conditions :

   LSTRA and MBL2 are lymphoid tumor cell lines induced by MoMulV, and obtained from Balb c and C57-B6 mice, respectively.

   Molt4;KE37 and JM are human T lymphoma cells. The cells were grown in RPMI-1640 supplemented with 10% FCS and 10 uM2-mercaptoethanol.

   Cell lines rich in haematopoietic precursors were obtained from DBA-2 mice infected with a new viral isolate (MPLV). One of the lines, rich in megakaryocytes cells (LINA) at various stages of differentiation has been established.

In vivo labelling of cells (with $^{i}P32$ or ^{35}S-Methionine); preparation of subcellular fractions, immunprecipitation with antibodies, Western blots, P-aa analysis and the assay for tyrosine kinase activity are all methods currently used in our laboratory.
Whole cell RNA was prepared by the guanidium thiocyanate method and probed with $^{32}p\text{-}cDNA^{lck}$ (Northern blots).

2. RESULTS :

A - High levels of tyrosine kinase activity (TPK) have been detected in human lymphomas (Molt4;KE37;and JM) and in murina lymphoma LSTRA; The functional domains of this kinase-p56- have been studied by the use of antibodies generated against peptides from the amino terminal region and from the tyrosine autophosphorylation site. The amino terminal antibody had higher affinity for the p56 than the antibody directed against the phosphotyrosine site. However, the phosphorylation of exogenous substrates by p56 was lower when the tyrosine kinase was immuncomplexed by the antibody against the amino terminal region than when the kinase was complexed by the phosphorylation site antibody. This suggest that in the N-terminal region exist structures which modulates the tyrosine kinase activity of the p56.

A similar protein-p56- has been found in human T-lymphocytes, largely augmented by PHA treatment; by various criteria we suspected that the same protein was present in each of these cells.

This was definitively shown by hybridization of total cellular RNA of these cells followed by hybridization with the specific $cDNA^{lck}$ probe (Northern blot). These results suggested that the product of lck is frequently expressed in cells of lymphatic origin. The functional role of the p56 remains to be established. The fact of beeing membrane associated makes its role in signal transduction and or phosphorylation of membrane receptors an attractive working hypothesis.

Some of the function of the p56 may be related with the ability of the $p56^{lck}$ to phosphorylate specific cellular protein. For this we used the monospecific anti phosphotyrosine antibody prepared by Dr Comoglio group (partner in the joint project). By either immunprecipitation or by Western blots decorated with the anti P-tyr antibody we showed the presence of a 78;50 and 35 kDa P-protein in the lymphoma of human origins.

B - Purification of the LSTRA tyrosine protein kinase ($p56^{lck}$)

We have purified $p56^{lck}$ to homogeneity by electrofocusing followed by two step SDS-polyacrylamide gel electrophoresis. The pure protein gave a single band by silver staining and was identical to p56 characterized in lymphoma cells. It was used to raised antibodies in rabbits. This simple

procedure is applicable for the rapid purification of minor proteins. (paper submitted for publication).

C - $p60^{src}$ is elevated in megakaryocyte cell lines.

Several haematopoietic cell lines have been established from mice infected with a new virus isolate : the MPLV (myeloproliferative leukemia virus) (cell lines and virus isolates were established by F. Wendling and P. Tambourin). Plasma membrane fractions were obtained from culture rich in primitive megakaryocytes (2n-4n cells) and from more differentiated ones (more than 8n) High levels of $p60^{src}$ were detected in each of these lines; but membranes from more differentiated cells had higher level of p60. P-tyr was the only aa detected. p60 was immunprecipitated by monoclonal anti src antibodies and the P-peptides generated by Cleveland gels were identical to $p60^{src}$. From metabolically labelled cells (^{32}Pi) $p60^{src}$ has also been detected, besides several other P-tyr containing proteins, which might be target protein of this kinase.

3. DISCUSSION :

A - We have shown that antibodies against the NH2 terminal region and the tyr phosphorylation site recognize p56 with different efficiency; secondly autophosphorylation of the p56 is higher when bound to the P.S. site and thirdly, the p56 bound to the P.S. Ab showed higher kinase activity towards exogenous substrates. These findings indicate that the p56 has modulatory regions that modify the TPK activity. Persuing the studies of the TPK regulation might help in the understanding of its role in cell proliferation.

B - We have established a very simple procedure for the purification of very minor proteins (showed for the $p56^{lck}$) which can be applied to allmost any protein.

C - With a new virus isolate, the MPLV, lines from several haematopoietic cell lines have been established. In the megakaryocyte rich lines, high level of $p60^{src}$ were detected. This is consistent with the hypothesis that some of these kinases (in particular $p60^{src}$)might be important in normal cell differentiation. The megakaryocyte system might also be helpful to define the potential target proteins of $p60^{src}$.

In general, it is very attractive to view all of the protein-tyrosine kinases, those that are integral membrane proteins as well as those that are simply membrane associated as components of signal transduction systems regulating cell proliferation.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1 - PUBLICATIONS IN SCIENTIFIC JOURNALS :

1- FISCHER, S., FAGARD, R., BOULET, I., and GESQUIERE, J.C.
The amino terminal region of the p56 lck from LSTRA exerts negative modulation on the Tyrosine Kinase activity
Biochem.Biophys. Res. Comm., 1987, 143, n°3, 819-826

2- Huynh van Tan, FISCHER, S., and Fagard, R.
Purification of the LSTRA Tyrosine Protein Kinase ($p56^{lck}$)
submitted for publication, July 1987

IV.2 - SHORT COMMUNICATIONS :

- CEC Meeting, May 24-27 (1987) at Seillac (France)
- Biology of Growth Factors, June 17-19 (1987) - University of Toronto Toronto (Canada).

IV.4 - DOCTORATE THESIS :

- I. BOULET
Purification and study of functional domains of the LSTRA tyrosine protein kinase $p56^{lck}$
University of Paris, 1987.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Dr Comoglio - Turin (Italy)

Carcinoma cells cultured under different conditions (growths factor ; metabolites-specially VO4-) in Dr Comoglio lab, will be studied by us for changes in P-tyr proteins and modification in the expression of c.onc gene product.With the perspective to establish a rational for optimal and controlled culture conditions.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: G.B.F., Braunschweig

Contract no.: BAP - 0130 - D

Project leader: J. LEHMAN

Scientific staff: E. Fraune, B. Röder, J. Vorlop

Address: Gesellschaft für Biotechnologische Forschung mbH (GBF)
Mascheroder Weg 1
D - 3300 BRAUNSCHWEIG

Telephone no.: +49.531.6181102

Telex no.: 952667 GEBIO D

Other contractual partners in the joint project:

J. Hache, Bertin & Cie (Plaisir)
J.M. Engasser, E. N. S. A. I. A. (Vandoeuvre lez Nancy)
K. Schügerl, Universität Hannover
P. Nabet, Université de Nancy

Title of the research activity:
Biochemical-physical knowledge, control, optimization of animal cell cultures in bioreactors.

Key words:
Membrane stirrer, Bubble free oxygenation, Carrier mixing, Reactor scale up

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Reactor scale-up and automatic control for animal cell culture is still problematic. The long term objective of this study is thus to design more efficient bioreactors and operation modes for the mass culture of different kinds of animal cells. More precisely one aims at reactors with a more physiological environment of the cells. This can be obtained by a better control of the limiting physical, chemical and biochemical parameters. Improved scale-up technologies are also expected, specially with respect to aeration, automatic control and feeding strategies. To achieve these technological objectives it is imperative to first obtain a better qualitative and quantitative understanding of cell behavior in reacotrs: nutritional requirements, sensitivity to the physical and chemical environment, control of cell physiology.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

In 1985 and 1986 a new reactor for mammalian cell-culture, employing a moving membrane stirrer has been developed and successfully operated in 1 ltr. and 2 ltr. scale in our group. The reactor then has been extended to 20 and 150 ltr. The specific objective of our group was to measure and to compare the oxygen transfer rate and mixing pattern of microcarrier in suspension, respectively, and a further development of the stirrer regarding to membrane segmentation and stirrer geometry.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Methods

Oxygen transfer measurements

The dynamic method for measuring the oxygen transfer rate OTR from the membrane into the culture medium was used. For this purpose the reactor was filled with pure water, as the concentration of O_2 is well known as a function of pressure, temperature and the dissolved oxygen. Oxygen was first removed from the water with nitrogen and then the parameters for the aeration conditions have been fixed. During the oxygen flow through the membrane the response of the dissolved oxygen probe was registered. With the help of this curve it is easy to calculate the OTR and the k_L value.

Membrane segmentation

In 1 ltr scale, the membrane fibre could be immersed in one piece but in 20 l and even in larger scale the membrane fibre has to be divided into segments to lower the pressure drop in the lumen of the membrane fibre. The influence of the mode of the segment connection - in parallel or in line - on the oxygen transfer rate has been estimated.

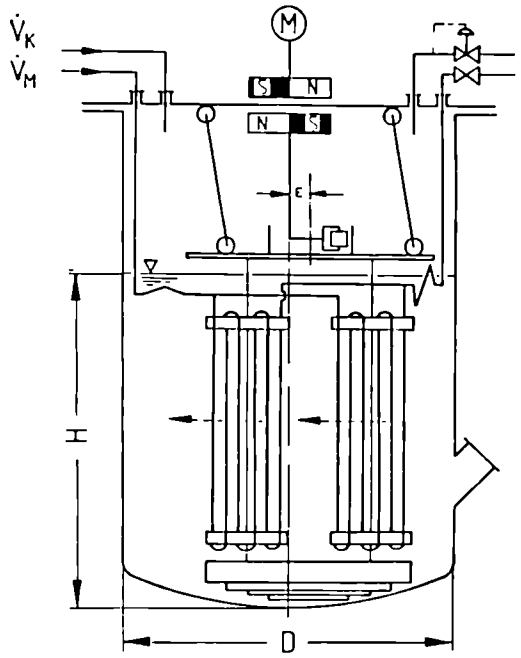


Fig. 1: Membrane stirrer geometry for 20 and 150 ltr. scale reactors*

For a better carrier mixing in larger reactors a turbulence generating spiral has been mounted underneath the membrane segment stirrer.

Microcarrier homogeneity measurement

To estimate the microcarrier distribution in the reactor, samples of 50 ml (PBS + carriers) were taken from the reactor at different heights, followed by sedimentation for 15 minutes in a 50 ml graduated cylinder. Then the portion of carrier in relation to the liquid was measured. The initial weight of microcarrier (Cytodex® 3, Pharmacia, Sweden) used for the measurements was 3 g/l dry carrier in PBS (Phosphate Buffered Saline). Those measurements have been made at different stirrer speeds.

Results

Oxygen transfer

The experiments with the 20 l reactor should give the relation between the OTR and the parameters $\dot{V}_M$, n and the number of segments. Fig. 2 shows that there is no significant relation between the kind of segmentation and the OTR_{max} values. After the OTR_{max} value increases up to 20 $mgO_2/(l \cdot h)$ with a flow of $\dot{V}_M$ = 150 ml_N/min a plateau is reached. This is a point where there seems to be no O_2 gradient inside the whole membrane. The $\dot{V}_M$ value of 150 ml_N/min related to the total area of mass transfer equals $\dot{V}_M/A$ = 0.86 $ml_N/cm^2 min$. Above this value of $\dot{V}_M$ there is no further increase of the OTR value. The influence of the numbers of revolutions (fig. 3) could be correlated well with $n^{0.5}$. There is no difference between the OTR values if a spiral is used or not.

*Membrane fibre: ACCUREL® 2,6/18 ENKA, Wuppertal

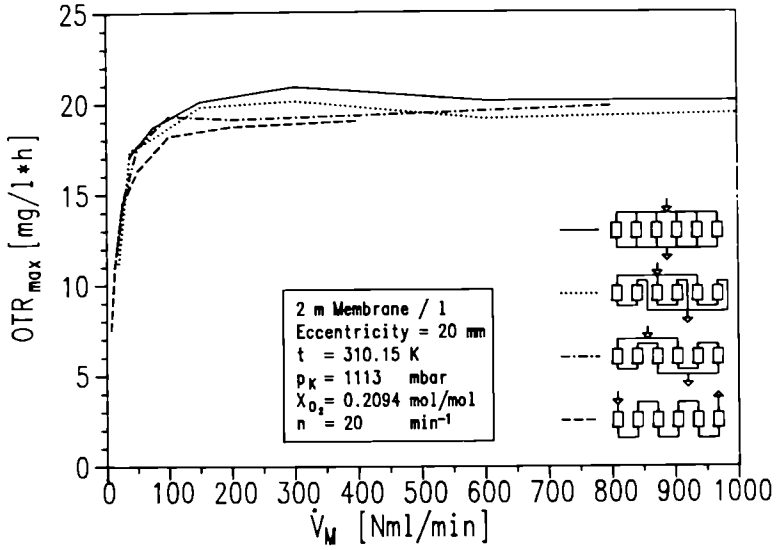


Fig. 2: Maximum oxygen transfer rate as a function of the gas flow into the membrane of the 20 l reactor with 18 l measuring volume. Maximum means: O_2 concentration c_L=0.

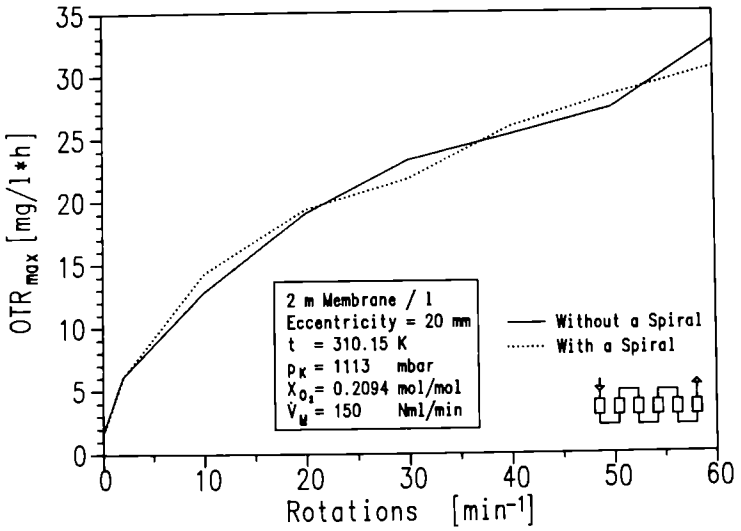


Fig. 3: Maximum oxygen transfer rate as a function of the number of revolutions with 18 l measuring volume. Maximum means: O_2 concentration c_L = 0.

Comparison of the different scales

A comparison between the model and the two reactor systems of 20 and 150 l is fundamental and shows that the scaled systems reached the same oxygen transfer values as the model. The scaling factors are 1.0 : 6.0 with an H/D of 1.0. The experiment in the 150 l reactor could only be operated with 3 m/l of tubing. The 1 l and the 20 l scale have been used with 2 m/l. The diameter of the tubing was the same in each case. The results are shown in fig. 4 with the Sh and Re numbers which are the results of a dimension analysis. The only parameter which was changed was the membrane speed u. Thus the figure shows the relation between k_L and the speed u.All points in this

double logarithmic graph are approximately linear. So k_L seems to be independent of the choosen scale and only a function of the membrane speed u.

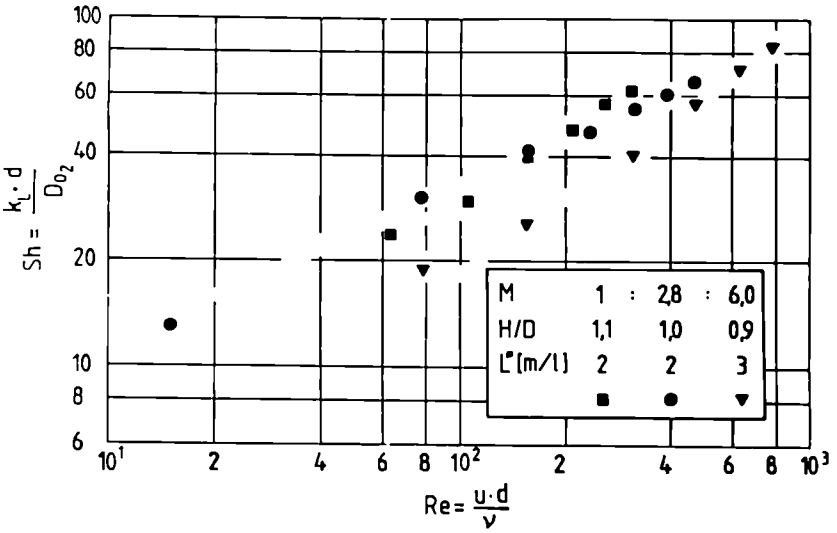


Fig. 4: Comparison between 1, 20 and 150 l reactor with regard to the mass transfer at the membrane. M: scale; H/D: proportion between liquid level and reactor diameter; L^* specific membrane length; d: diameter of the membrane outside; Sh: Sherwoodnumber; Re: Reynoldsnumber.

Mixing of microcarriers

In the 1 l reactor homogeneity occured at a tumbling frequency of 30 rpm. This was estimated optically as the small reactor volume prevents accurate measurement. The homogeneity in the 20 l reactor was determined systematically as a function of the viscosity, the spiral height, the distance of the spiral from the bottom of the reactor and the number of revolutions. For the evaluation of the homogeneity the 1 sec criterion was used. It is fulfilled if no microcarrier is more than 1 sec at the bottom of the reactor or if the standard deviation

$$\sigma^2 = \frac{1}{n} \sum_{i=1}^{n} \left(\frac{\varphi_{Vi}}{\varphi_V} - 1\right)^2$$

of a series of samples is less than 0.95, where n is the number of samples, φ_V is the microcarrier contents per sample in ml/l and φ_V is the initial value of microcarrier input in ml/l. If the standard deviation decreases the homogeneity will increase. For mass transfer from the liquid to the cells on the microcarriers $\sigma^2 = 0.95$ is an optimum /3/. The influence of n on the different parameters up to $\sigma^2 = 0.95$ are shown in tab. 1 to tab 3.

dextran g/l	viscosity Pas·10^{-3}	revo-lutions min^{-1}
0	0.70	23.5
1	0.80	22.5
3	0.86	22.5
5	0.93	22.0

Tab.1: Number of revolutions where the 1 sec criterion as a function of the viscosity is reached.

spiral height mm	revo-lutions min^{-1}
0	--
10	31.0
20	27.0
30	26.0
40	23.5
50	21.5

Tab.2: Number of revolutions where the 1 sec criterion is reached as a function of the spiral

distance mm	revo-lutions min^{-1}
1	24.5
5	24.5
15	29.0

Tab.3: Number of revolutions where the 1 sec criterion is reached as a function of the spiral distance from the reactor bottom.

The microcarrier homogeneity showed the best values if the spiral height was at a maximum, in our case it was 50 mm. The distance to the bottom should be minimized. If the geometric parameters are at an optimum only 22.5 rpm is sufficient to achieve standard deviation of 0.95.

The geometrical data of the spiral, where the best mixing caracteristic was achieved with n (σ^2 = 0.95) = 22.5 1/min for the 20 l reactor (H = 340 mm, D = 297 mm), ϵ = 20 mm, w = 40 mm, l_{spiral} = 1000 mm, h_{spiral} = 50 mm.

Comparison of the reactors

The microcarrier homogeneity was determined in the 150 l reactor using a mean value of 3 g dry carriers per litre PBS as well as in the 20 l reactor. Only a direct comparison between these two systems was possible, as

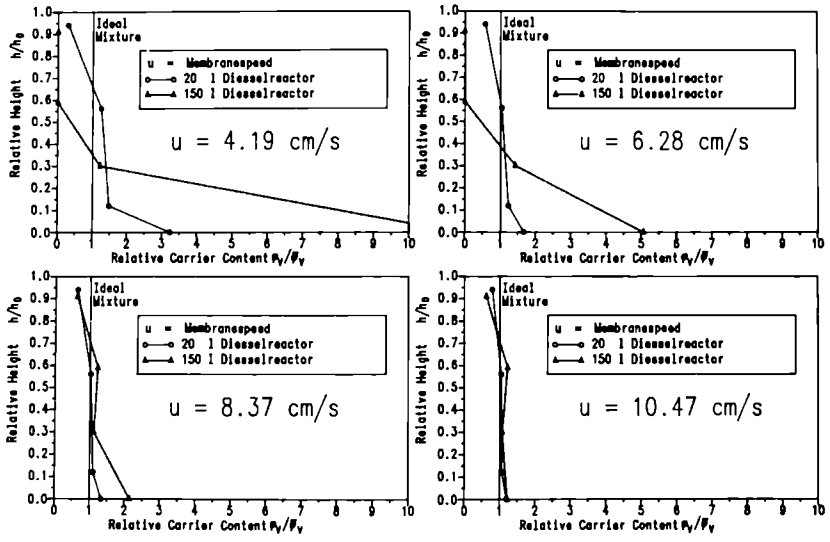


Fig. 4: Comparison between 20 and 150 l reactor with regard to the carrier concentration.

the spiral agitator could not be scaled up proportionally, although the same principle was used. If the same membrane speed u is used (fig. 4) the reactors reached different homogeneity values. The 20 l reactor achieved the 1 sec criterion with u = 4.7 cm/s, the 150 l reactor instead of u = 7.6 cm/s.

It is only useful to measure the microcarrier homogeneity in model media such as PBS without cells, if it is possible to correlate these results with experiments with cells on microcarriers in culture media. As a criterion the speed w_{SS} of the sinking uncovered microcarriers in PBS and the microcarriers covered with cells in culture medium were compared. The carrier concentration was 60 ml wet carriers per litre liquid. Both experiments with carriers in 0.175 mm diameter had w_S = 3.6 cm/min.

Conclusion

A scale up of a reactor was carried out which was equipped with the Accurel® membrane tubing. Instead of the oscillating movement of the 1 l reactor membrane stirrer the new construction used an excenter at the top of the membrane carrier to cause the membrane to travelthrough the culture medium. A fast rotating impeller was not used because of the high shear forces. A spiral agitator was attached beneath the membrane carrier to achieve sufficient values for the homogeineity. This causes vertical motion of the liquid stream and a good mixing of the microcarriers.

If the k_L-values of different reactors are compared it could be shown that the scale up criterion $a = A/V_R$ const. is sufficient to give almost the same oxygen transfer rates. Dividing the total length of the membrane into several segments allows the pressure drop to be reduced without a negative influence on the OTR.

Although mixing characteristics of this reactor system have not been completely investigated, it is possible to mix the microcarriers with a relatively low revolution rate (22.5 rpm for the 20 l scale with an eccentricity of 20 mm; 18.5 rpm for the 150 l scale with an eccentricity of 40 mm), if the 1 sec criterion is used.

The 20 l scale was used for several fermentations of different cells (CHO, BHK, mouse L 929 tk- and hybridomas). These fermentations each run more than 4 weeks. The 150 l is in the testing phase now.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

POSTER

J. Vorlop, E. Fraune, J. Lehmann: Development of a new reactor for mammalian cell-cultures, Protein Purification Technologies, 2nd European Symposium, Palais de Congrès, Nancy/Frankreich (Sept./Okt. 1986)

E. Fraune, J. Lehmann, J. Vorlop: Serum-free production and purification of β-Interferon, Symposium on Protein Purification Technologies, Nancy/Frankreich (Sept./Okt. 1986)

DOCTORATE THESIS

E. Fraune: Produktion und Aufarbeitung von humanem β-Interferon, Univ. Hannover (1986)

/. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

During the reporting period Hybridoma cells have been transferred from Prof. Mabet's laboratory to our group.

Miss Laurayne HELBERT from Laboratoire des Sciences du Génie Chimique, Nancy, spent the March 1987 in our group doing a joint experiment with BHK 21 cells in a 3 l reactor on microcarriers Cytedex® 3. Three joint meetings in Braunschweig, Hannover and Nancy have been held with all contractance.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Bertin & Cie Contract no.: BAP - 0127 - F

Project leader: J. HACHE
Scientific staff: F. Dufau, D. Duval, I. Geahel

Address: Bertin & Cie
Chemical and Biochemical Engineering Division
F - 78373 PLAISIR Cédex

Telephone no.: +33.1.34.81.8692

Telex no.: 690231 F

Other contractual partners in the joint project:

J. M. Engasser, E. N. S. A. I. A. (Vandoeuvre lez Nancy)
K. Schügerl, Universität Hannover
P. Nabet, Université de Nancy
J. Lehman, G. B. F. (Braunschweig)

Title of the research activity:
Biochemical-physical knowledge, control, optimization of animal cell cultures in bioreactors.

Key words:
Animal cells, High density, Ultrasound, Hybridoma

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The long term objective of this study is to design more efficient bioreactors and operation modes for the mass culture of different kinds of animal cells. More precisely, one aims at reactors with a more physiological environment of the cells. This can be obtained by a better control of the limiting physical, chemical and biochemical parameters. Improved scale-up technologies are also expected, specially with respect to aeration, automatic control and feeding strategies. To achieve these technological objectives, it is imperative to first obtain a better qualitative and quantitative understanding of cell behavior in reactors : nutritional requirements, sensitivity to the physical and chemical environment, control of cell physiology.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The final purpose of the programme is to obtain a high cell density during animal cell cultures and to maintain it during a long period of time. For that, the possibility to act on the environment by physical means is tested, namely by using an ultrasonic field. Indeed, under the action of low intensity ultrasounds, surface localized enzyme systems, which regulate the permeability of membrane, can be liberated, and the increase in the permeability of the cell walls can exert a positive influence on the synthesis of cellular components.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

III.1. **Methodology**

Today, most workers associate the action of ultrasound waves with the phenomena of cavitation. In fact, two domains of ultrasonic range can be determined :

- the first domain may be called the physical domain, where effects are generated by the mechanical action of ultrasound or by acoustic cavitation created in the liquid medium surrounding the cells. It corresponds to comparatively low ultrasonic frequencies, with high acoustic intensity and significant exposure time. In this domain, mechanical breakdown of cells is observed ;

- the second domain may be called the biological domain, where ultrasound produces subtile morphological and functional changes. We may have a transformation of the membranes by the ultrasound and a modification of transport conditions.

Contrary to the conventional cell culture systems where transport is strictly diffusive, application of ultrasound in the "biological range" will make the transport system involve both diffusion and

convection.
The ratio convection over diffusion can be characterized by the Peclet number :

$$Pe = \frac{3\ K\ h^2\ A^2}{8\ D\ c}$$

where K is the wave number, h is a characteristic length, A is the amplitude of the pressure fluctuation, D is the diffusion coefficient and c is the speed of sound. When this number increases, that is to say when the frequency or the amplitude increases, the mass transport increases.

The "biological domain" corresponds to comparatively low ultrasonic intensities and exposure times not greater that 50 - 100 sec. So the investigation range for ultrasounds action is the following:

frequency	from	0.5	to	3	MHz
intensity	from	0.1	to	0.3	W/cm^2
time exposure	from	30	to	300	sec

Our approach is the following :

1) First, a non-strategic cell line is chosen. For the first tests hybridoma F 34 of Professor NABET is chosen.

2) Second, we intend to perform a parametric study in a small system including the ultrasound generation.

3) Third, we will confirm the results in a 2 l fermenter type reactor. At the end of our project, we could test on a larger reactor, in collaboration with Dr LEHMAN if agreeable to him.

4) The last part consist in the establishment of criteria for a system using an ultrasonic field. We will give specifications and make numerical simulation for the design.

III.2. **Results**

The study began by a phase of bibliography and theoretical considerations while experimental work is still limited. However, the following points can be mentioned :

1) The kinetic studies on the retained cell line have been performed in Nancy in the laboratory of Professor ENGASSER. These studies continue in our new cell laboratory operational since May 1987.

2) We chosed and characterized the transducers required to obtain the ultrasonic field. For each of them, we determined the response and the radiation diagram. The transducer is put either outside the culture reactor (box) or directly within the culture medium.

3) The analysis of the results will take into account glucose, ammonia, lactate, cell density and cell viability in addition to the acoustic parameters. We want also to use the markers developed by Professor NABET, namely the alcaline phosphatase,

membrane bound enzyme, and the lactic deshydrogenase which can be a marker of cell death.

III.3. **Discussion**

It is too soon to give any reliable results and discuss them. Nevertheless, we think that a system including ultrasonic action would permit high cell density. Numerous results will be given in a future paper.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

No publications during this period.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	No
Exchange of staff	No
Joint experiment(s)	No
Joint meeting(s)	Yes

Descriptive information for the above data.

At this stage of the programme, transnational cooperation essentially involves coordination and mutual information meetings. Four of these have taken place so far.

It is the intention that the use of the ultrasonic generation system and the study of its influence on cell culture be tested on Dr LEHMAN's cultures at GBF.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: C. N. R. S. Contract no.: BAP - 0128 - F

Project leader: J.-M. ENGASSER
Scientific staff:

Address: Institut National Polytechnique
de Lorraine
E.N.S.A.I.A.
2, Avenue de la Forêt de Haye
F - 54500 VANDOEUVRE-LEZ-NANCY

Telephone no.: +33.83574841

Telex no.: 260024

Other contractual partners in the joint project:

J. Lehman, G. B. F. (Braunschweig)
J. Hache, Bertin & Cie (Plaisir)
K. Schügerl, Universität Hannover
P. Nabet, Université de Nancy

Title of the research activity:
Biochemical-physical knowledge, control, optimization of animal cell cultures in bioreactors.

Key words:
BHK cells, Microcarriers, Kinetics, Bioreactor, Aeration

Reporting period: July 1986 - June 1987

I . GENERAL OBJECTIVES OF THE JOINT PROJECT:

The long term objective of this study is to design more efficient bioreactors and operation modes for the mass culture of different kinds of animal cells. More precisely, one aims at reactors with a more physiological environment of the cells. This can be obtained by a better control of the limiting physical, chemical and biochemical parameters. Improved scale-up technologies are also expected, specially with respect to aeration, automatic control and feeding strategies. To achieve these technological objectives, it is imperative to first obtain a qualitative and quantitative understanding of cell behavior in reactors : nutritionnal requirements, sensitivity to the physical and chemical environment, control of cell physiology.

II . SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Due to the high sensitivity of mammalian cells to their environment, the optimization of the cell growth requires a controled and regulated bioreactor as well as a basic knowledge about cellular metabolism. The first objective is to set up a laboratory bioreactor with both an adequate aeration device to regulate the dissolved oxygen and to prevent foam formation, and a perfusion system to remove the metabolites and to supply the nutrients. The second objective is to improve the basic knowledge of cell cultures with different types of cells. Anchorage dependent BHK cells are first investigated, with a detailed kinetic analysis of their growth and metabolism in batch operated bioreactor.

III . SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT

1.METHODOLOGY

BHK cells were cultivated in DMEM completed with 10% tryptose phosphate broth (TPB), 10% NCS and 4mM glutamine. They were grown on microcarriers with 3 $g.l^{-1}$ Cytodex 3 (Pharmacia).

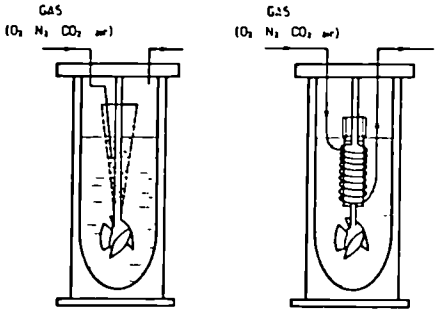
The working volume in the Biolafitte bioreactor was 1.5 liter. pH and dissolved oxygen set points were respectively 7.3 and 50% air saturation .

Glucose and lactate were analysed by enzymatic reactions (Boehringer) whereas ammonium was determined by the Berthelot reaction.

2. RESULTS

2.1. Development of two aeration systems

As headspace aeration was found to be limiting at high cell densities, two bubble-free systems were developped and compared (Fig.1). The first device consists of a stainless steel conic filter (63 µm) fixed on a rotative stirrer, with gas injection inside the filter . In the second bubble-free aeration system gas are injected through a microporous polypropylene membrane supported on a moving basket. Both devices resulted in an excellent dissolved oxygen regulation and prevented foam formation .The highest oxygen transport coefficient is obtained with the polypropylene tube. The conic filter system is simpler to design and to operate.



filter microporous tube

Figure 1:
Aeration systems

2.2. Kinetics of BHK growth on microcarriers

Kinetic studies were performed in a batch mode with a relatively low inoculum of 1.10^5 cells.ml^{-1} in order to observe the cell metabolism during the three consecutive growth phases (Fig 2).

During the lag phase, the specific glucose consumption rate is found constant at 0.2 µmol/10^6 cells.h. Meanwhile the specific lactic acid production rate increases and the specific ammonium production rate decreases. An high ammonium production yield per mole of consummed glucose is also observed: 0.5 mol NH_4^+/mol Glucose.

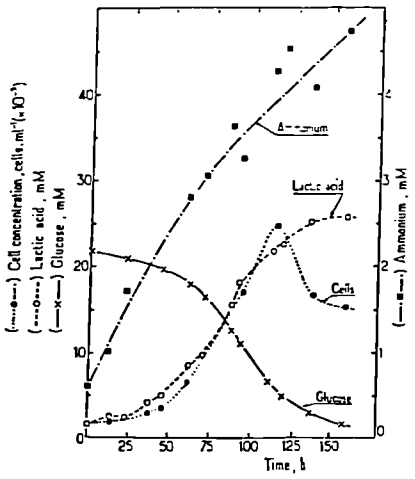


Figure 2:Batch kinetic of BHK cells on Cytodex 3 (3 $g.l^{-1}$)with an innoculum of 1.10^5 $cells.ml^{-1}$.

During the log phase, the glucose molar conversion yield to lactic acid is found constant at 60 %. The ammonium production per mole of consummed glucose is lower than during the lag phase : 0.12 mol $NH4^+$/mol glucose .The different specific consumption and production rates decrease to 0.1 µmol /$h.10^6$ cells for glucose and lactate and to 0.01µmol $.h^{-1}.10^6$ $cells^{-1}$ for ammonia . Overall yields between glucose consumption, lactic acid or ammonium production and cell growth are determined: $Y_{GLU/X}$= 0.6µmol.10^5 $cells^{-1}$, $Y_{LAC/X}$ = 1.1 µmol.$10^5$$cells^{-1}$, $Y_{NH4^+/X}$ = 0.13 µmol.$10^5$$cells^{-1}$.

During the death phase, the lactic acid concentration remains constant whereas the ammonium still increases because of the cell lysis.

Without the addition of TPB in the medium, both glucose consumption and ammonium production are higher. This shows that the utilization by cells more amino acids than glutamine has a strong influence on the cell basic metabolism.

3.PERSPECTIVES

Kinetic studies will be extended to continuous and perfused cultures. Conditions for reaching highest cell densities will be established. In order to obtain a more complete description of the cell metabolism additionnal medium components will be analyzed: glutamine, organic and amino acids. The obtained kinetic data will then be used to build a kinetic model for the growth of BHK cells on microcarriers.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.2.ORAL COMMUNICATOIN

"Culture of BHK cells on micrcarriers in laboratory bioreactors:optimization of aeration system and kinetic studies"
L.HELBERT, A.MARC and J.M.ENGASSER
C.E.C.Meeting on "New methods in animal cell cultures", Seillac,FRANCE, May 24-27, 1987

POSTER COMMUNICATION

"Kinetics of anchorage dependent BHK cells in bioreactors"
L.HELBERT, A.MARC and J.M.ENGASSER
Proc. 4th European Congress on Biotechnology, p 588, vol 3, 1987, Amsterdam, NETHERLAND

V . TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material (s)	No
Exchange of staff	Yes
Joint experiment (s)	No
Joint meetings (s)	Yes

Descriptive information for the above data

- EXCHANGE OF STAFF

Miss Laure HELBERT, PhD studient in INPL of Nancy, staid one month (March 1987) in GBF Institute (Dr. J.LEHMAN).

- JOINT MEETINGS

Three joint meetings took place during the reporting period:

. May 1986 in Hannover and Braunschweig

. November 1986 in Nancy

. June 1987 in Hannover

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Universität Hannover

Contract no.: BAP - 0132 - D

Project leader: K. SCHÜGERL

Scientific staff:

Address: Universität Hannover
Institut für Technische Chemie
Callinstrasse 3
D - 3000 HANNOVER

Telephone no.: +49.511.7622253

Telex no.: 923868 UNIHN D

Other contractual partners in the joint project:

P. Nabet, Université de Nancy
J. M. Engasser, E. N. S. A. I. A. (Vandoeuvre lez Nancy)
J. Hache, Bertin & Cie (Plaisir)
J. Lehman, G. B. F. (Braunschweig)

Title of the research activity:

Biochemical-physical knowledge, control, optimization of animal cell cultures in bioreactors.

Key words:

Animal cells, Shear forces, Pressure load, Hydrodynamic stress, Interfacial forces

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The long term objective of this study is to design more efficient bioreactors and operation modes for the mass culture of different kinds of animal cells by better control of the limiting physical, chemical and biochemical parameters. Improved scale-up technologies are also expected, especially with respect to aeration, automatic control and feeding strategy as well as by better qualitative and quantitative understanding of cell behavior in reactors: nutritional requirements, sensitivity to the physical and chemical environment, control of cell physiology.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

Investigation of the influence of mechanical stresses on the behaviour of animal cells. The following mechanical stresses have been applied:

a) steady state and periodical shear forces in a rotation viscosimeter, b) periodical pressure load in roller bottles, c) hydrodynamic stress in a nozzle and free jet, d) gas/liquid interfacial forces in a bubble column.

These investigations were carried out with insect cells from batch cultures by sampling them at different cultivation times.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. Methodology

The cells are exposed to four different types of stresses:

a) steady state and periodical shear stresses in a rotation viscosimeter, b) periodical pressure load in roller bottles, c) hydrodynamical stresses in a nozzle and free jet, d) gas/liquid interfacial forces in a bubble column.

Single-gap Searle viscosimeter was used with different bobs (with cylindrical symmetry, elliptical cross section and harmonically variable radius). Double-gap Searle viscosimeter was applied with cylindrical symmetry. Both of them were aerated by pure oxygen gas through a silicon membrane at the bottom of the cup to avoid the cell damage by the lack of oxygen. The shear stress was varied by choosing different gap-widths and applying different rotational speeds.

The age of the cells and the exposition times were varied.

In plastic roller bottles cylindrical metal bars were used to exert periodical pressure load on the cells attached to the inner surface of the roller bottles. The diameter, i. e. the weight of the bars as well as the exposition time of the cells were varied.

The cells were cultivated in a spinner flask. After the desired cell density has been obtained, the cell suspension was transferred to a vessel, in which the pressure could be varied up to 30 bar by increasing the air pressure above the cell suspension. By opening the valve between the pressurized vessel and the spinner the cell suspension was forced through a nozzle back to the spinner. The free jet was formed in the liquid bulk, not in the gas phase. By variing the pressure and the number of repetitions of this treatment the cells were exposed to different degree of stresses in the nozzle and the free jet.

The cells were cultivated in a spinner. After the desired cell concentration has been obtained, the cell suspension was transferred to a vessel equipped by a sparger and silicon tubing for bubble free aeration. As sparger a single nozzle or a gas distributor plate was used. Two different operation modes were applied. The cell suspension was aerated by forming the bubbles which rised in the column. During the disengagement of the bubbles the gas/liquid interface is destroyed and by that strong interfacial forces arise.

When applying a paraffin layer at the top of the bubble column, the bubbles pass the oil layer with a thin water film, which is destroyed above the paraffin layer. The strong interfacial forces which arise during the destruction of the gas/liquid interface can not damage the cells, since the cells are stripped off from the bubbles at the water/paraffine interface, thus they are not present when the bubbles burst above the paraffin layer. In the first case the cells can be damaged during the bubble formation, bubble rise and bubble burst. In the second case the cell damage by bubble burst is eliminated. The cell damage was investigated as a function of the aeration time.

2. Results

This testing sytem was built and set in operation. At first the cell line Spodoptera frugiperda was tested in these equipments. These investigations are going on, however, some results have already been obtained. No cell damage was observed during the pressure loading experiments. The shear stress, as well as the hydrodynamical stresses in the nozzle and the free jet quickly destroy the cells. The fraction of the dead cells were determined as a function of the shear stress and exposition time. In the bubble column without paraffin layer the cells are destroyed, only viable cells or cell debris

was present. In bubble columns with paraffin layer viable cells and whole dead cells were found. This indicates that the destruction force acts on the cells during the bubble disengagement is much larger than that during the bubble formation and rise.

3. Discussion

The methods worked out during the reported period are suitable to investigate the cell damage under well controlled conditions. The analytical methods to characterize the cell damage are the viability test, size determination of cells and cell debris, investigation of cell growth as well as measurement of intracellulary components and leakage of the cells. The development of these methods are going on.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

IV.1

M. Wudtke, K. Schügerl

Investigations of the influence of physical environment on the cultivation of animal cells.

8th ESACT-Meeting (European Society for Animal Cell Technology).

A Joint Meeting on Modern Approaches to Animal Cell Technology

Tiberius, Israel 6 - 10 April 1987

and

in "Rheologie und mechanische Beanspruchung biologischer Systeme"

Reprints, GVC-Vortragstagung, Tübingen 25. - 26. Mai 1987.

159 - 173

. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff	Yes	
Joint experiment(s)		No
Joint meeting(s)	Yes	

Descriptive information for the above data.

We met in Nancy on 25. and 26. November 1986 and in Braunschweig and Hannover on 11. June 1987 to exchange cell lines, experimental methods and ideas on the common project as well as reported on our research activities. Mrs. Wudtke spent two weeks in Nancy to analyse the enzyme activities of cells after exposing them to mechanical stresses.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Univ. Nancy I Contract no.: BAP - 0129 - F

Project leader: P. NABET
Scientific staff: J.M. Bour, M. Maugras, J. Capiaumont, A. Derouiche, C. Legrand (Thesis)

Address: Université de Nancy
INSERM
Faculté de Médecine
F - 54505 VANDOEUVRE-LEZ-NANCY CEDEX

Telephone no.: +33.83565656 EXT.314

Telex no.:

Other contractual partners in the joint project:

J. Lehman, G. B. F. (Braunschweig)
J. Hache, Bertin & Cie (Plaisir)
J. M. Engasser, E. N. S. A. I. A. (Vandoeuvre lez Nancy)
K. Schügerl, Universität Hannover

Title of the research activity:
Biochemical-physical knowledge, control, optimization of animal cell cultures in bioreactors.

Key words:
Eukaryotic cell culture, Enzymatic markers, Metabolism markers

Reporting period: January 1987 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The long term objective of this study is to design more efficient bioreactors and operation modes for the mass culture of different kinds of animal cells. More precisely, one aims at reactors with a more physiological environment of the cells. This can be obtained by a better control of the limiting physical, chemical and biochemical parameters. Improved scale-up technologies are also expected, specially with respect to aeration, automatic control and feeding strategies. To achieve these technological objectives, it is imperative to first obtain a better qualitative and quantitative understanding of cell behaviour in reactors : nutritional requirements, sensitivity to the physical and chemical environment, control of cell physiology.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

When cells are grown in mass bioreactors it is often difficult to appreciate their proliferation and physiological status. If an immobilized cell reactor is used one cannot reached the cells, even in a suspension culture repeted sampling is not recommanded. In order to solve these problems we investigate new biochemical markers able to give on line informations. The cells we are used to work with are hybridomas and because they are made with tumour cells we looked first for cancer markers. We also determine some carbohydrates, amino acids and ammoniac quantities as well as enzymatic activities for several membrane bound enzymes, cytoplasmic enzymes and lysosomal enzymes, which, if they are shown to be good markers, could be suitable for all types of cells.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. METHODOLOGY

1.1. Cell culture : In order to show which possible markers are able to give informations on cell condition and which are not, we carry out a batch wise experiment. Seventy 25 cm^2 plastic flasks are inoculated at day 0 with 20.10^4 cells/ml in 9 ml RPMI 1640 supplemented with 10 % FCS and 2 g/l glutamine. Flasks are incubated at 37°C with 5 % CO_2. Each day 7 flasks are picked up, cells are counted and determinations are done in the culture supernatant. This experiment was repeated with 3 different murine hybridomas strains : 3C2 and A49 which were obtained in our laboratories and TSH F021 D6 which was given us by the italian society SORIN.

Another experiment is realized in order to achieve best culture conditions, nearer from the bioreactor ones. A spinner flask filled with 100 ml of the same medium is inoculated with 6×10^4 cells/ml of the A 49 strain. The spinner is kept in an incubator at 37°C with 5 % CO_2 and stirred at about 50 rpm. Each day 50 ml of the medium is removed, cells are counted in a haemocytometer, then centrifuged and resuspended in 50 ml fresh medium, finally reintroduced into the spinner. Determinations are done in the culture supernatant.

1.2. Determinations

1) Metabolites : glucose and lactic acid are determined by enzymatic methods on a centrifuge automatic analyser ; pyruvate is determined by enzymatic method with manual method ; ammoniac is bound on an ion exchange resin, eluted by NaCl then determined by

BERTHELOT's reaction ; aminoacids (except glutamine) are determined by gaz chromatography ; glutamine is bound on a specific resin, free ammonia is washed, then glutamine is eluted. Determination is done by ammonia dosage after glutaminase action.

2) Monoclonal antibodies : Determinations are done by E.L.I.S.A.

3) Cancer markers : Carcino Embryonic Antigen, α fœto protein, β_2 microglobuline are determined by R.I.A. ; Lipid bound sialic acid is determined by the resorcinol colorimetric method, after lipid extraction.

4) Enzymatic activities : Membrane bound enzymes : γ glutamyl transferase, alkaline phosphatase, 5' nucleotidase ; Cytoplasmic enzymes : lactate dehydrogenase, glutamyl oxaloacetic transaminase, creatine phosphokinase.

All these enzymes are determined by kinetic methods on a centrifuge automatic analyser.

Lysosomal enzymes : N acetyl glucosaminidase, β glucuronidase.

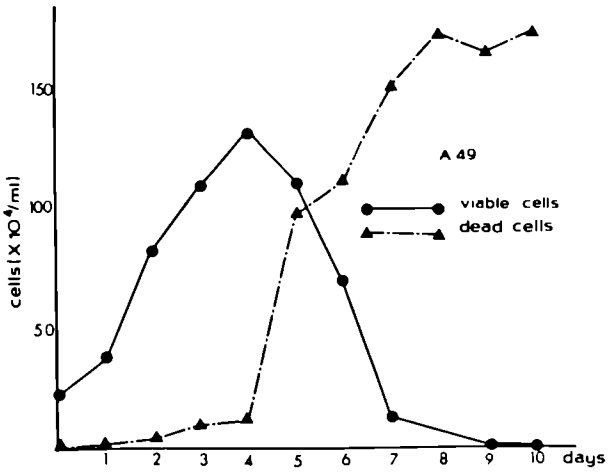
These determinations are done by a manual technique : fluorometric determination of 4 methyl umbelliferol released by artificial substrates.

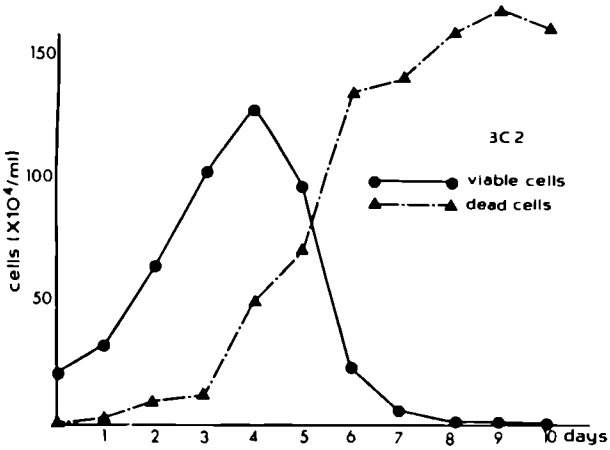
2. RESULTS

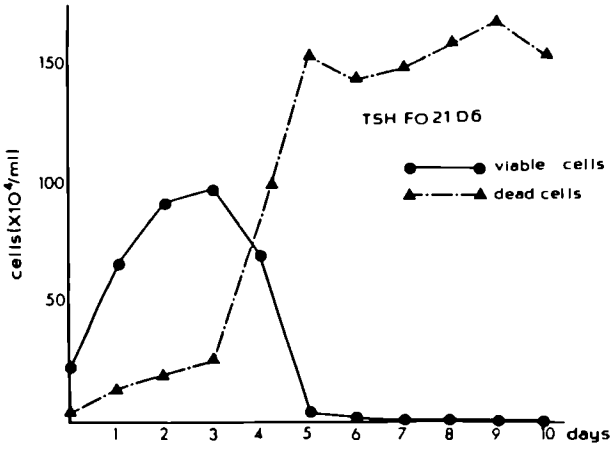
2.1. Cell growth

In the plastic flasks experiment A 49 and 3C2 strains grow rapidly until the fourth day and they reach the density of 130 x 10^4 viable cells/ml (see fig 1 & 2). TSH FO21 D6 grow more rapidly during the first two days, the third one growth decrease and they reach the peak of 97 x 10^4 viable cells/ml (see fig 3). After these three or four days the cells die rapidly.

fig 1 | 2 | 3







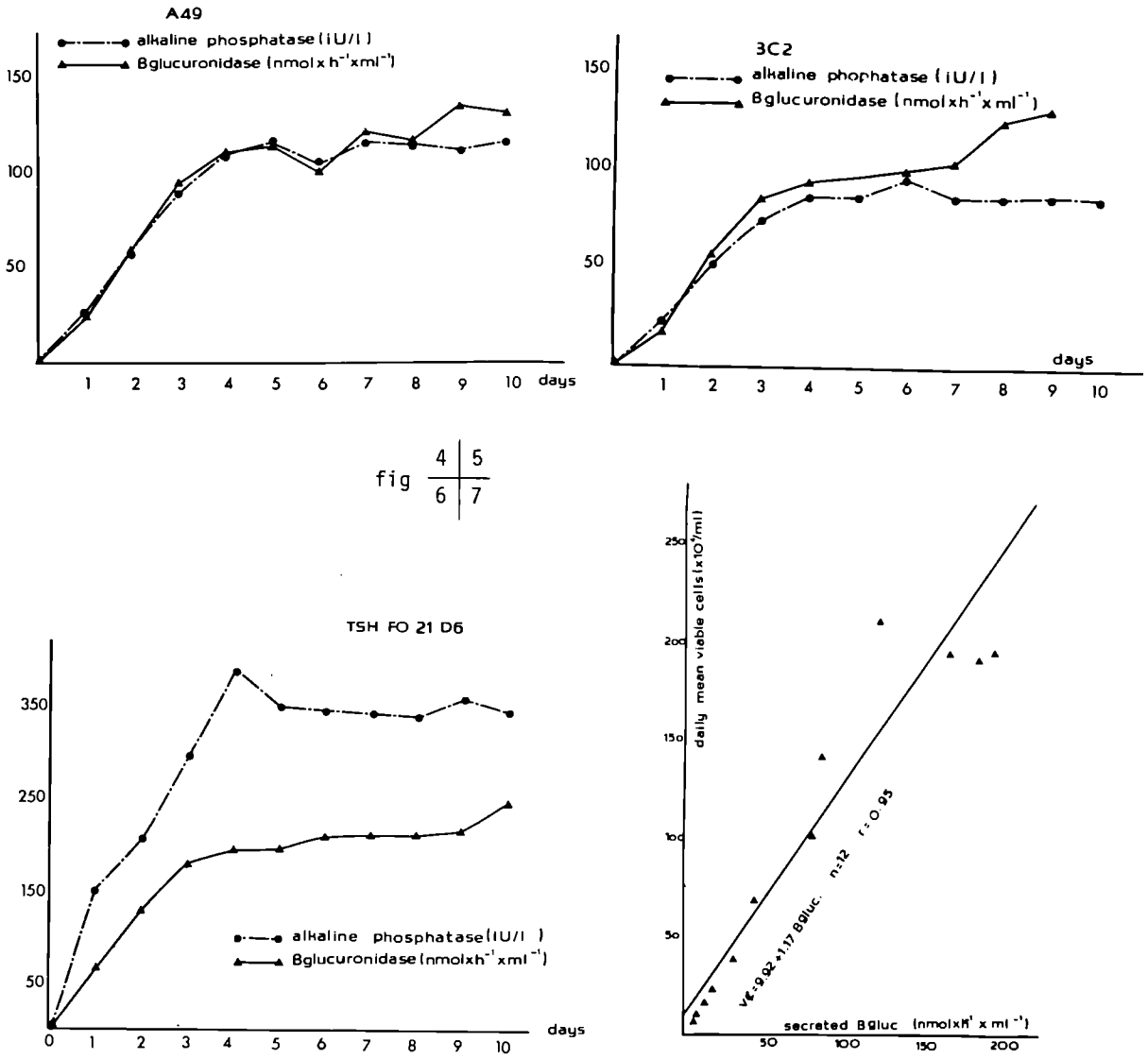
In the spinner experiment cell number increase until the ninth day, then stay at a plateau of 200.10^4 viable cells/ml for four days, afterwards cells die.

2.2. Markers : Cancer markers determination are negative of of no interest (data not shown). In the plastic flasks experiments we find that for the three cell lines alkaline phosphatase and β glucuronidase activities increase with the cell number and are possible markers of cells proliferation (see fig 4, 5 & 6).

In the spinner experiment we are able to establish correlations between average cell number and daily enzyme production for alkaline phosphatase and β glucuronidase as long as the cells grow so up to the twelfth day (see fig 7). Glucose consumption is generally used for the estimation of cell number, in this assay we show that it is a good marker

as long as there is no glucose depletion which happened here the seventh day, but correlation became very bad since this day.

Cell death is characterized by a rapid increase in lactate dehydrogenase activity.



3. DISCUSSION

We have shown that β glucuronidase activity is a good marker of cell proliferation in culture. This can be explained by the fact that at least one part of lysosomal enzymes are excreted out of the cell as soon as they are synthetised. They are then fastened to special membrane bound receptors and enter the cell by pinocytosis. Probably only a few amount of the excreted enzyme is captured by the cell. So the higher cell concentration is, the higher enzyme activity is in the culture supernatant.

Alkaline phosphatase is a membrane bound enzyme and is normally not excreted out of the cell. However even if there is actually no physical stress, we assume that the turnover of membrane proteins is the cause of the release of some amount of the enzyme into the medium.

Lactate dehydrogenase is a cytoplasmic enzyme so it is not be wondered that its activity increases in the medium when cells die.

For the three enzymes the concentrations are very near from each other in the supernatant of the 3C2 and A 49 lines but are much higher in the supernatant of the TSH FO21 D6 line. This clearly show that a caracterisation must be done for each cell line prior to the utilization of these markers.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

1	Exchange of material(s)	Yes
2	Exchange of staff	Yes
3	Joint experiment(s)	Yes
4	Joint meeting(s)	Yes

Descriptive information for the above data.

1 : We gave several hybridomas strains to Pr. SCHUGERL's team (Institut für Technishe Chemie - Hannover)

2 & 3 : Marita WUDTKE who's working in Pr. SCHUGERL's laboratory on insect cell culture, came in Nancy to measure enzymes excretion during schear stress in a viscosimeter. We found that lactate dehydrogenase, alkaline phosphatase and probably creatine phosphokinase are excreted in the medium as a result of shear forces.

4 : Nancy November 25th & 26th 1986
Pr. ENGASSER
Pr. SCHUGERL
Pr. NABET

Hannover June 11th 1987
Pr. SCHUGERL
Pr. LEHMAN
Pr. NABET

For the two meetings program was :
- Visit of the laboratories (Pr. ENGASSER's and Pr. NABET's in Nancy, Pr. SCHUGERL's and Pr. LEHMAN's in Hannover)
- Report of results obtained by the different teams
- Planing of the work to do in the future

RISK ASSESSMENT

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Warren Spring Lab. Contract no.: BAP - 0110 - UK

Project leader: G. LEAVER
Scientific staff: I.W. Stewart, R.H. Watson

Address:
Warren Spring Laboratory
P.O. BOX 20
Gunnels Wood Road
UK - STEVENAGE, HERTFORDSHIRE SG1 2BX

Telephone no.: +44.438.74.11.22

Telex no.: 82250

Other contractual partners in the joint project:

I. Rousseau, I. T. C. - T. N. O. (Zeist)

Title of the research activity:

Assessment of environmental risks and containment of biotechnological scale up processes.

Key words:

Biotechnology risk assessment, Aerobiological monitoring, Rapid detection, Containment, Droplet Size measurement

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

1. Assess and compare existing methods for the detection of containment breach.
2. Assess containment of process components and unit operations.
3. Assess failure modes in order to improve design.
4. Assess the relevance of existing reliability and risk data bases to bioprocessing.
5. Make information available so that equipment selection can be based on reliability, containment, asepsis and safety.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

1. Establishment of standard techniques and methodology for the evaluation and comparison of aerobiological monitoring methods.
2. Comparison and evaluation of current aerobiological monitoring methods.
3. Identification and evaluation of a process marker to be used during the programme and later adopted as a means for the validation of process integrity.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Methodology

A containment cabinet equipped with inlet and outlet HEPA filters was designed by WSL and constructed by Bassaire Ltd, Southampton, UK. An aerosol injection port and a number of sampling ports were incorporated such that simultaneous sampling with several detectors could take place. Initially the cabinet was validated by injecting sterile air and sampling with five samplers - three viable monitors and two clean room monitors (Reference 1,2). Next the same five samplers were challenged with an aerosol of Bacillus subtilis var niger spores in phosphate buffer solution at known concentration. The samplers were operated for different lengths of time according to the manufacturers' instructions and for the viable monitors, the number of colonies formed on a solid substrate (Tryptone Soya Agar) after incubation were counted manually. The results were normalised by dividing the number of colony forming units (CFU) by the amount of air sampled. The droplet size distribution of the challenge aerosol was varied by altering the atomiser operating conditions. Comparative measurements of the droplet size distributions were made with a six stage Andersen Microbial Sampler and a ten stage California Measurements Quartz Crystal Monitor.

Preliminary tests to develop a process marker for more rapid detection have been carried out. It was decided to use spores of the organism B. subtilis v. niger. Two approaches were considered - first the property of natural pigmentation and second the use of fluorescent labelling techniques. Five different growth media designed to exploit the natural pigmentation of B. subtilis v. niger were inoculated and incubated for various periods of time. Where solid media were employed the colonies formed were examined by eye for colour development and in liquid media a spectrophotometer was used to measure the absorbance. Test organisms were also grown in nine liquid cultures containing different fluorescent stains. Aliquots of the cultures were examined using an epifluorescence microscope and a spectrofluorimeter.

Results

Some of the results of aerobiological sampling produced to date are summarised in References 1 and 2 and several features have been examined including the reproducibility, droplet size effect and loading. Duplicate tests performed on consecutive days with mean droplet sizes of approximately 3.5 micron and the same droplet size distribution yielded droplet counts (#/litre) within 10% and airborne concentrations (microgram/litre) within 10%. The viable monitors showed differences of between 50–100%. Similar tests with a mean droplet size around 1.2 micron gave reproducibilities within 25%. When results were expressed as a percentage recovery (airborne spore concentration divided by the normalised result for the viable monitors) smaller mean droplet sizes gave higher values. When the differences between viable monitors were considered a positive ranking was observed. The Millipore Membrane Filter always gave the lowest percentage recovery; the Andersen Microbial Sampler the highest.

The natural pigmentation studies yielded ambiguous and in some cases unreproducible results. On solid media different colour colonies were observed but when these were subcultured they either lost their colour or changed colour. At low spore concentrations in liquid media the amount of pigmentation was indistinguishable from unpigmented controls. In the fluorescence studies under microscopic examination only three of the nine stains gave clear fluorescing spores. When examined using the spectrofluorimeter it was found that for the remaining six stains the excitation frequency of the microscope did not cause the stained spores to fluoresce. Further examination of the stained spores and an unstained control using the spectrofluorimeter showed that for seven of the stains

their emission frequency was close to that of the natural fluorescent emission frequency (autofluorescence) of the spores. Only the stains Acridine Orange and Rhodamine-b-amine gave unambiguous results free from autofluorescence interference.

Discussion

It has been shown that there are a number of problems interpreting data from viable aerobiological monitors where the aerosol droplets are collected by impaction on a solid substrate. When monitoring contained bioprocesses for the emission of viable organisms, it is likely to be important to know the absolute number of organisms emitted. The results to date have shown that aerosol droplet size effects are important for the viable monitors tested. Larger droplets are likely to contain more than one spore leading to low percentage recovery figures. Also, with impaction type viable monitors, impaction of more than one droplet at the same point on the solid surface can lead to low percentage recoveries. Currently corrections are being estimated to allow for these two effects knowing the droplet size distribution. Further experimental work will be carried out to confirm the corrections. Further work on aerobiological monitoring will also involve the use of two different types of impinger where the airborne spores are collected in a liquid. It is hoped that this method will make the above corrections unnecessary although other errors, for example, in dilution, are anticipated.

In order to rapidly identify the chosen marker organism, B. subtilis v. niger, in tests on process equipment and components, it has been shown that it can be stained with one of two possible fluorescent chemicals. Further development of the technique will involve attaching the fluorescent stain to an antibody which is specific to the surface antigens of the chosen spore. Successful outcome of this part of the programme will allow the rapid detection of the stained spores by either microscopic, spectrofluorimetric or opto-electronic means.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

1. Stewart, I.W., Watson, R.H. and Leaver, G., Aerobiological Monitoring for Containment Validation. Proceedings 4th European Congress on Biotechnology 1987, Vol. 1, pp 163-166, Elsevier, Amsterdam.

2. Stewart, I.W., Watson, R.H. and Leaver, G., Evaluation of Aerobiological Monitors. Paper to the SCI Conference "Separations for Biotechnology", University of Reading, September 1987.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	No
Exchange of staff	No
Joint experiment(s)	No
Joint meeting(s)	Yes

Descriptive information for the above data.

Representatives from Warren Spring Laboratory visited the MT-TNO laboratory in Zeist, the Netherlands on 2-3 February 1987 to review the progress of the risk assessment project and identify areas of future co-operation. Warren Spring presented their work to the members of the Dutch Working Party representing users and manufacturers of biotechnological equipment.

Full details of the visit were reported to the commission. (Copy enclosed with report).

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: T. N. O., Zeist Contract no.: BAP - 0109 - NL

Project leader: I. ROUSSEAU
Scientific staff:

Address:
ITC-TNO
Institute of Applied Chemistry
P.O. BOX 108
NL - 3700 AC ZEIST

Telephone no.: +31.3404.55444

Telex no.: 40022 CIVO NL

Other contractual partners in the joint project:

G. Leaver, Warren Spring Laboratory (Stevenage)

Title of the research activity:

Assessment of environmental risks and containment of biotechnological scale up processes.

Key words:

Biotechnology risk assessment, Aerobiological monitoring, Rapid detection, Containment, Droplet Size measurement

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The objective of this project can be summarized as follows:
1. To develop a risk assessment method for bioprocess activities
2. To design novel or improved monitoring methods to detect the release of organisms from bioprocesses into the environment at an early stage
3. To identify the failure modes and mechanisms of various components or unit operations, leading to improved design and manufacturing criteria
4. To develop laboratory test procedures to validate and assess the containment and aseptic integrity of components or unit operations
5. To develop methods for life testing, maintenance procedures and reliability data of bioprocessing equipment

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The objective of TNO during this period was to identify problematic unit operation(s) or component(s) in biotechnological processes. Identification proceeded via consultations with manufacturers of bioprocess equipment and application of risk analysis techniques (failure mode and effect analysis) to evaluate the reliability of the equipment.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Methodology

During the first year of this project TNO has concentrated in one activity, a targeting exercise. The objective of the targeting is to identify most problematic areas (components and unit operations) in biotechnological processes with respect to loss of containment. To execute the targeting, we have initially proposed to adopt the following approach:

1. The use of questionnaires to users and manufacturers of different bioprocessing equipment.
2. A search and evaluation of current reliability data bases and evaluation of their relevance for bioprocessing equipment.
3. On the basis of questionnaires results several unit operations will be chosen for a more detailed analysis.

Consultations at an early stage with Dutch biotechnological companies have clearly indicated that the use of questionnaires would be less effective in this project.
The major reasons for this have been given in the section Discussions.
Two suggestions emerged from the consultations: One company has offered

to provide their "engineering expertise" in evaluating the problematic areas. A second suggestion was to approach the Working Party on Safety in Biotechnology of the European Federation of Biotechnology - EFB. Representatives of the different countries were personally requested to act as a contact person between TNO and relevant companies in the corresponding countries.

In order to intensify our contacts with the Dutch industry, a Working Party was formed to include both users and manufacturers of bioprocessing equipment in the Netherlands. The main tasks of the Working Party are to give technical guidance/advice during the project and to disclose relevant (confidential) information within biotechnological companies. Separately, a Steering Committee was formed with representatives from the industry institutes and government ministries, to guide and direct the non-technical aspects of the project.

Results

Our contacts with members of the EFB Working Party on Safety in Biotechnology resulted in positive reactions from two countries: Sweden and Switzerland. These positive reactions led to initial contacts with leading equipment manufacturing companies in aforementioned countries. These contacts will be further consolidated.

During the reported period, two meetings were held with the Dutch Working Party. The first meeting was devoted to the formulation of principles of operation, terms of confidence and exchange of information.
Following the first meeting, three equipment manufacturing companies from the Working Party were approached individually with the request to provide detailed information with respect to equipment design. They all agreed to provide such information under strict terms of confidentiality.

The provided information includes pilot-plant fermentor and cell components and is currently being analyzed by the TNO Department of Industrial Safety, using the technique of Failure Mode and Effect Analysis (FMEA). At the moment of reporting the following equipment parts have been analyzed:

1. Pilot-plant fermentor:
   * lipseal stirrer (top-mounted shaft)
   * magnetic coupled stirrer (top-mounted and bottom-mounted shafts)
   * sampling device
   * inoculation system
   * air inlet system

2. Cell disruption equipment:
   * cylinder block
   * homogenizer valve assembly

A preliminary evaluation of the analysis of the above components led to the following major conclusions:

1. Single static seals do not provide a high level of containment integrity. It is therefore recommendable to apply double static seals where a high level of containment is required (e.g. pathogens).

The two seals should preferably be separated by a compartment which is purged with a sterilizing medium (e.g. steam, biocidic fluid).

2. The probability of leakage of a dynamic seal is judged relatively high. Hence, in cases where the containment of micro-organisms or products thereof has to be warranted to a certain degree, use has to be made of multiple dynamic seals. If a high level of containment is required the use of an inactivating agens should be considered, either by direct introduction to the system (e.g. steam) or by addition of a biocide to the lubricant.
3. For operation with pathogens or classified r-DNA micro-organisms it is recommended to equip the fermentor with a top-mounted agitator.
4. Interfaces between different pieces of equipment may jeopardize the process's integrity with respect to containment. This is especially the case for high pressure - low pressure interfaces (e.g. equipment downstream cell-disruption apparatus). However, such interfaces are not restricted to the situation where several pieces of equipment are connected with each other, but may also occur within one single unit operation (e.g. lubricating system of a cell-disrupture apparatus).
5. Complete inactivation of remaining (pathogenic) micro-organisms is a prerequisite to any cleaning or maintenance operation. It is recommended to investigate in more detail whether this condition can be obtained under all circumstances with the current sterilization procedures.
6. In the currently available sampling systems, procedural errors may not be excluded.

The above points and others have been discussed extensively in the second meeting of the Working Party.

The Failure Mode and Effect Analysis will be extended further to include micro- and ultrafiltration and possibly centrifuges and large-scale fermentors.

Finally, during the reported period, a literature review has been carried out on safety aspects of biotechnological processes. A detailed report of the literature review is currently being prepared and will be issued in September 1987.

Discussion

This discussion is devoted to two topics, namely our contacts with the biotechnology industry, and future work by TNO in this project.

The innovative characteristics of the biotechnology industry and the versatility of processes involved made it difficult to apply the approach of using questionnaires. Therefore, we have amended our strategy in executing the targeting activity. It was soon realized that the users of bioprocessing equipment would be reluctant to discuss biosafety of processes involving genetically engineered organisms. This attitude may continue as long as the process of formulation of guidelines or regulation for large-scale utilization of recombinant DNA are not well established, either in individual countries or on European scale.

This led us to concentrate our targeting efforts to consultation with equipment manufacturers. In this sector of industry there is clearly the need for guidelines for design of equipment conform with containment requirements.

TNO has established working relationships in this sector with Dutch, Swedish and Swiss producers of biotechnological equipment. It is important that TNO contacts are extended to include British and German companies. In the next six months our efforts will be concentrated in this direction.

The dialogue with Dutch industry will be continued to include, where possible, users of bioprocessing equipment. In this way TNO is in a better position to evaluate the results of the Failure Modes and Effect Analysis.

Finally we woud like to enlighten the future work in this project:

1. Activity 1 - Targeting:
   This activity will be continued by examining the design of additional equipment. Efforts will be made to analyze the current reliability data bases for their relevance for bioprocessing equipment.

2. Activity 3 - Component Testing:
   In the next half year TNO, in consultation with Warren Spring Laboratory (WSL), will construct clean-air facilities to test various components. Monitoring methods developed by WSL will be adopted by TNO.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

In preparation: "Safety aspects of biotechnological processes - a literature review". TNO internal report.

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS

Exchange of material(s)	No
Exchange of staff	No
Joint experiment(s)	No
Joint meeting(s)	Yes

Descriptive information for the above data.

During February 2 and 3, 1987, a meeting was held at ITC/TNO, Zeist, between Warren Spring Laboratory and TNO.
A copy of the minutes of this meeting is enclosed.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: I.N.R.A., Dijon
Contract no.: BAP - 0108 - FR

Project leader: N. AMARGER
Scientific staff: A. Hartmann, S. Mazurier

Address:
Laboratoire de Microbiologie des Sols
I.N.R.A.
17, rue Sully
B.P. 1540
F - 21034 DIJON CEDEX

Telephone no.: +33.80633000

Telex no.: 350507 INRADIJ F

Other contractual partners in the joint project:

W. Klingmüller, Lehrstuhl für Genetik (Bayreuth)
P. Hirsch, Rothamsted Experimental Station (Harpenden)

Title of the research activity:

Assessing the risks involved in the release of genetically manipulated microorganisms.

Key words:

Genetic transfer, Rhizobium, Symbiotic plasmid

Reporting period: November 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

Le risque principal de la mise en circulation de bactéries génétiquement manipulées est de créer des nouveaux organismes par transfert de gênes à des bactéries indigènes. La recherche proposée vise à voir si de tels échanges sont possibles en conditions naturelles d'environnement et dans des environnements différents. Une bactérie du genre Rhizobium a été retenue comme organisme modèle car c'est une bactérie non pathogène, génétiquement bien connue, que l'on sait introduire dans les sols et qui peut être récupérée à partir des sols en utilisant la plante hôte.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

- Mise en place de l'expérimentation au champ commune aux trois laboratoires.
- Etude en conditions de laboratoire du transfert de plasmides de Rhizobium : mesure de la fréquence de transfert d'un plasmide marqué en fonction de différentes variables expérimentales, recherche de la présence de plasmide symbiotique transmissible dans une population naturelle de Rhizobium et essai d'estimation de la fréquence de transfert de ces plasmides.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Méthodologie

- Essai au champ.

La souche de Rhizobium leguminosarum biovar viceae a été sélectionnée au laboratoire de Rothamsted. C'est une bactérie qui porte la résistance à 200 µg ml^{-1} de sulfate de streptomycine et à 100 µg ml^{-1} de rifampycine sur le chromosome et dans le plasmide symbiotique de laquelle un transposon Tn 5, qui confère la résistance à 50 µg ml^{-1} de sulfate de kanamycine, a été inséré.

Le protocole d'expérimentation commun au trois laboratoires associés du projet a été établi au cours d'une réunion à Rothamsted.

La parcelle de 12 x 24 m a été subdivisée en deux sous-parcelles. L'une a été semée avec du Pois var Solara, l'autre avec du blé. L'inoculum a été apporté au semis réalisé le 13/3/87. Une suspension bac-

térienne contenant 2,7 x 10^8 bactéries par ml a été épandue dans le lit de semence à raison de 20 ml par m^2 ce qui était voisin du nombre de Rhizobium de la même spécificité contenu dans les 30 premiers cm de sol.

Un prélèvement de 10 plantes en 4 endroits de la parcelle de pois a été réalisé le 15.6.87 (d'autres prélèvements seront réalisés ultérieurement). 1200 nodosités ont été séparées des racines, stérilisees en surface et écrasées dans de l'eau stérile. Les suspensions ainsi obtenues sont repiquées sur milieu témoin, milieu contenant de la rifampycine, milieu contenant de la kanamycine et milieu contenant rifampycine et kanamycine. Les souches résistantes à un seul des antibiotiques qui ne correspondent donc pas ni aux souches naturelles ni à la souche inoculée, seront conservées pour être identifiées ultérieurement.

- Etudes au laboratoire.

Les expériences de conjugaison ont été réalisées en utilisant une souche de R. leguminosarum biovar viceae donnée par A. Johnston, curée de son plasmide symbiotique donc non nodulante, résistante à la streptomycine et à la rifampycine. Les conjugaisons ont été réalisées sur filtre de diamètre 25 mm.

Pour l'étude de la fréquence du transfert en fonction des conditions d'expérimentation, une souche de R. leguminosarum biovar phaseoli (obtenue par A. Hartmann) contenant un plasmide de 180 kb marqué par insertion d'un Tn 5 a été utilisée comme souche donneuse. Les transconjugants sont sélectionnés par leur résistance à la streptomycine et à la kanamycine.

Pour l'estimation de la fréquence de transfert de différents plasmides symbiotiques provenant d'une population naturelle de R. leguminosarum biovar phaseoli, des dilutions sont réalisées après les conjugaisons. L'incubation en présence de streptomycine et rifampycine permet l'élimination de la souche donneuse et la multiplication des transconjugants et de la souche receveuse. Seuls les transconjugants étant capables de noduler le haricot, il est possible de les détecter en inoculant des plantules de haricot avec le mélange de bactéries.

Dans tous les cas la vérification du transfert est faite par observation des profils plasmidiques.

Résultats

- Expérience au champ : les résultats sont en cours d'acquisition.
- Expérience en laboratoire : Les résultats sur l'influence du nombre

de bactéries donneuses et receveuses sur la fréquence de transfert du plasmide 180 kb marqué au Tn 5 de R. phaseoli Ro 84 sont donnés dans le tableau suivant :

Nombre total de bactéries	$1,4x10^6$	$1,4x10^7$	$1,4x10^8$	$1,4x10^9$
Rapport D:R*	0,8	0,8	0,8	0,8
Rapport T:R	$1,2x10^{-4}$	$5,0x10^{-4}$	$5x10^{-3}$	$6x10^{-3}$

Nombre total de bactéries	$6x10^8$			$14x10^8$	$8x10^8$		
Rapport D:R	$8x10^2$	$8x10^1$	8	0,8	$8x10^{-2}$	$8x10^{-3}$	$8x10^{-4}$
Rapport T:R	$1x10^{-3}$	$1x10^{-3}$	$7x10^{-3}$	$6x10^{-3}$	$7x10^{-3}$	$3x10^{-3}$	$8x10^{-6}$

* D = nombre de bactéries de la souche donneuse R = nombre de bactéries de la souche receveuse T = nombre de transconjugants.

En ce qui concerne l'existence de plasmide symbiotique transmissible dans une population de R. leguminosarum biovar phaseoli provenant d'un champ, la première expérience faite sur une cinquantaine de souches a montré qu'environ 20 % des souches possédaient un plasmide symbiotique capable d'être transféré à la souche de R. leguminosarum biovar viceae utilisée comme receveur. La fréquence de transfert est de 10^{-5} à 10^{-6}. Des expériences sont en cours avec d'autres souches receveuses.

Discussion

Les premièrs résultats au laboratoire obtenus avec un plasmide transmissible à fréquence comprise entre 10^{-2} et 10^{-3} nous indiquent que cette fréquence est constante si elle est exprimée par rapport à l'espèce la moins représentée et pour des nombres totaux égaux ou supérieurs à 10^8 pour une surface de 5 cm^2. Par contre cette fréquence diminue rapidement dès que le nombre total de bactéries diminue c'est-à-dire dès que les bactéries ne sont plus en contact. Les expériences ultérieures nous permettront de voir ce qu'il en est lorsque les bactéries sont dans une phase liquide et à la surface de particules c'est-à-dire dans des milieux plus complexes et plus proches d'un sol.

La fréquence de transfert des plasmides symbiotiques étudiés semble faible mais les résultats restent à vérifier en fonction d'autres souches receveuses et donneuses.

English translation of the report by N. Amarger (BAP-0108-FR)

I. GENERAL OBJECTIVES OF THE JOINT PROJECT.

The main risk involved in releasing genetically manipulated bacteria is of creating new organisms through gene transfer to native bacteria. The aim of the research is to ascertain whether such transfers are possible in natural environmental conditions and in different environments. A member of the Rhizobium genus was chosen as a model microorganism, since it is a genetically well-known non-pathogenic bacterium that can be introduced into soils and recovered therefrom using the host plant.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

- Setting-up of the field trial common to the three laboratories.
- Study in laboratory conditions of the transfer of Rhizobium plasmids : measurement of the frequency of transfer of a marked plasmid as a function of different experimental variables, search for the presence of symbiotic plasmids transmissible in a natural Rhizobium colony and an attempt to estimate the frequency of transfer of such plasmids.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

Methodology

- Field trial.

The Rhizobium leguminosarum biovar viceae strain was selected at the Rothamsted laboratory : it is a bacterium which carries resistance to 200 ug ml^{-1} streptomycin sulphate and 100 ug ml^{-1} rifampycin on the chromosome, into the symbiotic plasmid of which a Tn5 transposon, which confers resistance to 50 ug ml^{-1} kanamycin sulphate, has been inserted.

The experimental protocol common to the three laboratories taking part in the project was drawn up at a meeting at Rothamsted.

The 12 x 24 m plot was subdivided into two stips ; one was sown with pea var Solara and the other with wheat. The seeds, sown on 13 March 1987, were inoculated. A bacterial suspension containing 2.7 x 10^8 bacteria per ml was applied to the seed bed in quantities of 20 ml per m^2, similar to the number of Rhizobia of the same specificity contained in the first 30 cm of soil.

A sample of ten plants was taken on 15 June 1987 from four different places on the pea strip (other samples will be taken at a later date). 1,200 nodules were separated from the roots, their surface sterilized and crushed in sterile water. The suspensions thus obtained are being cultured on a control medium, a medium containing rifampycin, one containing kanamycin and another containing rifampycin and kanamycin. Strains that are resistant to one of the antibiotics only and therefore correspond neither to the natural strains nor to the inoculated strain will be kept for subsequent identification.

- Laboratory studies.

Conjugation experiments were run using a R. leguminosarum biovar viceae strain given by A. Johnston, from which the symbiotic plasmid had been removed and which was therefore non-nodulating and resistant to stroptomycin and rifampycin. The conjugations were carried out on a 25 mm diameter filter.

For studying transfer frequency as a function of experimental conditions, a R. leguminosarum biovar phaseoli strain (obtained by A. Hartman) containing a 180 kb plasmid marked by Tn5 insertion was used as a donor strain. The transconjugants are selected by their resistance to stroptomycin and kanamycin.

In order to estimate the transfer frequency of different symbiotic plasmids from a natural colony of R. leguminosarum biovar phaseoli, dilutions were made after the conjugations. Incubation in the presence of streptomycin and rifampycin enables the donor strain to be eliminated and the transconjugants and the host strain to multiply. Since only transconjugants are capable of nodulating the bean, they can be detected by inoculating bean plantlets with the bacteria mixture.

Transfer is checked in all cases by observing the plasmid profiles.

Results.

- Field trial : the results are being gathered.
- Laboratory experiment : the results concerning the influence of the number of donor and host bacteria on the frequency of transfer of the Tn5-marked 180 kb plasmid of R. phaseoli Ro 84 are set out in the following table :

Total number of bacteria	$1,4x10^6$	$1,4x10^7$	$1,4x10^8$	$1,4x10^9$
D/R ratio*	0,8	0,8	0,8	0,8
T/R ratio	$1,2x10^{-4}$	$5,0x10^{-4}$	$5x10^{-3}$	$6x10^{-3}$

Total number of bacteria	$6x10^8$			$14x10^8$	$8x10^8$		
D/R ratio	$8x10^2$	$8x10^1$	8	0,8	$8x10^{-2}$	$8x10^{-3}$	$8x10^{-4}$
T/R ratio	$1x10^{-3}$	$1x10^{-3}$	$7x10^{-3}$	$6x10^{-3}$	$7x10^{-3}$	$3x10^{-3}$	$8x10^{-6}$

* D : number of bacteria of the donor strain ; R : number of bacteria of the host strain ; T : number of transconjugants.

As regards the existence of transmissible symbiotic plasmids in a colony of R. leguminosarum biovar phaseoli taken from a field, an initial experiment conducted on some fifty strains demonstrated that approximately 20 % of the strains had a symbiotic plasmid capable of being transferred to the strain of R. leguminosarum biovar viceae used as host. The transfer frequency ranges between 10^{-5} and 10^{-6}. Experiments are in progress with other host strains.

Discussion

The initial laboratory results obtained with a plasmid transmissible at a frequency of between 10^{-2} and 10^{-3} demonstrate that such a frequency is constant if expressed in terms of the least represented species and for total numbers of not less than 10^{-8} for a surface of 5 cm^2. On the other hand, the frequency decreases rapidly as soon as the total number of bacteria falls, i.e. as soon as the bacteria are no longer in contact. Further experiments will enable us to see what happens when the bacteria are in a liquid phase and on the surface of particles, i.e. in more complex media that resemble soil conditions more closely.

The frequency of transfer of the symbiotic plasmids studied appears low, but the results still have to be checked on other host and donor strains.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes	
Exchange of staff		No
Joint experiment(s)	Yes	
Joint meeting(s)	Yes	

Descriptive information for the above data.

La souche de Rhizobium utilisée au champ a été fournie par le laboratoire de Rothamsted.

L'expérimentation au champ est commune au 3 laboratoires contractants.

Le plan expérimental a été arrêté lors d'une réunion à Rothamsted regroupant les 3 responsables scientifiques le 23/10/86.

The Rhizobium strain used in the field trial was supplied by the Rothamsted laboratory.

The field trial is common to the three contracting laboratories.

The experimental programme was drawn up by the three project leaders at a meeting at Rothamsted on 23 October 1986.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: University of Bayreuth

Contract no.: BAP - 0107 - D

Project leader: W. KLINGMÜLLER

Scientific staff: K. Döhler

Address:

Lehrstuhl für Genetik
Universitätsstrasse 30
Postfach 3008
D - 8580 BAYREUTH

Telephone no.: +49.921.552701

Telex no.:

Other contractual partners in the joint project:

N. Amarger, I. N. R. A. (Dijon)
P. Hirsch, Rothamsted Experimental Station (Harpenden)

Title of the research activity:

Assessing the risks involved in the release of genetically manipulated microorganisms.

Key words:

Enterobacter, *Rhizobium*, *nif* genes, Tn*5*, Releasing of bacteria

Reporting period: May 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The project is designed to assess the risks of gene transfer from a genetically marked soil bacterium to other members of the soil microflora by monitoring the persistence of genetically manipulated bacteria introduced into agricultural soils and by screening for the spread of genes carried by these microorganisms to other members of the soil flora. The selected strains for releasing experiments contain either a conjugative plasmid with Tn5 as marker gene (Rhizobium leguminosarum biovar viceae RSM 2004) or the nif genes of Enterobacter agglomerans 339 cloned into a suitable safety vector plasmid.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

The properties of the selected strain RSM 2004 and its qualification for the monitoring of the persistence of genetically manipulated bacteria were to be studied. All methods necessary for the recovery, selection and identification of the released strain as well as of the strains which were expected to get the transposon marker via genetic recombination were to be performed on a small scale in pots with soil. The releasing experiment was planned to start in May 1987. In addition the single center experiment was to be prepared by the construction of a non selftransmissible but mobilizable plasmid containing cloned nif genes.

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

METHODOLOGY: All methods necessary for the recovery, selection and identification of the released strain as well as of the strains which were expected to get the transposon marker via genetic recombination were performed on a small scale in pots with soil. The methods including seed sterilization, nodulation of leguminosae, nodule sterilization, sampling for bacteria in soil, isolation and identification of Rhizobiaceae and other Gram negative bacteria, isolation of large plasmids, and both DNA-DNA hybridization and colony hybridization with biotin-labeled Tn5 were done as described in the appropriate literature. Cloning of nif genes and conjugation experiments were performed by published methods. About 10^{11} rhizobia RSM 2004 per m^2 equivalent to 10^9 per pea seed were applied to the experimental field in the middle of May. One third of the field (about 100 m^2) was sown with peas, another third with wheat and barley. To check the spreading of bacteria in the field, the third part was inoculated with

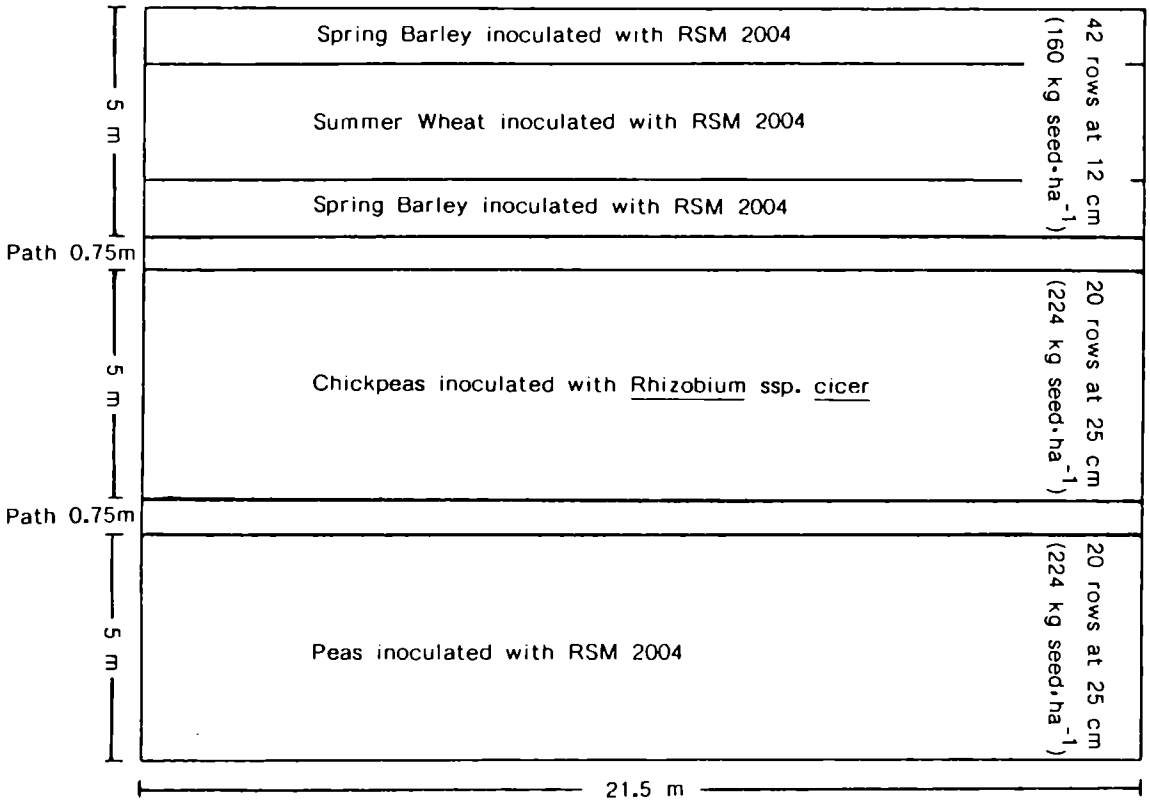
Rhizobium ssp. cicer (2×10^9 per m^2) and sowed with chickpeas.

RESULTS: Nif genes from plasmids of Enterobacter agglomerans were cloned into suitable safety vector plasmids which were non selftransmissible but mobilizable by helper plasmids. The hybrid plasmids were then checked for transfer in the laboratory. Bacteria isolated from soils around Bayreuth did not show any ability to mobilize the new nif-plasmid constructs.
The analysis of the persistence of the Rhizobium strain RSM 2004 in pots with soil showed an approximately tenfold decrease of survivors over a period of eight weeks. Although there is a relatively high background of kanamycin-resistent microorganisms in natural soil, no hybridization with Tn5 could be found.
The conjugative plasmid pRL1JI in RSM 2004 has been shown in the laboratory to transfer to other Rhizobiaceae and some strains of Enterobacter which were isolated from fields around Bayreuth. However, in the exconjugants with Enterobacter we could not determine pRL1JI itself. Rather, in all cases labeled Tn5-probes hybridized with the chromosomal DNA of the exconjugants. With three strains of Escherichia coli we got a very low level of exconjugants (about 10^{-9}) which also had inserted Tn5 into the chromosome. Transfer to other genera has not yet been detected and we are not able to demonstrate transfer of the Tn5 marker to strains of Klebsiella and Pseudomonas.
Before sowing the seeds and running the inoculations with Rhizobium in the field, the soil parameters and the indigenous Rhizobiaceae in the experimental field were determined. We found Rhizobium leguminosarum biovars viceae and trifolii at 10^4 to 10^5 per g soil.
The experimental field, surrounded by potatoes and oats, was protected from mammals and birds with wire-fence and nets.

DISCUSSION: The genetically marked strain RSM 2004, constructed in Rothamsted by Dr. P. Hirsch's group was chosen because it contains the conjugative plasmid pRL1JI that can transfer to other members of the Rhizobiaceae, and Tn5 that can insert and express antibiotic resistance in a wide range of Gram negative bacteria. Therefore it can be used to monitor plasmid transfer, but also transient genetic interactions, if these result in transposition of Tn5 from pRL1JI. The experiments of the first year are still in progress, results will not be available before autumn.

PLANTING DIAGRAM FOR FIRST YEAR

Experimental field: Vereinshof, Universität Bayreuth



IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

None under contract

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

In the middle of May J. Spokes from Rothamsted was in Bayreuth, to assist in the releasing of the rhizobia, according to the Rothamsted experimental protocol. He brought along the pea and chickpea seeds as well as the granular inoculant with the Rhizobium strains RSM 2004 and ssp. cicer for spreading in the furrows and coating the seeds.

The core experiment, the releasing of Rhizobium RSM 2004 was and will be done according to the common protocol with Dr. P. Hirsch's group in Rothamsted. The seeds and strains used were identical to theirs. What differs are the climatic conditions and the soil parameters of the Bayreuth experimental field.

To come to agreements in techniques and concepts, a visit to Rothamsted, to Dr. P Hirsch's group was carried through in October 1986. At this visit, Dr. Amarger from Dijon was also present, and points of mutual interest were discussed.

BIOTECHNOLOGY ACTION PROGRAMME

Progress Report

Contractor: Rothamsted Experim. Station, Harpenden

Contract no.: BAP - 0024 - UK

Project leader: P. HIRSCH

Scientific staff: J. Spokes, A. Latham, M. White, D. Gibson

Address: Rothamsted Experimental Station
UK - HARPENDEN, HERTS AL5 2JQ

Telephone no.: +44.5827.63133 EXT.2669

Telex no.: 825726 REXPST G

Other contractual partners in the joint project:

N. Amarger, I. N. R. A. (Dijon)
W. Klingmüller, Lehrstuhl für Genetik (Bayreuth)

Title of the research activity:

Assessing the risks involved in the release of genetically manipulated microorganisms.

Key words:

Rhizobium, Risk assessment, Gene transfer, Plasmid transfer, Environmental release

Reporting period: July 1986 - June 1987

I. GENERAL OBJECTIVES OF THE JOINT PROJECT:

The aim is to obtain data on the extent of gene transfer between bacteria in soil. This information can be used to help assess the risks involved in the deliberate or accidental release of genetically manipulated micro organisms into the environment, which could lead to transfer of genes to native soil bacteria forming novel hybrids with potentially hazardous properties. The project uses Rhizobium as a model soil microorganism and the transposon Tn5 as a marker gene.

II. SPECIFIC OBJECTIVES OF THIS LABORATORY FOR THE REPORTING PERIOD:

1. Construction of R. leguminosarum strain carrying Tn5 to use in release experiments

2. Obtaining clearance from ACGM (U.K. Advisory committee on genetic manipulation) for release of strain in field

3. Testing strain for symbiotic properties and preparation of inoculant for use by participating countries

4. Setting up and monitoring field trial

III. SUMMARY OF THE MAIN RESULTS OBTAINED DURING THE PERIOD UNDER CONTRACT:

1. R. leguminosarum biovar viceae strain RSM 2004 was constructed for use in field trials from field isolate JI248 which carries pRL1JI, a self-transmissible symbiotic plasmid. The chromosome was marked by selecting spontaneous mutants resistant to both streptomycin and rifampicin, and Tn5 was inserted onto pRL1JI using a suicide plasmid vector pJB4JI and selecting for kanamycin resistance. RSM2004 was picked from several Tn5 insertions on pRL1JI since it was the most competitive for nodulation of the host plant, pea, when co-inoculated with its parent strain JI 248. It formed effective nodules, indistinguishable from those of the parent, and transferred pRL1JI (and Tn5) at high frequency. Although RSM 2004 was constructed using "natural" bacterial genetics, it is an interspecific hybrid since Tn5 was derived originally from Klebsiella and is thus subject to new guidelines in the U.K. concerning environmental release.

2. The ACGM group advising on release of genetically manipulated organisms had only recently been formed when we submitted our proposal and since it was the first proposal involving bacteria it was subject to close scrutiny and lengthy debate since it would both set a precedent, and also provide data relevant to their future deliberations. After detailed discussions and some alterations to our plans, the ACGM said they saw no reason why our release experiment should not go ahead.

3. Reconstruction experiments using soil from the proposed field site in pots in the glasshouse indicated that about 10 RSM 2004 added to one plant in 600 g soil formed c. 30% of the nodules. The native rhizobia were estimated at about 10^3 R. leguminosarum bv viceae per g. dry soil, indicating that RSM 2004 is not very competitive with native strains, but can form nodules in their presence. We decided to use both seed-coating and granules of inoculant to increase the chances of RSM 2004 forming nodules. Peat based inoculant containing RSM 2004 were prepared for use at Rothamsted and Bayreuth, along with Cicer rhizobial inoculant to use to monitor spread of the rhizobia from the field site (Cicer rhizobia do not occur in Rothamsted or Bayreuth soils naturally).

4. The field experiment was planted in three strips each 100 m , the first planted with peas inoculated with RSM 2004 at about 10^{10} per m^2 (approximately equal to the number of native rhizobia) i.e. 10^8 per seed (as in the reconstruction experiment in pots). The second strip was inoculated with RSM 2004 at the same level, but sown with barley and wheat, and the third with Chickpea Cicer arietinum inoculated with Cicer rhizobia, to act as a marker for spread of inoculum from the site. The plot was covered with a net to keep out birds and surrounded with a low electric fence to keep out rabbits. The plants have grown and first sampling of root nodules, which is not yet complete, indicates that RSM 2004 may form only about 10-20% of the nodules, less than the reconstruction experiment predicted. No transfer of Tn5 to other rhizobia has been detected so far.

The exeriment has aroused interest not only from local and national U.K. media but also the international press.

IV. PUBLICATIONS ISSUED DURING THE PERIOD UNDER CONTRACT:

NONE

V. TRANSNATIONAL COOPERATION WITH OTHER BAP CONTRACTORS:

Exchange of material(s)	Yes
Exchange of staff	Yes
Joint experiment(s)	Yes
Joint meeting(s)	Yes

Descriptive information for the above data.

Exchange of materials

RSM 2004 was provided for the Dijon experiment, and inoculant containing RSM 2004, Cicer rhizobia, and seeds were taken to Bayreuth.

Exchange of staff

In May, John Spokes travelled to Bayreuth to assist with planting the field experiment.

Joint experiments

The same experimental design using peas inoculated with RSM 2004 is being utilized by Bayreuth, Dijon and Rothamsted.

Joint meetings

A preliminary meeting to discuss the experimental strategy was held at Rothamsted in October 1986 and attended by Noel Amarger, Walter Klingmüller Charly Döhler, John Spokes, John Day and Penny Hirsch.

European Communities — Commission

EUR 11138 — Biotechnology Action Programme — Progress report 1987
Volume 2: Scientific reports from participating laboratories

Edited by: *E. Magnien*

Luxembourg: Office for Official Publications of the European Communities

1987 — X, 1 167 pp., 105 fig., 26 tab. — 21.0 x 29.7 cm

Biological sciences series

EN

Volume 1 : ISBN 92-825-7671-X
Volume 2 : ISBN 92-825-7672-8
Volumes 1 + 2: ISBN 92-825-7676-6

Catalogue number: CD-NB-11138-EN-C

Price (excluding VAT) in Luxembourg
ECU 66.20 BFR 2 850 IRL 51.50 UKL 46.50 USD 75.50

The Biotechnology Action Programme (BAP) was adopted on 12 March 1985 for a period of five years and led to the conclusion, throughout the year 1986 and early in 1987, of a total of 261 research contracts. The purpose of this action is to allow the continuation of a previous 'Biomolecular Engineering Programme' (BEP) and its extension to new areas considered as essential for the development of biotechnology in the Community. Further support has been given to the sectors on second generation bioreactors, on genetic engineering — covering the whole range of methods applying to animal husbandry, veterinary medicine, plant improvement, crop protection, beneficial soil micro-organisms — and on risk assessment. New domains which BAP now also addresses include contextual measures for the pooling and improvement of infrastructures for R&D (storage and treatment of biological data, collection of biotic materials), as well as specific projects for protein design, the applications of genetic engineering to industrial micro-organisms, the development and upscaling of novel methods for *in vitro* cell cultures. A special effort is also being initiated in the areas of *in vitro* systems for the screening and assessment of pharmacological properties and toxicological effects of new molecules.

Due to a step-wise start of contractual activities, this first annual report brings together the individual contributions of little more than 200 laboratories out of 261, reviewing in all cases very preliminary research achievements still in the initial phase of execution. However, there is an achievement of a certain significance in the fact that activities of these many groups are being carried out in an integrated manner, in the framework of 93 multi-partners European Laboratories Without Walls (ELWW) with provisions for exchanges and division of tasks. This is how the Community hopes to be able to circumvent some of the academic and geographical barriers which penalize a field with a high multidisciplinary level.

In addition to this research programme, a parallel training programme is run with 60 grants a year covering all biotechnology-related fields. Periodical reports on the training programme are issued separately.

Venta y suscripciones · Salg og abonnement · Verkauf und Abonnement · Πωλήσεις και συνδρομές
Sales and subscriptions · Vente et abonnements · Vendita e abbonamenti
Verkoop en abonnementen · Venda e assinaturas

BELGIQUE / BELGIE

Moniteur belge / Belgisch Staatsblad
Rue de Louvain 40-42 / Leuvensestraat 40-42
1000 Bruxelles / 1000 Brussel
Tél 512 00 26
CCP / Postrekening 000-2005502-27

Sous-dépôts / Agentschappen

Librairie européenne / Europese Boekhandel
Rue de la Loi 244 / Wetstraat 244
1040 Bruxelles / 1040 Brussel

CREDOC
Rue de la Montagne 34 / Bergstraat 34
Bte 11 / Bus 11
1000 Bruxelles / 1000 Brussel

DANMARK

Schultz EF-publikationer
Montergade 19
1116 København K
Tlf (01) 14 11 95
Telecopier (01) 32 75 11

BR DEUTSCHLAND

Bundesanzeiger Verlag
Breite Straße
Postfach 10 80 06
5000 Köln 1
Tel (02 21) 20 29-0
Fernschreiber ANZEIGER BONN 8 882 595
Telecopierer 20 29 278

GREECE

G.C. Eleftheroudakis SA
International Bookstore
4 Nikis Street
105 63 Athens
Tel 322 22 55
Telex 219410 ELEF

Sub-agent for Northern Greece

Molho's Bookstore
The Business Bookshop
10 Tsimiski Street
Thessaloniki
Tel 275 271
Telex 412885 LIMO

ESPANA

Boletín Oficial del Estado
Trafalgar 27
28010 Madrid
Tel (91) 446 60 00

Mundi-Prensa Libros, S.A.
Castelló 37
28001 Madrid
Tel (91) 431 33 99 (Libros)
431 32 22 (Suscripciones)
435 36 37 (Dirección)
Télex 49370-MPLI-E

FRANCE

Journal officiel
Service des publications des Communautés européennes
26, rue Desaix
75727 Paris Cedex 15
Tél (1) 45 78 61 39

IRELAND

Government Publications Sales Office
Sun Alliance House
Molesworth Street
Dublin 2
Tel 71 03 09

or by post

Government Stationery Office
Publications Section
6th floor
Bishop Street
Dublin 8
Tel 78 16 66

ITALIA

Licosa Spa
Via Lamarmora, 45
Casella postale 552
50 121 Firenze
Tel 57 97 51
Telex 570466 LICOSA I
CCP 343 509

Subagenti

Libreria scientifica Lucio de Biasio - AEIOU
Via Meravigli, 16
20 123 Milano
Tel 80 76 79

Libreria Tassi
Via A. Farnese, 28
00 192 Roma
Tel 31 05 90

Libreria giuridica
Via 12 Ottobre, 172/R
16 121 Genova
Tel 59 56 93

GRAND-DUCHÉ DE LUXEMBOURG
et autres pays / and other countries

Office des publications officielles des Communautés européennes
2, rue Mercier
L-2985 Luxembourg
Tél 49 92 81
Télex PUBOF LU 1324 b
CCP 19190-81
CC bancaire BIL 8-109/6003/200

Abonnements / Subscriptions

Messageries Paul Kraus
11, rue Christophe Plantin
L-2339 Luxembourg
Tél 49 98 888
Télex 2515
CCP 49242-63

NEDERLAND

Staatsdrukkerij- en uitgeverijbedrijf
Christoffel Plantijnstraat
Postbus 20014
2500 EA 's-Gravenhage
Tel. (070) 78 98 80 (bestellingen)

PORTUGAL

Imprensa Nacional
Casa da Moeda, E. P.
Rua D. Francisco Manuel de Melo, 5
1092 Lisboa Codex
Tel. 69 34 14
Telex 15328 INCM

Distribuidora Livros Bertrand Lda.
Grupo Bertrand, SARL
Rua das Terras dos Vales, 4-A
Apart. 37
2700 Amadora CODEX
Tel 493 90 50 - 494 87 88
Telex 15798 BERDIS

UNITED KINGDOM

HM Stationery Office
HMSO Publications Centre
51 Nine Elms Lane
London SW8 5DR
Tel. (01) 211 56 56

Sub-agent:

Alan Armstrong & Associates Ltd
72 Park Road
London NW1 4SH
Tel (01) 723 39 02
Telex 297635 AAALTD G

UNITED STATES OF AMERICA

European Community Information Service
2100 M Street, NW
Suite 707
Washington, DC 20037
Tel (202) 862 9500

CANADA

Renouf Publishing Co., Ltd
61 Sparks Street
Ottawa
Ontario K1P 5R1
Tel Toll Free 1 (800) 267 4164
Ottawa Region (613) 238 8985-6
Telex 053-4936

JAPAN

Kinokuniya Company Ltd
17-7 Shinjuku 3-Chome
Shinjuku-ku
Tokyo 160-91
Tel (03) 354 0131

Journal Department
PO Box 55 Chitose
Tokyo 156
Tel (03) 439 0124

10 86

NOTICE TO THE READER

All scientific and technical reports published by the Commission of the European Communities are announced in the monthly periodical '**euro abstracts**'. For subscription (1 year: BFR 3 300), please write to the address below.

Price (excluding VAT) in Luxembourg
ECU 66.20 BFR 2 850 IRL 51.50 UKL 46.50 USD 75.50

OFFICE FOR OFFICIAL PUBLICATIONS
OF THE EUROPEAN COMMUNITIES
L-2985 Luxembourg

ISBN 92-825-7672-E

9 789282 576724