Commission of the European Communities
Directorate-General XII
Science, Research and Development

BRIDGE

*B*iotechnology *R*esearch for *I*nnovation, *D*evelopment and *G*rowth in *E*urope

Progress Report 1993

Edited by
A. Vassarotti and U. Kirchheim

EUR 15111 EN

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Directorate-General XII
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*B*iotechnology *R*esearch for *I*nnovation, *D*evelopment and *G*rowth in *E*urope (1990-1993)

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INTRODUCTION

A glance at recent history:

As a means to reinforce the scientific and technological bases of agriculture, industry, health care and environment protection, the Commission of the European Communities has been conducting several priority actions in biotechnology over this last decade. One of these actions aims at the establishement of a Community network for training and research. From 1982 to 1989 two successive programmes, the Biomolecular Engineering Programme (BEP; 1982-1986) and the Biotechnology Action Programme (BAP; 1985-1989) had been implemented. With a budget of 15 million ECU, BEP supported 91 training contracts and 103 cost-shared research contracts with public and private laboratories. Successor of BEP, BAP included 262 laboratories with a budget allocation of 55 million ECU. To further reinforce the Community R&D effort in Biotechnology and to enable Spanish and Portuguese laboratories to join the activities after the adhesion of the new Member States, the BAP programme was revised in 1988 and its budget brought up to 75 million ECU, bringing the overall number of laboratories to 378 (with 116 new contracts, covering the period 1989-1990).

BRIDGE:

The current programme BRIDGE (**B**iotechnology **R**esearch for **I**nnovation, **D**evelopment and **G**rowth in **E**urope), with a budget of 100 million ECU, covers research activities extending from 1991 to 1994 in the following sectors:

1) Information infrastructure for the processing and analysis of biotechnological data;

2) Enabling technologies (protein design, macromolecular modelling, biotransformation, genome sequencing);

3) Cellular biology (industrial microorganisms, plant and associated organisms, animal cells);

4) Pre-normative research including the *in vitro* evaluation of the activity of molecules, and safety assessments associated with the release of genetically modified organisms.

In the framework of the BRIDGE programme, as many as 579 laboratories are engaged in transnational collaborations, and till now, after 2-3 years of work (depending on the project), over 800 publications have been produced. The research activities are conducted via two different types of projects: N-projects (N for Network) and T-projects (T for Targeted).

The N-projects, implemented by European Laboratories Without Walls (ELWW's — an instrument for organising transnational research with the joint participation of several contracts), are carried out by groups of European laboratories which, through complementary approaches, are devoted to the removal of commonly experienced gaps in knowledge and know-how. On average, each N-project under BRIDGE involves 5-6 laboratories in 3-4 different countries. The progress reports in the following pages provide sound illustration of the "value-added" resulting from those interactive approaches.

The T-projects, larger and targeted towards the elimination of specific bottlenecks resulting from structural or scale constraints, have been initiated under BRIDGE. They bring together an average of almost 30 laboratories and 100 staffs, drawn from all European Community Member States. Seven T-projects are implemented in this programme:

— Sequencing of the yeast genome;
— Molecular identification of new plant genes;
— Biotechnology of lactic acid bacteria;
— Industrial lipases;
— Regulation of plant cell regeneration;
— High resolution automated microbial identification, and
— Animal cell technology.

For the T-projects, the reader will have an opportunity in the following pages not only to appreciate the progress achieved but also the kind of underlying specific organisational arrangements required to carry on such large and complex projects with an abundance of interacting partners. A monitoring unit with representatives of the contractors, of the programme committee "CAN-BRIDGE" and of the Commission is attached to each of the T-projects to help in the implementation and to facilitate communication both internally, within the project, and externally with, for instance, industrial platforms. These platforms are open independent structures, called upon the initiative of any interested European industry willing to approach the research carried out in a given T-project. They communicate freely with the contractors and, in certain cases, discuss specific results with a view to identifying laboratories suitable to carry on further work (on the basis of direct and independent arrangements) on specific aspects with a potential for industrial applications.

Two novelties introduced in BRIDGE and now shared by all specific programmes under the IIIrd Framework Programme of Community R&TD activities need to be underlined:

— Closest ties with EFTA countries — Although not funded by the EC, 17 laboratories from 5 EFTA countries participate in the different projects on their own financial resources.

— Internal coordination — Project coordinators are now responsible for integrating the work and stimulating the interface between laboratories participating in the same project. Their role, already instrumental at the level of the preparation and submission of proposals to the Commission, also covers several other administrative and scientific duties once a proposal is selected and thereafter implemented.

Finally, this report describes the full complement of the research projects in the BRIDGE programme. The individual progress reports are presented in 7 sections: objectives, results, highlights/milestones, cooperative activities, European dimension, list of joint publications, and other publications. In addition, each report contains a list of names and brief addresses of each of the participants. Indexes of participants and keywords as well as contractors' names and addresses are given at the end of the volume.

ACKNOWLEDGEMENTS

The annual reports in the following pages have been prepared under the responsibility of the different project coordinators who have centralised and integrated the information provided by the partners in the project. In this respect, the Commission Services wish to thank all the participants and, in particular, the project coordinators, for their valuable contribution and the demonstration they have made of an active and lively community of scientists across Europe.

The research action of the BRIDGE programme, as well as the other activities such as training and concertation, have been implemented by the Commission with the help of the advisory committee CAN-BRIDGE (see table, page XIII) composed on national delegations from all Member States. The delegates have made their broad range of competences available to the Commission Services and taken an active part in all discussions which have accompanied the implementation steps of the programme. Their positive influence is hereby acknowledged.

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Director of Life Sciences
and Technologies
Directorate-General for Science,
Research and Development

E. Magnien,
Head of Biotechnology Unit
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Further reading:

On ELWW:

— E. Magnien, A. Aguilar, P. Wragg and D. de Nettancourt. (1989) Biofutur, November, 17-30

On BAP:

— Biotechnology R&D in the EC. Cataloque of BAP achievements — (1990). A. Vassarotti and E. Magnien eds., Editions Scientifiques Elsevier, Paris, 242 pp.
— Biotechnology R&D in the EC. Cataloque of BAP achievements on Risk Assessment for the period 1985 — 1990. (1991) I. Economidis ed., Printéclair, Brussels, 92 pp.
— Biotechnology R&D in the EC. Cataloque of BAP achievements on Protein Design/Bioinformatics for the period 1989 — 1990. (1992) B. Nieuwenhuis ed., Office for Official Publications of the EC, Luxembourg, 96 pp.

On BRIDGE:

— **B**iotechnology **R**esearch for **I**nnovation, **D**evelopment and **G**rowth in **E**urope (1990 — 1993). Cataloque of contracts with project descriptions. (1992) B. Nieuwenhuis ed., Office for Official Publications of the EC, Luxembourg — II, 346 pp.
— The T-projects of BRIDGE, a new tool for technology transfer in the Community. (1991). D. de Nettancourt, Agro-Industry Hi-tech, April, 3-9.
— **B**iology **R**esearch for **I**nnovation, **D**evelopment and **G**rowth in **E**urope (1990-1993). Progress Report 1992. (1992). A. Vassarotti ed., Office for Official Publications of the EC, Luxembourg — VIII, 528 pp.

— E. Magnien, and D. de Nettancourt (1993). What drives European biotechnological research?, in E.J. Blakely and K.W. Willoughby eds., Biotechnnology Revisew No. 1: The Management and Economic Potential of Biotechnology, pp. 47-58.

Many additional publications on training, sectoral activities, specific projects and other Biotechnology related issues have been produced by the Unit Biotechnology of DG XII. To obtain an updated listing please write to:

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BRIDGE

N-PROJECTS

AREA A:

INFORMATION INFRASTRUCTURE

- **PROCESSING AND ANALYSES OF BIO(TECHNO)-LOGICAL DATA**
  (from page 5 to page 39)

— **CULTURE COLLECTIONS**
  (from page 40 to page 48)

Continuation of the expanded services of the EMBL Data Library (BIOT CT-910254)

COORDINATOR:
G. CAMERON, the EMBL Data Library, Heidelberg, D
PARTICIPANT:
The EMBL Data Library

OBJECTIVES

During the reporting period we planned four releases of the databases on magnetic tape and CD-ROM as well as the continuous updating of the copies at EMBnet nodes and accessible via our Fileserver.
Also anticipated was a meeting of the European Advisory Committee.

RESULTS

The data

The nucleotide sequence database grew 55% during 1992, the number of sequences increasing from 57,765 to 89,100 and the nucleotides increasing from 75,400,487 to 111,413,979. This growth is summarised in figure 1.

Data management

A significant upgrade to our RDBMS server machine plus workstation purchases have improved our data throughout rates during 1992.
Much software development has been concentrated on our submission processing procedures, allowing us to achieve 100% automation of processing of submissions created with the Authorin software, and also to improve our procedures for matching published and submitted sequence.

Data Distribution

CD-ROM and magnetic tape are still used for distribution of quarterly releases of the databases, while daily updates of the EMBnet nodes and other network access methods make the data between releases available.

Although the electronic mail server continues to be very popular, we have added a number of new services in 1992. An anonymous FTP server supplements the e-mail server, giving access to complete EMBL databases and software repository including the releases and weekly updates. Despite the short period of its existence the FTP server has gained immense popularity with more than 100 downloads every day. The weekly updates to the EMBL database are very popular allowing remote users to keep their local database copy up-to-date.

Additionally, we now offer FTP access via the Gopher protocol. Gopher clients simplify the use of computer networks by hiding their complexity behind a simple graphical user interface. EMBL's Gopher is used by people all around the world — a huge proportion of requests come from outside Europe. Being part of the EMBnet Biogopher network EMBL's, Gopher provides links to other information resources within Europe and elsewhere.

The EMBL servers continued to play their role as important archives for molecular biological software, seeing a constant flow of molecular biological programs submitted to the Data Library from many authors.

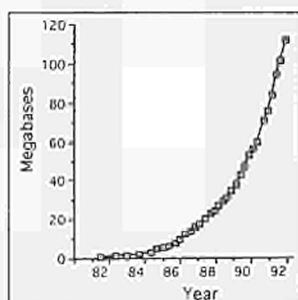

Figure 1. Nucleotide Sequence Database growth

Data from genome projects

1992 has seen a dramatic increase in data coming from genome projects. In collaboration with several European genome projects the Data Library has implemented procedures to allow these researchers to load data directly into the relational database. The stream of this kind of submission is a major factor in the average growth rate of the database of more than 10% per release. Table 1 summarises the data received from various projects.

	Entries	*bp*
Genexpress Genethon	3266	998074
C. elegans nematode project	640	408511
S. cerevisiae yeast project	4	327317
French Arabidopsis cDNA project GDR	903	271501
Genexpress Munich	239	64870

Table 1. Data from Genome projects

It is interesting to note that, although the nucleotide sequence database contains data from more than five thousand species, as can be seen from figure 2, almost half the data come from the ten most commonly sequenced species. It will be interesting to see how this balance changes as the genome projects deliver increasing quantities of data.

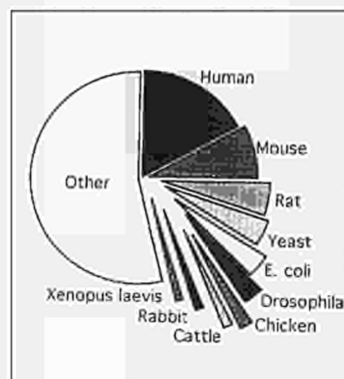

Figure 2. Proportion of Nucleotide Sequence Database from various species

Sequence data in patents

In 1992 a contract was finalised with the European Patent Office for the incorporation of sequence data from patents into the database. The software development is complete, and we are now entering the data.

International Collaborations

The cooperation between EMBL and the DDBJ and GenBank groups in Japan and the United States continues. Our 1992 collaborative meeting was held in Mishima, Japan, and was devoted to discussions of new data exchange systems being implemented following the transfer of GenBank to the National Centre for Biotechnology Information (NCBI) in the USA.

We now have access to a comprehensive database of published sequences as a result of our collaboration with NCBI, and work very closely with DDBJ to ensure that sequences submitted by Japanese researchers are being matched against their published versions as quickly as possible.

The collaboration with MIPS, the European partner in the PIR international protein sequence database continued. EMBL forwards protein coding nucleotide sequences to MIPS and the protein and nucleotide sequence databases pursue a co-ordinated data submission policy.

Research and Development

EMBL-Search
A new database retrieval system has been developed to allow rapid access to the databases on the EMBL CD-ROM. It is based on index files that allow quick lookups of entry names, accession numbers, keywords, species, author names and free text searches. These index files are platform-independent and allow any software developer to build database retrieval tools. As an example, we have developed a Macintosh application called EMBL-Search that allows queries of the EMBL, SWISS-PROT and PROSITE databases on our CD-ROM. It complements our CD-SEQ software available for MS-DOS computer systems. An interesting aspect of EMBL-Search is its utilisation of cross-references between databases, allowing users to navigate easily between databases to explore related information.

MacPattern
The popular Apple Macintosh program MacPattern for the functional analysis of newly determined protein sequences has been improved considerably to support block searches with scoring matrices derived from S. Henikoff's BLOCKS database as well as the identification of statistically significant sequence segments according to the maximal segment score method of S. Altschul and S. Karlin and, of course, the original Prosite patterns.

MacT
In a collaboration with A. Lüttke (Cologne), R. Fuchs has developed Apple Macintosh programs for the construction and evaluation of phylogenetic trees which integrate several algorithms.

Utilisation of parallel architecture
EMBL has recently acquired a massively parallel Maspar computer. In a collaboration with John Collins in Edinburgh we now provide a service allowing very fast database similarity searches using the sensitive Smith and Waterman algorithm on this machine.

CLUSTAL V
The CLUSTAL V multiple alignment program continues to be improved. The main changes are to allow for more flexible input and output formats and to improve the scoring system for the alignments. It has been distributed via e-mail and ftp servers to thousands of users worldwide.

Novel database approaches
The Data Library's exploration of object-oriented methodologies has made significant progress in the past year with the acquisition of the database management system Object Store. Utilising this system work has begun on a prototype databases for molecular biology.

HIGHLIGHTS/MILESTONES

The most exciting developments in 1992 were enhancements to the CD-ROM format and the production of software to exploit them.
Also striking was the improved automation of inclusion of data from genome sequencing projects. Such data are already beginning to heavily affect the emphasis of the nucleotide sequence database.

COOPERATIVE ACTIVITIES

International advisory committee
The European members of the International Advisory Committee for Nucleotide Sequence Databases met in October and made a number of recommendations aimed at ensuring a smooth transition to a collaboration with NCBI and encouraging good links to literature databases.

EUROPEAN DIMENSION

The European Bioinformatics Institute
Throughout the year EMBL and the EC have been exploring ways to:
- ensure that the highly-valued services of the EMBL Data Library can continue and develop into the future.
- reinforce areas which, under the present level of support, have been neglected, particularly training and user support.
- make the voice of European bioinformatics heard in the global arena and by other European organisations.
- increase the effectiveness of dispersed, high-quality, European bioinformatics research and service by extensive collaboration.

The culmination of these deliberations was the decision, by the EMBL Council to found a new Outstation of EMBL — the European Bioinformatics Institute (EBI) — at Hinxton near Cambridge in the UK. The new Outstation will incorporate and extend the mandate of the Data Library.

LIST OF JOINT PUBLICATIONS WITH TRANS-NATIONAL AUTHORSHIP

Bairoch, A. and Data Library Staff (1991) SWISS-PROT Release Notes and User Manual, Releases 22, 23, 24.
Data Library Staff (1992) EMBL Data Library Release Notes and User Manual, Releases 31, 32, 33, 34.
Fuchs, R., Rice, P. and Cameron, G.N. (1992) Molecular biological databases — present and future. *Trends in Biotechnology* **10**, 61-66.
Geber, A., Higgins, D.G., Waters, A.P., Bennett, J.E. and McCutchan, T.F. (1992) Small subunit ribosomal RNA of *Blastomyces dermatitidis:* DNA sequence and phylogenetic analysis. *J. Gen. Mic.* **138:**395-399.
Higgins, D.G. and Stoehr, P. (1992) Fast approximate DNA sequence database searches on compact disc. *CABIOS* **8**(**2**):137-139.
Higgins, D.G., Bleasby, A.J. and Fuchs, R. (1992) CLUSTAL V: improved software for multiple sequence alignment. *CABIOS* **8**, 189-191.
Higgins, D.G., Fuchs, R., Stoehr, P. and Cameron, G.N. (1992), *The EMBL Data Library. Nucl. Acids Res.* **20** (Supplement), 2071-2074.
Shields, D.C., Higgins, D.G. and Sharp, P.M. (1992) GCWIND: a microcomputer program for identifying open reading frames according to codon positional G+C content. *CABIOS* **8**(**5**):521-523.

OTHER PUBLICATIONS

Higgins, D.G. (1992) Sequence ordinations: a multivariate analysis approach to analysing large sequence data sets. *CABIOS* **8(1)**, 15-22.
Luettke, A. and Fuchs, R. (1992) MacT: Apple Macintosh programs for constructing phylogenetic trees. *CABIOS* **8**, 591-594.
Sibbald, P.R. (1992) Approximate string searching algorithm: technical note. *Comm. ACM* **35**(**4**):132-137

CarbBank — a complex carbohydrate structured database (BIOT CT-900184)

COORDINATOR:
K. BOCK, Carlsberg Lab., Copenhagen, DK
PARTICIPANTS:
H. PAULSEN, Univ. Hamburg, Hamburg, D
J.F.G. VLIEGENTHART, Univ. Utrecht, Utrecht, NL

OBJECTIVES

The objective of the research program is to establish a *Complex Carbohydrate Structural Database* (CCSD) containing published oligosaccharide structures larger than disaccharides and the accompanying database management program CarbBank. The main goal of the reporting period (01.02.92-31.01.93) was to increase the number of oligosaccharide structures included in the database to bring the CCSD more up to date. Additionally, the development of tools to link the structural database to spectroscopic information like ^{1}H- and ^{13}C-NMR data should be continued.

MAJOR PROBLEMS ENCOUNTERED

Due to the growth of the database, the distribution medium of *CarbBank* and the CCSD was changed from floppy disks to compact disk (CD-ROM) released by the US National Center of Biotechnology Information (NCBI). Unfortunately, the release of CCSD6 was delayed by several months. Data supplied by Chemical Abstract Service (CAS) during 1992 included only oligosaccharide structures previously not described. Structures which were cited in the literature before were not included in the dataset we obtained. For a complete coverage of the literature another procedure has to be established to access the missing oligosaccharide structures, probably by scanning the relevant journals manually.

RESULTS

1. The Complex Carbohydrate Structural Database

Release number 5 of the CCSD containing 8,000 records was distributed in April 1992 to over 500 customers at 350 sites worldwide. In February 1993, the latest version of the *CarbBank* program and the structural database CCSD was released with 22,333 records of which 10,893 were unique structures. So far, 14,476 records have been examined by curators, 7,857 entries are still unverified. Most of the published literature on carbohydrates up to 1990 has been included. Thus, the backlog problem has almost been solved. Therefore, future releases of the database will be more up to date, i.e. only about six months to one year behind the current literature.

Starting with release 6 of the CCSD the distribution has been changed from floppy disks to compact disk. Among other databases, the CCSD is included in the Data Repository CD-ROM distributed by the US National Center of Biotechnology Information (NCBI). Furthermore, the database is also accessible over the Internet by anonymous FTP from a public NCBI server.

The main focus of the efforts of the last year was to continue building the CCSD by adding new records. Therefore, a large part of the 20,000 records supplied by

Chemical Abstracts Service (CAS) were reviewed and corrected, where necessary. The data subjected to the validation process were divided under the 3 participating groups and verified against the original literature. Detected errors like misspellings in the author and title fields and structural errors were corrected. Structural errors consisted of incorrect specification of the reducing end as free aldose or alditols or pyridylamino derivatives, wrong anomeric configuration, altered linkages between sugar residues, additional or missing glycosyl residues. Keywords and additional structures found in the articles were added to the database. Some structures which were not sufficiently characterized were not incorporated into CCSD. A detailed error report was sent to the Complex Carbohydrate Research Center (CCRC) and CAS. In total 4,847 records could be verified, whereas the literature of another 3,765 records was not readily available to the curators.

The group in Copenhagen worked on unverified records from the publication years 1983-1986. Of a total of 2,450 records 1,481 records were accessible and were verified against the original literature. 670 entries were considered to be correct. In 811 cases either errors were detected or additional structures were found in the articles.

The group in Hamburg was working on unverified database records of the publication years 1987-1991. From a total of 4,297 records the literature of 1988 entries have been verified so far. In 537 records errors were detected and 1,361 additional structures were found. Furthermore, NMR data from complex carbohydrate structures synthesized in the laboratories of Prof. H. Paulsen and Prof. J. Thiem, University of Hamburg were collected to be included in *SUGABASE*, the NMR database program developed and managed from the Utrecht group.

In Utrecht unverified database records of the year 1982 and older were compared to the original literature. From 2,865 records, the journals of 1,487 records were not present at the Utrecht library and could not be verified. Thus, 1,378 records could be verified against the original articles. 680 records were passed unmodified. 276 records were modified and 422 records were marked for removal from the database. In the majority of these records the structure is not clearly (or not at all) defined. Sometimes only the carbohydrate composition is given (e.g. Man7GlcNAc), or a hydrolysate of a polysaccharide is used. This results in ambiguous structures.

2. CarbBank program

The US group responsible for maintenance of the CCSD has added new features to the database management program CarbBank. The database files can handle now up to 26,000 records. Additional non-carbohydrate substances, like primary alcohols, carboxylic acids and hydroxy-carboxylic acids are supported when linked to glycosyl residues. The new version of CarbBank uses Expanded Memory (EMS). Therefore, less DOS RAM is required and the program will run now under most network operating environments.

3. The NMR database SUGABASE

At the laboratory in Utrecht (NL) a computer program *SUGABASE* was developed to add ^{1}H- and ^{13}C-NMR data to the structural information included in *CarbBank*. During the reporting period new ^{1}H-NMR and ^{13}C-NMR tables have been added to the database, resulting in a total of 734 ^{1}H-NMR and 258 ^{13}C-NMR records. Furthermore, the program, which was running only on IBM-PC computer, has been successfully ported to Silicon Graphics workstations. The

database of NMR tables of carbohydrate structures has been extended, and the corresponding management program has been improved. The database can be searched for carbohydrate structures, by entering a list of chemical-shift values. The resulting carbohydrate structures and NMR tables are displayed concurrently, whereby the matching monosaccharide residues in the carbohydrate structures, and the matching chemical-shift values in the NMR tables are highlighted. During the reporting period the work on *SUGABASE* has been focused on two items: improvement of its modular structure and implementation of a new graphical user interface.
Internally, the program is divided into separate modules. This modular structure has been enhanced to make porting of the program to other operating systems and other computer platforms relatively easy. The replacement of the user-interface module made it possible to port *SUGABASE* to the X-Window environment of Silicon Graphics workstations. The database module has been modified to allow the use of the same binary database files on different computer platforms. This allows for one copy of the database files in a network environment. Some problems still exist due to the use of typical MS-DOS characters by CarbBank (and also by *SUGABASE*) that are not available on other platforms.

HIGHLIGHTS

The efforts to build the CCSD have been very successful. With 22,333 records, the size of the database is much higher than anticipated at this point of time. The database is well accepted by more than 500 customers worldwide.

WIDER CONSIDERATIONS

The work performed on data obtained from Chemical Abstract Service has demonstrated the importance of controlling the quality of entries derived from existing databases. Many errors were detected during the verification process.

COOPERATIVE ACTIVITIES

Meetings:

July 1992:
Copenhagen (DK) — CarbBank meeting
K. Bock, R. Stuike-Prill, A. van Kuik, A. Kleen, S. Doubet (CCRC), B. Bossenbroek (CAS).
July 1992: Paris (F) — Meeting of the Board of Overseers in connection to the XVIth International Carbohydrate Symposium
K. Bock, J.F.G. Vliegenthart, H. Paulsen, P. Albersheim, S. Doubet, T. Ogawa, B. Bossenbroek.
November 1992: Hamburg (D)
K. Bock, H. Paulsen, A. Kleen
November 1992: Utrecht (NL)
K. Bock, J.F.G. Vliegenthart, A. van Kuik

The communication between the participating laboratories and the collaborating group in the USA is done by electronic mail, fax and phone without any major problems.

PUBLICATIONS:

1) *CarbBank* Version 2.3 with the database CCSD6.
2) J.A. van Kuik and J.F.G. Vliegenthart (1992), *Databases of complex carbohydrates*, Trends Biotechnol., **10,** 182-184.
3) J.A. van Kuik and J.F.G. Vliegenthart (1992), A *^{1}H-NMR database computer program for the analysis of the primary structure of complex carbohydrates*, Carbohydr. Res. **235**, 53-68.
4) R. Stuike-Prill, K. Bock, A. Kleen, H. Paulsen, J.A. van Kuik, J.F.G. Vliegenthart, S. Doubet, D. Smith and P. Albersheim (1992), *CarbBank and the Complex Carbohydrate Structure Database*, Bioinformatics **1**, 12-15.

Protein Sequence Databank (BIOT CT-900170)

COORDINATOR:
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OBJECTIVES

- Decrease of the backlog of the Protein Sequence Database.
- Development of efficient annotation procedures.
- Development of software for sequence comparisons and sequence data distribution.

MAJOR PROBLEMS ENCOUNTERED:

Poor performance of the European Academic Networks.

RESULTS:

The Martinsried Institute for Protein Sequences (MIPS) at the Max Planck Institute for Biochemistry has developed resources to support the data collection and distribution as part of the tripartite collaboration of the Protein Sequence Databanks (PIR-International). The work funded by the BRIDGE project concentrates on the development of the Protein Sequence Database, particularly on its standard of annotation and the data distribution on networks.
Sequences in the Protein Sequence Database are derived from publications in books or scientific journals, or from submission of the sequence to a sequence database.

(i) Database Progress

In the year 1992 the PIR-International Protein Sequence Database increased by 30% to 47,234 sequences in release 35.0, Dec. 1992. These figures testify the success of the project[1]. The major effort focused on the development of annotation strategies, the key problem of sequence data processing. An elaborate format (CO_2), designed to facilitate consistent full annotation[2], is advanced and the initial steps for an objectoriented database system have been taken. The new format that will allow the symmetric data exchange between PIR and MIPS and the implementation of a truly distributed database.

Services provided have been extended. A BLAST server was implemented in addition to the existing file services, and the availability of uptodate protein sequence data was largely improved by loading intermediate releases of the PIR-International database. The database is now distributed on CDROM for VMS, ULTRIX and MSDOS systems, including the ATLAS software for concurrent access to multiple databases.

(ii) A database of sequence similarities

The project of an exhaustive database of sequence similarities (FASTA database) was completed[4]. The continuous update of the FASTA database allows immediate access to similar sequences of any sequence in the dataset. The FASTA database proved to be extremely useful both for the scientific evaluation of the data and for the daily use by the annotation staff.

Every protein sequence added to the dataset is compared to, all other sequences available. The result of the comparison is the key information for the classification of the protein and its annotation. Although it is easy to run a sequence comparison against the current data collection, its result will soon be outdated by more recent sequence data. In the FASTA database system, the results of all possible sequence comparisons are saved in compressed form and the dataset is updated with any incremental change of the data collection (addition or modification of sequences). The resulting database is a useful resource for the work of the annotation staff as well as for any user interested in the family relationship among individual proteins. The database facilitates cluster investigations as well as detailed statistical analysis of the sequence database growth.

The FASTA searches carried out in order to load the database were time consuming: it took a total time of 2100 hours to complete the comparisons for the 36.150 entries of the PIR-International dataset (Release 31.0) on a DECstation 5125. Recently, the computing time was reduced by factor 2.5 by porting the FASTA program to ALPHA/VMS.

The database was weekly updated and contains now (April 1993) 77.880 entries. It also contains FASTA results for sequences before their inclusion into the PIR-International dataset.

(iii) Progress in the annotation of protein sequences

Standardization of data, tools for data processing and data flow control are necessary in order to create a correct, complete and comprehensive protein sequence database.
A comprehensive protein sequence database must provide
— reliable sequence data
— complete coverage of published protein sequences
— high quality of annotation: the biological information related to a protein sequence should be complete and consistent
— organization of data: data should be represented in a well defined form that is suitable for database operations

Defined lists of terms were compiled and the database entries were compared with these lists for the following items:

— ***species names***
The taxonomy list, is applied to the source field of the database entries.

— ***enzyme names***
The nomenclature of the Enzyme Commission is applied. The correspondence between EC number and 'recommended protein name' is checked regularly for all entries in the database.

— ***non enzyme protein names***
In contrast to the enzyme names, no nomenclature for nonenzyme proteins exists. To achieve standardization for nonenzyme protein names, lists of approved, undecided and inadmissible names have been compiled.

— ***keywords***
a list of selected keywords has been developed. Major releases of the database contain only valid keywords. Keywords yet missing in entries are continuously being added. Since keyword lists do not describe the application of individual keywords, a set of rules has to be developed to describe the relation between the set of defined keywords and the properties of proteins.

— *superfamily names*
  The list of superfamily names has been generated and all entries are checked for the occurrence of new or invalid superfamily names. Major releases contain valid superfamily names.

— *features*
  The representation of features was very inconsistent until 1992. Most of the features have been standardized since. Completion of the feature information for all entries in the database is a task that only progresses slowly due to the enormous number of entries that have to be revised. A concept is under development to annotate features in the entire protein families using multiple alignments.

(iv) Progress in protein classification

One of the handicaps in the usage of the present PIR data bases is the lack of a complete sequence classification: only 12% of the sequences contained in the Protein Sequence Databank have already been classified. The remaining sequences are gathered in the two databases PIR2 (partially annotated) and PIR3 (preliminary entries).
In order to achieve this goal, the development of two sets of procedures was necessary:
— Automatic migration of the alignment and relationship data base to a new release of the PIR-International sequence data base
— Automatic classification of sequences according to the classification rules

In close cooperation with Dr. Pardowitz (MPI f. Exp. Medizin, Göttingen), a set of programs is applied to create Family Alignment Data Base (FAMALNDB) which now consist of 3 major phases:
— Migration to new release
— Scanning the sequence data base for new homologies
— Multialigning new or modified families

By the insertion of new members into existing protein families, complete annotation is largely facilitated.

(v) Data distribution on wide area networks

In 1992, MIPS started to contribute to the activities of the EMBnet. As a special node, MIPS will forward the most recent protein sequence data to the network of European national nodes. Active data distribution on wide area networks is not a trivial task and is inhibited by the current deficiencies of the European data networks[3]. A long distance cooperating network for data distribution requires several properties that are not provided by commercial systems.

A prototype of a propagating network to update remote nodes was implemented with the Biozentrum Basel. This work will be extended to an advanced data model for the exchange of data between the nodes of PIR-International. We intend to redesign the application interface to perform efficient database updates.

As shown in figure 1, data distribution can be described by a directed graph, where the nodes represent data bases and the arrows represent updating operations between them.

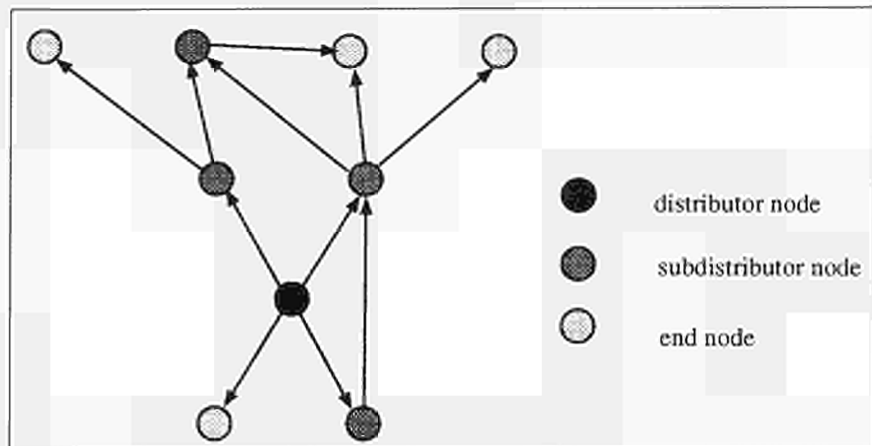

Figure 1. A sample data distribution graph

Data bases at all nodes must be consistent within a given time interval. This global property of the graph is realized by a distributed transaction protocol, in which the transaction cycle is triggered by the main distributor and is propagated to the end nodes by subdistributing nodes. The following aspects must be considered at an early stage of software design:

— The communication partners form a heterogeneous system. They use different hardware platforms, different operating systems, different network software, different application programs, etc...
— The network links between the nodes are abstract endtoend connections.

A neat separation between the network, the data distribution and the application software is mandatory (Fig. 2) to allow the data distribution layer to be used as a general purpose tool.

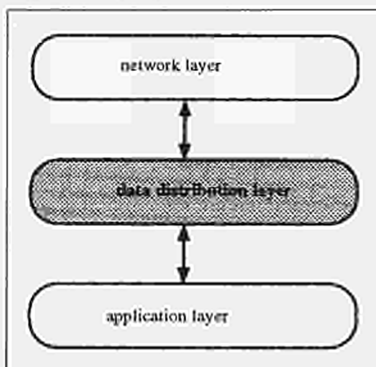

Figure 2. Layered software architecture for data distribution in heterogeneous systems

HIGHLIGHTS/MILESTONES

The unique goals of the Protein Sequence Database are

(1) to dynamically maintain a complete and comprehensive set of published protein sequences and related information in accordance with current biological understanding
(2) to organize these data by similarity and evolutionary relationship
(3) to add biochemical information that is displayed in a consistent and comprehensive form

The development of the Protein Sequence Database now copes with the increasing data flow from the literature and the submissions to the nucleic acid sequence

databanks. Currently more than 50.000 protein sequences are represented in the database, approximately 50% of the worldwide data input is provided by MIPS.

WIDER CONSIDERATIONS

Protein sequence data are of major importance for the basic research in biomedical sciences as well for the biotechnological infrastructure. The collection and distribution of protein sequences in Europe has been subject of our work. MIPS is the European contribution to the international network or protein sequence databanks. The growing data streams from large scale genomic sequencing projects will furthermore increase the workload to perform a comprehensive annotation of the information related to the sequences. Structuring the database into protein families and development of new concepts for the organization of biological data will improve the efficiency of the annotation process and largely ameliorate the usefulness of the database in the future.

COOPERATIVE ACTIVITIES

A number of cooperative efforts have been undertaken with European labs, partially in conjunction with the BRIDGE Yeast Sequencing Project. MIPS serves European laboratories by providing most up-to-date protein sequence data collections, distribution of sequence data, training in sequence data analysis, and user support. As part of the collaboration with the EMBL Data Library, the latest nucleic acid sequence data collected are forwarded to MIPS.

PUBLICATIONS

Barker W.C., George D.G., Mewes H.W. and Tsugita A.; 'The PIR-International Protein Sequence Database', *Nucleic Acids Res. (1992),* **20**: 2023-2026

George D.G., Orcutt B.C., Mewes H.W., and Tsugita A.; 'An ObjectOriented Sequence Database Definition Language (SDDL)', Protein Sequences and Data Analysis (1993, accepted)

Heumann, K.; 'Spezifikation und Implementierung einer Datenverteilinfrastruktur für Proteinsequenzdatenbanken', Diploma Thesis, Univ. of Munich (1992)

Liebl, S., Heumann K., Mewes H.W.; 'A dynamic database for sequence similarities'; Manuscript in preparation.

Promotion of EMBnet: Computer network for bioinformatics in Europe (BIOT CT-910273)

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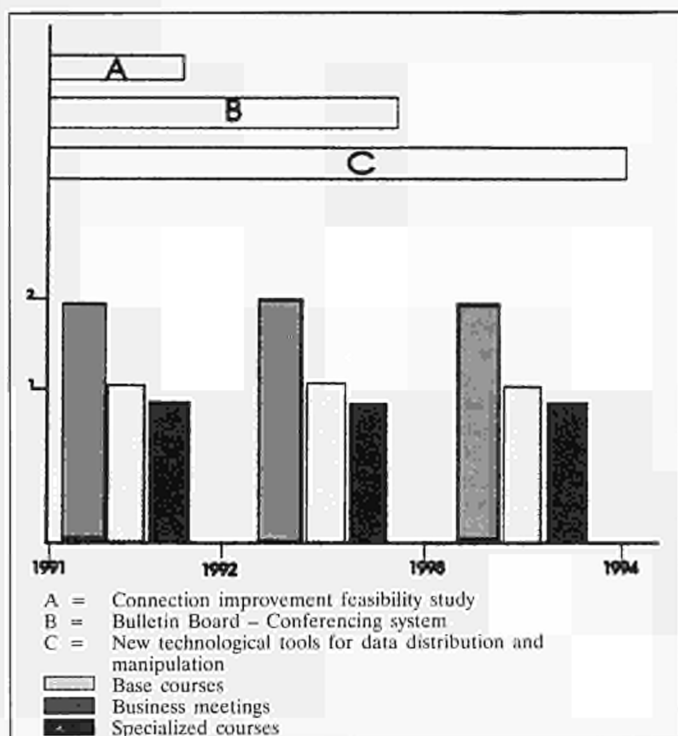
OBJECTIVES

The major objective of the present project is essentially the promotion of EMBnet as an European computer network for bioinformatics. The main topics for the development of the network are:
a) setting up a bulletin board
b) study the technological tools for data distribution
c) planning of specialized courses and workshops

With respect to the above topics, in the second year a greater emphasis has been put on the technical tools, and the exchange of knowhow, in order to streamline harmonization between nodes. The scheme which follows, illustrates the development of activites in the various years of the project and was annexed to the original project workplan.

Progress has been good.
A Steering Committee (SC) was nominated and established during the EMBnet business meeting held in Nijmegen in July 1992.The SC is composed of 3 members and its role is to promote new projects to be funded, and to stimulate inter-node cooperation. The tasks of the Steering Committee were confirmed at the sixth business meeting, held in Lisbon last January and a fourth member was appointed beside those already nominated at the previous meeting. Members of the Steering Committee are at present Jan Noordik (CAOS/CAMM, Netherlands), Alan Bleasby (Daresbury Laboratory, UK), Chris Sander (EMBL, Germany) and Rob Harper (CSC, Finland).

The EMBnet project has recently been joined by two more European countries, Portugal, the Gulbenkian Instituto de Ciencia (Oeiras), and Belgium, the ULB-VUB Brussels Free Universities Computing Center (Brussels).



RESULTS

EMBnet resource project assistant

Dr. Albertus M. Kroon was hired as project assistant from February/March 1992 to January 1993. He has worked for the promotion of EMBnet with success, and as has produced two main documents. The first is a Strengths and Weaknesses Analysis of the network as he found it when he accepted the position, and the second, a 'proposal' for the further development of EMBnet.

His major point of emphasis was the need for more structure in the group, which would allow faster decision-making at all levels. His suggestion was taken with the consequent appointment of a Steering Committee and a Funding Committee.

He also helped re-define the tasks for the project assistant. Indeed, through his action, it became clear that EMBnet did not simply need an assistant, but a manager, to help the project work into the latest developments in bioinformatics.

The tasks of the BRIDGE project manager were therefore defined as:

1. *Support for the scientific coordinator:*
   — drafting the yearly report for BRIDGE.
   — lend support to the activities of the funding committee.
   — control and help for running projects.

2. *Support for the Steering Committee* in implementing the decisions taken by the EMBnet business meeting. Preparing the EMBnet business meeting.
3. *Extending and reinforcing the network.* This will imply visits to the nodes, research of sponsorships and any kind of activity aimed at the enlargement of the group.
4. *Reinforcing the strategic importance of EMBnet* and preparing for further and future funding.
5. *Drafting reports* from documents to be submitted by individual nodes or project managers.
6. *Supporting the EMBnet working groups* in reaching their aims.

Dr. Kroon also suggested that It would be desirable the manager spends at least 1 to 2 days per week on this project for the remaining period of the project (April 1993 - March 1994).

In the light of this workplan, Dr. Kroon proposed the hiring of Dr. Jack Franklin for the remaining period of the contract. The proposal was agreed to by the Scientific Coordinator and the whole community, and Jack Franklin has been hired as the new manager of EMBnet/BRIDGE for the period from February 1993 to March 1994, with a committment of two days a week.

Training programm for EMBnet/Bridge

A Training Working Group (TWG) was appointed during the business meeting in Nijmegen last June, to promote EMBnet training and to help in the organization of inter-node courses. Members of this group are Rodrigo Lopez (Norway), Jack Leunissen (The Netherlands) and Martin Bishop (UK).

The following courses, and participation to courses, were organized and financed through the BRIDGE grant:
- Travel grant for Network Services Conference in Pisa,
- 2nd EMBL Practical course on exploring genome information;
- Short collaborative node visits, visiting scientist program at CAOS/CAMM (The Netherlands)
- Course on molecular analysis held at Istituto di Tecnologie Biomediche Avanzate in Milan by the HGMP (UK),
- Biocomputing course organized by the TWG in Vienna,
- Biocomputing course organized by the TWG in Madrid
- Itinerant course on ACNUC organized by the CSMME (Italy),
- User training course organized in Trieste with the ICGEB (Italy)
- Collaborative node visit between the Swiss and Greek EMBnet nodes.
- A place was made available to an EMBnet user with travel and accomodation funded on the computing courses of the UK Human Genome Mapping Project.

EMBnet business meetings

As detailed in the original project description, two business meetings were held one in Nijmegen (The Netherlands), in June 1992 and another in Lisbon (Portugal) in January 1993. A point was made that business meetings are held twice a year, if necessary.
The 5th EMBnet workshop was held in Berg-en-Dal, Nijmegen, June 19-20th 1992. It was organized by the CAOS/CAMM centre, the Dutch regional EMBnet node. Sequence data distribution was reviewed, and alternative mechanisms for updating

and data handling were also proposed. Node services and service charging policies were evaluated.

At the meeting, the project assistant presented a paper which stimulated a long discussion about the current and future directions of EMBnet. Finally, it was decided that an EMBnet Steering Committee would cooperate with the project assistant to set out lines to reach the newly identified objectives. and the goals set earlier at the Uppsala and Crete meetings. In this workshop, time was allocated for the first time for a Poster session on 'node achievements' and/or 'organization'.

The 6th EMBnet business meeting was held in Lisbon, Portugal in January 29-31 1993. It was jointly organized by the Steering Committee of EMBnet and the Instituto Gulbenkian de Sciencia of Oeiras, Portugal. The choice of Portugal as guest country for the meeting actually helped the appointment of the Instituto Gulbenkian de Sciencia as Portugese National node.

The Berg-en-Dal meeting had marked the raising of a more political and 'managerial' awareness among EMBnet members, which led to the election of a Steering Committee. EMBnet business meetings should have been annual, yet this meeting was scheduled shortly after the previous one to review the way the organizational structure created in Nijmegen had been working; and to examine possible amendments to it. Most of the topics discussed in Lisbon focussed on the organizational structure needed for the promotion of EMBnet, mainly in view of the creation of an European Bioinformatics Istitute (EBI) sponsored by the EC. The general opinion was that if EMBnet is going to play a role in this upcoming scenario, as its long-standing committment in bioinformatics would suggest, then it should obtain a better organizational structure and higher PR.

Improvement of data distribution

a) Several new projects have been presented for data distribution.

Peter Gad (Swedish node) has presented the NNTP protocol, which is a data selection and distribution mechanism. The main advantage of this protocol is that it allows the change of a single item, without re-transmission of the whole database, thus saving a lot of disk space.

Reinhard Dölz (Switzerland) proposes a different protocol (HASSLE) exploiting the idea of polling. The software would check the entry name, and version number and date, thus transfering only the most recent data. This would have the advantage of an intelligent system together with that of sending out compressed files with a great saving in disk space. Another advantage would be the feedback software provided for each transfer. Both NNTP and HASSLE protocols will be tested at the Norwegian node, and then by the EMBL Data Library.

b) A project has also been funded through BRIDGE grant for protein sequence data distribution on Wide Area Networks from MIPS (Germany). The novelty of this protocol is in its layered structure, which allows not only single node to node updates, but also node to many nodes.

c) Several projects were also aimed at the development of EMBnet software, both for databases and for interface:

— A feasibility study is underway for the creation of a database of biologists. This will be carried out as a cooperation between EMBL the Finnish, and the Swiss nodes.
— A project has been funded for the design and development of an EMBnet user interface, or menu system.

— A workshop will be organized for the release of data manipulation software in cooperation with the Swiss, Dutch and Norwegian nodes, and the EMBL Data Library.

d) With respect to the new nodes, TCP-IP software is being further distributed and some assistance is also being provided for those who want to install it.

Bulletin board

A NEWS system has been set up to ease discussion on technical matters. And a public list is being set up, aimed at making EMBnet known to everybody on research networks not only in Europe, but also in the USA.

Several GOPHER clients/hosts have been installed. In the near future, EMBnet Gopher will provide a complete database, not only of the programmes available in the group, but also of the official documents issued at the business meetings. Concerning the Gopher system, a project has been sponsored, through BRIDGE grant, for the creation of an EMBnet gopher interface to major biological ftp sites.

These recent implementations are not only aimed at improving communication and knowhow exchange between EMBnet nodes, but also to make the European and international scientific world aware of the scope and impact of this project, This advertising function is seen by the project nodes as being of great importance.

HIGHLIGHTS / MILESTONES

The major achievement during this year, is the creation of a structure within the group. Though it might seem a move into bureaucracy, it has proved of great spur in the presentation of new projects (21 presented projects in 1992-93 as against 7 projects presented in 1991-92) and has eased the management of the whole project. This has proved a great advantage, especially in defining guidelines to be applied to the future cooperation between the nodes.

This structure is also a clear mark of a new awareness in the EMBnet community. The community has realized its crucial role in bioinformatics in Europe, and wants to be approached by any agency who might be interested. There is therefore already some discussion on the reconstitution of EMBnet into an interest group or a foundation, which will be a better structure for approaching national and international agencies and assuring some independence to the project in its long-term future.

The creation of this structure has also led to a better definition of the scope of the project. The SC, with the project assistant, have drafted a document which identifies aims and scope of the EMBnet, specifies the criteria for the joining of new nodes, and fixes the rules for the funding of collaborative projects. This document will be part of the new image we intend to give EMBnet, as such points are also aimed at the public.

WIDER CONSIDERATIONS

EMBnet is now getting ready to become an appropriate partner for any structure operating in the European Bioinformatics field.

In this regard, the EMBnet community has been following the developments surrounding the formation of the new EBI with interest. While we realise that no formal decision has yet been taken by the Commission, it seems most likely that

Europe will soon have a central focal point for at least nucleotide and protein sequence databases. We see EMBnet as a complement to any central data facility, indeed in Germany, where the EMBL Data Library has its headquarter, we also have a national node, the DKFZ. Therefore, we feel even more committed to improving our network, evolving in parallel with any central facility so that Europe can benefit from both a strong central and a diverse peripheral nodal system.

COOPERATIVE ACTIVITIES

This project is, in its very structure, quite different from most other BRIDGE funded programs in that it is based completely on cooperation of different countries, EEC and EFTA, and Institutions. Every result is the fruit of this cooperation. Yet, EMBnet as an organization is open to contributions from any other institution and organisation; indeed, cooperation is under way between the so called 'special purpose nodes', that is the human Genome Mapping Project Resource Centre of the United Kingdom, the MIPS in Heidelberg and the CEPH in France, the ICGEB in Trieste (Italy), and Hoffman-La-Roche in Basel. Very recently, the Geneva Laboratory, coordinated by Amos Bairoch has also been proposed as a special purpose node.

EUROPEAN DIMENSION

Overall, we believe that the European dimension of EMBnet is very evident; in fact, we dare say that, without collaboration between different European countries, no European Molecular Biology network would exist.

Therefore we hope to be able to further emphasize the European scope of the project and present it as the European bioinformatic infrastructure in the future. In view of this, some cooperation has been started through ICGEB to Eastern European countries; there is already a possible EMBnet node in Hungary.

Integrated data and knowledge base of protein structure and sequence (BIOT CT-900271) (SMA)

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J. THORNTON, University College London, London, UK *(partner 3)*
P. GRAY, University of Aberdeen, Aberdeen, UK *(partner 4)*
C. RAWLINGS, Imperial Cancer Research Fund, London, UK *(partner 5)*
M. MOREAU, BIM, Everberg, B *(partner 6)*

OBJECTIVES

All partners: The main objective for this reporting period has been to finalize the work on the standard conceptual schema of the object types and relations describing protein structures and sequence data. Objectives of individual partners are spelled out in the results section.

MAJOR PROBLEMS ENCOUNTERED

The task of defining a machine-independent standard conceptual schema has proven to be more complex than originally anticipated. Progress on this task has therefore been slower than planned. Consensus has however been reached on the core schema, and it is hoped that this consensus can be extended to higher level descriptions of protein data items (supersecondary structures, families of folding motifs, descriptions of topological features, etc.), by the end of the 3rd quarter of 1993.

Several partners *(partners 2 & 3* here and *partner 1* previously) have been dealing with the problem of representing data on sequence alignments and families of related proteins. This turned out to be more time consuming than expected as it required the development of elaborate consistency check procedures. Providing services over electronic networks (ftp) has also been particularly labour intensive as it required installation of specific network procedures.

RESULTS

All partners:

Definition of the standard schema for the description of protein structure and sequence.
All *partners* in the project have taken part in meetings and discussions to help arrive at a consensus conceptual schema that integrates the data and requirements of all the consortium partners. This included the definition of object classes and relations with a particular emphasis on the determination of the primary keys allowing to uniquely identify individual objects. It also included plans for storing structural motifs, tertiary interactions, information about protein families, multiple versions of the same protein, revisions of the data, and links to other databases.

Schema Definition Language
A Schema Definition Language (SDL) for interchange of project database designs has been defined by *partners 1 and 5*. SDL is an abstract schema definition language for describing the attributes of each entity and its relations with other entities. In particular, the entity class, primary key, simple attributes, and relations

are defined separately using a block structuring convention. It was designed to facilitate the exchange of designs for the BRIDGE schema based on object-oriented semantic data models.
SDL permits maintenance of two inter-related schema definitions: (i) a network of abstract classes; (ii) a network of concrete classes. The same notation and graphics can be used in each case with greater ease and clarity. The abstract schema describes a meta-design: the abstract classes, attributes, and relations serve as templates which model the generic behaviour of the concrete schema. If a new concrete class is desired, the abstract schema can be consulted for a suitable template, so that the concrete schema is merely extended 'sideways'. The BRIDGE project domain of discourse is usefully modelled as homo- or heterogeneous collections of molecular components, whose behaviours are described generically in the abstract schema.
The SDL syntax permits some uncertainty and deferring of decisions to allow progressive consolidation of the design, but is formal enough to be parsed by validation programs.

Partner 1 (Brussels):

Porting SESAM to different hardware platforms and other RDBMS
The SESAM database and its user interface ALI have been ported onto three new hardware platforms: the DEC-station, Silicon Graphics IRIS and IBM compatible PC running under UNIX. Porting to the latter platform required a change in RDBM from Sybase to Oracle. Oracle does not have the buffering capacity of Sybase. This required to re-write the low level routines of ALI which are part of the sequence-structure pattern search programs.

Extension of the molecular dictionary of SESAM
SESAM contains a molecular dictionary which provides the chemical and topological description of the molecular building blocks encountered in the database. Such building blocks comprise the naturally occurring amino-acids and nucleotides as well as various organic molecules and groups that bind to proteins.
The large increase in the number of protein structures that has recently occurred, brought with it a proliferation of new types of chemical groups/molecules. The description of these groups was introduced into the molecular dictionary of SESAM. Furthermore, software was written to generate the required descriptions automatically from the Brookhaven atomic coordinates file. This will be extremely useful for coping with any future diversification in the types of non-standard chemical groups.

SESAM/ALI developments

(a) Generation of atomic coordinate outputs in the Brookhaven format; to be used as input to various peripheral programs.
(b) A one letter-code providing a description of backbone conformation in terms of allowed $\varphi\psi$ regions of the Ramachandran map was entered into SESAM.
(c) Preliminary tests were carried out on ways of storing information on spatial proximity of atoms, residues, secondary structure elements, with a minimum overhead in storage space. These involved combining a cubing procedure, where each residue is assigned the index of the cube in which it is contained, and a compact code representing the residue neighbour list.

Partner 2 (EMBL):

HSSP Database
The HSSP database of protein families contains a set of biological relations between protein structures and all homologous sequences. It now serves as an important link between the structure database (PDB) and the protein sequence database (SwissProt). Secondary structure information in HSSP is extracted routinely and reported in the corresponding SwissProt entry. In the near future, the SwissProt entries will include homology pointer to known structures, established through HSSP. This cross referencing has helped improve consistency of the sequence information. HSSP was also used to develop a new secondary structure prediction method using Neural Nets, which yields an average accuracy of 70.8% (three states: α, β, loop) for soluble globular proteins.
The program producing the HSSP-database was implemented on a wide range of parallel multiple-instruction-multiple-data (MIMD) computers, markedly reducing the time to update the database from weeks to a few days. Production runs are planned for the end of 1993.

Data extraction from flat-file databases
PickProt is a set of scripts based on the Awk language, developed to extract information from protein structure databases (PDB, DSSP, HSSP). It allows to select database entities subject to user defined constraints, such as categories of structures (DNA, Protein), secondary structure content, presence of cofactors, homology data (HSSP alignments), methods employed (X-Ray, NMR), and quality of experimental data (resolution, R-factor). Different sets of constraints yield different lists of protein chains that can be used for further analysis.

Graphical user interface to heterogeneous databases
ProtQuiz is a new program for browsing through molecular flat-file databases (PDB, DSSP, HSSP, FSSP, SwissProt), providing tools for extracting information and visualizing molecular structures and sequences. It is an object-oriented system implemented in C++ and X-Window, using the ET++ application framework (ETH, Zurich). An internal interpreted command language allows flexibility for user queries and extensions. The internal data structure of ProtQuiz reflects the proposed standard conceptual schema developed within this project, and will form a graphical user interface (GUI) for the database management systems developed by *partners 1* (SESAM-ALI) and *4* (P/FDM).

Network access to databases and programs
Access to the various flat-file databases (HSSP, DSSP, FSSP) to the ProtQuiz, and structure prediction programs is now provided via international electronic network. On internet, files can be downloaded using ftp (file transfer protocol) from *ftp.embl-heidelberg.de.* Electronic mail access is provided by the server *netserv@embl-heidelberg.de.* The secondary structure prediction network facility is used heavily (appr. 1000 requests per month).

Partner 3 (UCL):

Homologous and Analogous Fold Families
During the last year our major effort has been to develop a procedure for automatically classifying the Brookhaven databank into families of homologous and analogous structures. This is crucial if the database is to remain usable as there are now over 2000 protein chains in the databank. Initially pairwise comparisons are performed between all protein sequences and those having greater than 35% identities are clustered into homologous families. Subsequently representatives from each family are structurally compared using a flexible alignment program (SSAP).

Families whose representatives align with a high score have very similar folds and are combined into a single family. These contain proteins usually having related functions. A softer cutoff enables families of analogous proteins with related folds but often no functional similarity to be identified.

Using this approach we have been able to remove the degeneracy of the databank and identify the number of unique folds, which currently stands at about 160. The method is completely automatic and allows fold families to be updated and new families to be identified with each release of the Brookhaven databank. This takes less than two days on a SUN4. We are currently designing tables for storing information about fold families in our relational database IDITIS.

Structural Motifs
Within the laboratory, we are deriving algorithms to identify and classify secondary and supersecondary structural motifs. Recently we have completed an analysis of beta-hairpins, beta-bulges and Greek key motifs and generated database tables for all examples in the Brookhaven databank. We have also been analysing the interaction of alpha helices in different protein classes and the types of packing observed.

Data Validation — PROCHECK
We are continuing work on protein structural validation and including a stereochemical Quality Assessment for each structure in the Brookhaven databank. This work links in closely with the EC Biotechnology Data Validation Project, which will continue addressing other problems in this area.

Partner 4 (Aberdeen):

We have explored the possibility of using the SYBASE relational database system to provide persistence for a functional data model database, thus standardizing on SYBASE as a low-level storage environment. This enables partners wishing either relational or functional views of the same data to share the same physical data organisation. This has been achieved by coupling the P/FDM system developed in Aberdeen to SYBASE. Using the ProDBI interface between the logic-based environment of Quintus Prolog and SYBASE, we have implemented basic routines for retrieving data from a relational storage module, in accordance with the functional data model.
Basic routines for adding data about new entity instances and attributes have also been implemented for relational storage modules. These have enabled us to load data into SYBASE using P/FDM's bulk loading utility. As a trial, just before the November meeting, data for a few proteins, covering a subset of the schema agreed at that time, were loaded into SYBASE via P/FDM using these routines. Prolog, Daplex and SQL queries against this test database were demonstrated at the November meeting.
Daplex queries against a relational module can be answered by translating these into a series of small Prolog access requests. However, much better performance can be achieved for simple Daplex queries by translating these into single SQL commands. Therefore, we have implemented a program (DAPSTRA) which translates Daplex queries into SQL. Because SQL has less expressive power than Daplex, translation to SQL is not always possible, however, such queries can always be translated to Prolog access requests. Thus, this solution provides the full capabilities of Daplex, while giving the same performance as direct access using SQL whenever this is possible.
The P/FDM database has been coupled to the molecular graphics package Quanta, making it possible to display and highlight fragments of protein structure identified by a database search. This facility has been particularly useful in homology model-

ling. The coupling has been achieved using remote procedure calls from the database running on a Sun4 to a server process running on an Iris Indigo. While the coupling is to Quanta, the same coupling strategy could be used to interface P/FDM to any molecular graphics package with a command line interface.
While our main database development work is done using Quintus Prolog on Sun4s, we intend porting P/FDM to Sicstus Prolog. This Prolog can be run on a variety of platforms, and Sicstus licences are much cheaper. Progress has been made towards this, adding preprocessor macros to the source code, so that different versions of P/FDM can be built for specific Prolog environments.
A format for storing general schema descriptions has been developed. Using the P/FDM database, a file containing a schema description in this format can be translated automatically into the corresponding relational table declarations, or corresponding Daplex data definition statements, or a formatted document describing the schema. Once a conceptual schema is agreed, this will make it easy to produce consistent schemata for relational and functional implementations.
Prototype graphics-based interfaces are under development at Aberdeen, and these were demonstrated to partners at the meeting in November.

Partner 5 (ICRF):

Representing Protein Structure
Several geometries used to describe packed protein secondary structure element pairs abstracted as pairs of vectors have been compared to determine the best geometric features for describing both packed and non-packed elements in proteins. Within this framework various geometric and physico-chemical relationships have been examined and ranked to derive a 'feature space' within which protein secondary structure groupings can be plotted and quantitatively compared. This material has been applied in the area of protein structure comparison and will be of value in extending the earlier Topol deductive database of protein structure to describe three-dimensional relationships.

Integrating Databases
In collaboration with *partner 3* and Oxford Molecular, data files from the commercial database IDITIS containing restructured and validated data extracted from the Brookhaven database have been obtained. Programs to select subsets of the IDITIS data and to convert them into Prolog clausal form have been developed. Preliminary experiments in collaboration with the APPLAUSE project (ESPRIT EP-6708) using ElipSys/Megalog to store the subset of data necessary to describe beta sheet topologies have shown that the Megalog clause storage mechanism has an overall disk storage overhead of approximately 8 fold. The retrieval times for Prolog queries using ElipSys/MegaLog has been very encouraging with inference and retrieval times from ElipSys/Megalog being similar to interpreted memory-resident Prolog even though the ElipSys/Megalog database contains 100 times more data.

Partner 6 (BIM):

Expert problem solving approaches to protein data loading and validation
Loading protein data into SESAM has so far required frequent human intervention in the form of expert decision making, and problem solving. The problems that arise are often due to missing information or to inconsistencies in available information. Though some of these problems will disappear with the new PDB file format problems related to information consistency will remain. In close collabora-

tion with *partner 1* procedures based on expert problem solving approaches were introduced into the SESAM loading programs, with the effect of significantly reducing human intervention in the loading process.

Interfacing SESAM to visualization tools
SESAM was interfaced to the public domain visualization program FLEX (written by Mike Pique, USA) which runs on SUN computers, and allows to display structural objects. The FLEX routines are activated from within ALI.

HIGHLIGHTS/MILESTONES

Consensus has been reached on a standard way of describing the most commonly used protein 3D structure data items and the relations between them. This will greatly facilitate devising standard protocols for exchanging data on protein 3D structures, and for comparing the designs of different databases of protein 3D structures.

Object-oriented front-end tools have been mounted onto the relational protein structure database SESAM, and shown to provide a flexible and useful working environment. First prototypes of links and interfaces between protein structure databases and modelling programs, have been developed by *partner 4* and *partner 1*. Prototypes of object oriented C^{++} user interfaces allowing fast access to the Brookhaven Databank files, and providing graphic tools have been developed *(partner 2)*. Important progress has been made in representing protein structure data: a comprehensive classification of protein fold families has been carried out by *partner 3* and ways of storing information on spatial proximity of atoms and residues have been devised by *partner 1*.

WIDER CONSIDERATIONS

Work carried out by partners of this project addresses key issues concerning the problem of handling the ever growing amount of data on structures of biological macromolecules. In particular it addresses problems related to archiving, exchanging, accessing and analyzing protein 3D structure data. The work on defining the standards for representing protein 3D structure data items and the relationships between them (the standard conceptual schema) is of particular importance. Such standards will greatly facilitate exchange of protein data between different laboratories and programs. It will also make possible to carry out rigorous comparisons between the design of databases developed in different laboratories in Europe and in the US. The schema definition furthermore turns out to be extremely useful in defining the data structure for the macromolecular CIF dictionary (the dictionary for the Crystallographic Information File, which is due to replace the widely used Protein Databank Format) now being finalized by the international task force of the IUCr (International Union of Crystallography) headed by Dr. P. Fizgerald. Contacts established recently with this task force will result in incorporating much of the schema into the CIF dictionary.

COOPERATIVE ACTIVITIES

Several joint meetings and workshops were organized within this reporting period:
(a) Prof Gray and Dr Kemp *(partner 4)* visited EMBL in May 1992 for discussions on schema design.
(b) *Partner 2* was host to a 2-day meeting (22-23 May 1992). Discussion took place on the design of a conceptual schema for protein
(c) Dr Kemp *(partner 4)* visited UCL on 8 October 1992.

(d) Michael Scharf *(partner 2)* gave a seminar in Aberdeen on 'The ET++ toolkit for advanced object-based graphic applications' on 18 November 1992, and installed ET++ at Aberdeen.
(e) *Partner 4* was host to a 2-day meeting (19-20 November 1992). Discussions took place on the design of a conceptual schema for protein data, and demonstrated P/FDM, the coupling to SYBASE and Quanta, and some prototype graphical interfaces to the database.
(f) *Partner 5* was host to a 2-day schema design workshop (27-28 January 1993) and a 1-day project meeting. Discussions took place on the consolidation of the agreed concrete conceptual schema and on the generalization into abstract classes of the already defined concrete classes.

EUROPEAN DIMENSION

The complementary expertise in the different fields of protein modelling and structure analysis, object oriented databases and logic programming, provided by the different European partners of this project, has been essential in making the right decision about developing the appropriate computer tools and concepts to handle the very complex data of protein structure and sequence.

LIST OF JOINT PUBLICATIONS/PATENTS WITH TRANS-NATIONAL AUTHORSHIP

A joint publication by *all partners* describing the standard conceptual schema for protein structure data is in preparation.

OTHER PUBLIUCATIONS

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C. A. Orengo, T. P. Flores, D. T. Jones, W. R. Taylor and J. M. Thornton (1993). Recurring Structural Motifs in Proteins with Different Functions. *Current Biology* **3**, 131-139.

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D. N. Woolfson, P. A. Evans, E. G. Hutchinson and J. M. Thornton (1993). Topological and Stereochemical Restrictions in B-Sandwhich Proteins. *Protein Engineering*, **6**, 461-470.

C. A. Orengo, T. P. Flores, W. R. Taylor and J. M. Thornton (1993). Identification and Classification of Protein Fold Families. *Protein Engineering*, submitted.

Immunoclone and hybridoma database network for Europe (BIOT CT-910257)

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OBJECTIVES

1. Development of a scientific and technical database on immunoclone descriptions ('Immunoclone Database-ICDB') coming from:
   — Scientific literature currently published,
   — Patent applications to the European Patent Office,
   — Industrial and commercial catalogues,
   — Participants' collections of hybridomas.
   Altogether, the target was an input capacity of 2,000 descriptions per month after 18 months.
2. Implementation of a stable consortium agreement, as a model for biological database network in Europe.
3. Development of an end-user interface for improvement of the use of databases for biotechnology in Europe.

MAJOR PROBLEMS ENCOUNTERED

1. **Concerning the whole project:** A four month delay of the initial training party for several centres because of the delay of the shipment of EEC funds, and in consequence delay for the local implementation of input capacity;
   Lack of electronic communication in the institutions of laboratory partners;
   Development of software for the automatic conversion of ASCII format input into ORACLE format and vice versa;
   Difficulty for participating centres to reach the requested production.
2. **Concerning centres:** ECACC: Network communication; successive trained coders left the centre, which severely affected the level of input.
   IST: Delay for the acquisition of requested microcomputers; difficulty to reach the appropriate literature sources.
   VUB/IMB: Difficulty to select appropriate literature sources.
   CLB: Decision to retire at the beginning of the project.
3. CERDIC reviewed the communication problems and payed a visit to production centres in order to implement an appropriate solution for each case.

RESULTS

1. **Database management implementation**

(a) The *data input and validation software*, achieved during the first period, was improved during this period in order to integrate new functions related to the

ICDB Vocabulary list and the EMBASE Thesaurus viewing (with the kind authorisation of Elsevier Science Publishers), integrated help system and various extended validation routines (262,000 bytes in 20 C- language modules). Five built-in databases are used when the software operates (ICDB Vocabulary, EMTREE Subset, Rules file, Help file and Numbering data).

(b) The main database of ICDB is managed by ORACLE procedures and specific software (136,700 bytes in 12 modules) for insertion and extraction of records. The internal structure includes 17 information tables and 12 join tables. A pre-formatted Text table is added in order to facilitate text retrieval.

(c) An associated database was added for the treatment of journal article references, extracted from floppy disks containing a selection of bibliographical data. Automatic Redistribution of Bibliographic Reference Entries software (ARBRE) was developped in order to read the textfile as delivered by EMBASE, MEDLINE and PASCAL producers and to insert the reference information into the ORACLE database, including the respective database record number. ARBRE detects the country of origin of the 1st author and assigns each reference to one of the coding centre, according to a dispatching list. Each list of those references is sent automaticly by electronic mail to the appropriate coding centre.

(d) An original software for the automatic treatment of E-Mail from inputing centres was developped. Automatic Selection of Pertinent Input and Reporting software (ASPIR) reads incoming E-Mail, detects ICDB records of input centres, open the message, start the insertion programme, analyzes the results and reports to the sender and to the database manager. When encountered, the offending records are reproduced with error messages explaining the problem.

2. Hardware and software installation

The initial installation was done during the 1st phase. Updates of the database and of the input software were sent and implemented in each centre (except ECACC for the main database).

3. Communication procedures

Difficulties encountered during the first period were solved. Appropriate solutions have been found for each centre. The regular use of ASPIR solved most of file transfer difficulties.

4. Data collection

The coordinating centre dispatched among the partners: 15,560 bibliographic references, 1160 patents and 70 new catalogues from major companies.

5. Data processing implementation and optimisation

After the implementation during the first period, a third training session took place in January '93, at CERDIC for the improvement of input techniques and production rate.

6. User-friendly interface

(a) A *menu driven interface* was developped by DIMDI for Immunoclone Database, in order to attract a wider community of scientists to use the online database. For the ICDB user guidance the expert knowledge has been used to

optimize the internal search strategy for antibodies, either by its reactivity or by authors. Comprehensive description of each search feature of the user guidance has been integrated into the 'Help-function' of the system, in English and in German.

(b)A feature specific to ICDB user guidance is the *Hypertext function which links ICDB records with their literature sources* identified in Medline and/or Embase in order to enable the user to perform a simultaneous search.

HIGHTLIGHTS / MILESTONES

1. Production

9,175 records were produced during the first 12-month period and 11,118 records during the 2nd period. In addition CERDIC processed about 3,900 records on scientific literature for the years 1984 to 1988.

The monthly production met 2,020 records in March 1993.

In total, ICDB on-line contains at the end of the project 43,000 records.

2. Diffusion

The database is currently *accessible on-line via DIMDI, DataStar and the computer host of Conseil Général of Alpes- Maritimes* (Videotex on Minitel). ICDB is monthly updated. *More than 600 users worldwide* are using the database. Due to the very high performance of host software, the connection time is very short and the net royalty income is low for the producers with this type of access.

Monthly updates of ICDB are also distributed on floppy disks.

WIDER CONSIDERATIONS

1. The Immunoclone Database is now the *biggest database on monoclonal antibodies and hybridomas* and the most accessible, worldwide. Procedures and organization can be easily adapted to similar projects.
2. *This type of database related to the description of biological products may take advantage in future to be closely linked with the delivery of the bioproducts* themselves. The user of the information is willing to get directly the product selected by the information search. It is easier, from the user point of view, to include the cost of information processing into the purchase of the product than to be asked for a separate payment of the information service. The traditional 'free' catalogue marketing technique may apply to scientific and technical information, as well.
3. This project pointed out *the need for a strategy in the field of biotechnology scientific information* (and probably in many other connected areas) at the Community level and at each national level. If such, it could improve its perenniality, make the cooperation with similar projects in Europe, America or Japan more efficient and less hazardous, increase the feed-back from the research community and improve its integration in research infrastructure.

COOPERATIVE ACTIVITIES

1. Dr. Alan Doyle, Curator of ECACC, was elected Chairman of the Board of CERDIC (December 1992)

2. A 3rd training session took place at Sophia-Antipolis in January 1993, with attendees from each input centre.
3. The second international workshop on hybridoma and animal cell lines is planned for April 27th, 1993.
4. A joined participation of ECACC and CERDIC to BioExpo is planned in June 1993.

EUROPEAN DIMENSION

Biological data collection and information transfer are typically international activities. *The European dimension is probably the minimum required for such projects.* There is no particular problem induced by a multinational partnership but in many circumstances it provides complementary experiences and know-how, e.g. the improvement of electronic communication, resource identifications, language translation, scientific analysis, etc.

1. Electronic communication and data access in research community

Many biomedical research institution equipment and know-how are below the minimum required for a reliable communication infrastructure. This was in progress during these last 2 years, thanks to the *success of Internet Protocol networks.*

One can expect that it will improve the transfer of scientific and technical knowledge in biology and increase the role of databases in this transfer. It is important that those databases be validated and reliable sources of information, under scientific control. The European scientific community may turn the integration of this activity in its research infrastructure to great advantage.

2. Biological databases in the European research community.

The current budget of biological research laboratories cannot bear cost of scientific information produced on a commercial basis. Although such activity should be financed on a regular basis, most of the biological databases produced in Europe are in fact supported by special programmes, like BRIDGE. *The discontinuity of these programmes do not let any possibility to compete (or even cooperate on a equal basis) with foreign equivalent projects.* Some efforts are made for sequencing data but many other domains are concerned.

3. Availability of biotechnology products

Some biological products, like hybridomas, cell line cultures, DNA probes have a strategic importance for the worldwide competition in pharmacology research, genetic disease investigations, etc. But the research bioproduct resources are much more scattered in Europe than in the US or in Japan. More and more bioproducts used by the European laboratories are of foreign origin because the ones which have been developped in Europe are unknown and consequently not internationnally recognized. *The support of information networks and database management in that domain may have a very positive effect on the development of biotechnologies in Europe.*

PUBLICATIONS

Monthly publication of 'Immunoclones' (EC support aknowledged on the cover).

Electronic linking services for biotechnologists and microbiologists in Europe (Grant)

COORDINATOR:
E. ROSS (previously B. KIRSOP), MSDN, Cambridge, UK
PARTICIPANTS:
MSDN is a sole contractor.

OBJECTIVES

As outlined in the original workplan.

RESULTS

[Note: The MSDN received a single grant under the BRIDGE Programme and has no BRIDGE funded partners. Comments regarding progress in different laboratories are not relevant. All results described below refer to activities emerging from the MSDN Secretariat office in Cambridge. Activities are on-going.]

Database storage/hosting arrangements

The new database hosting system is now well established and the management is working smoothly. Databases are loaded onto the system very efficiently and collaboration between the Base de Dados Tropical and the MSDN in this work is very satisfactory.

New databases

The following databases have been added to the MSDN system during this reporting period:
- ATCC catalogues of bacteria and bacteriophages, cell lines, clones, vectors, libraries and hosts, plant viruses, animal viruses, filamentous fungi and yeasts, protozoa and algae.
- Additional UK BioIndustry Association databases on National Biotechnology Associations and up-dated EEC regulatory information.
- Czechoslovak catalogues of bacteria, filamentous fungi, algae and cyanobacteria.
- International Mycological Institute catalogue of Fungi.
- Animal Virus Information System.

The following IRRO databases have been made available:
- OECD BIOTRACK database of GMO releases worldwide.
- CABI BIOCAT database on insect control by the release of natural enemies.
- UNIDO's Code of Conduct database for the release of organisms into the environment.

Communications services for IRRO and other organisations

A communications facility has been set up for the IRRO (Information Resource for the Release of Organisms into the environment) and the first databases are available for searching through MSDN.

Negotiations have been held with various organisations to provide them with communications services and database hosting facilities. They include COBIOTECH's Biotechnology Information Service, the National Bioindustry Associations and the World Bank.

Internet connections

An interactive connection was set up between MSDN's commercial network, running under British Telecom, and the research-based Internet through CGnet who provide the bridge between the two systems. Subscribers may access the MSDN network from the Internet. MSDN users may access certain Internet databases identified as of potential interest to them. It is also possible to send and receive electronic mail between MSDN and the Internet. The link with the Internet is considered vital to the further expansion and development of MSDN. It is within the Internet that exciting new networking and software tools have/are being developed. The bridge between the MSDN and the Internet through CGNet is, however, an intermediary solution. CGNet charges are high and often too costly for subscribers. MSDN has, therefore, identified a new networking host that will replace British Telecom and enable access to MSDN over the Internet as well as the X.25 networks. Facilities through the new host will be available in the very near future.

New agreements have been reached to offer the following Internet hosted databases from the MSDN: Israel EMBnet node databases, Microbial Germplasm databases, APC Econet conferences and databases, Human Genome Mapping Project Resource Centre databases, and Interlab databases of cell lines and molecular probes. The World Data Center databases and those offered by Datastar will also be provided over the Internet rather than the X.25 networks as at present.

Biodiversity

The MSDN co-organised an international workshop to assess the needs and specifications for a biodiversity information network that would provide information on plants, animals, microorganisms, ecology and monitoring for the benefit of biodiversity studies. The workshop was held between July 27th — 31st 1992 in Brasil and was sponsored by the WFCC, IUBS, IUMS and UNEP. Funds were provided by UNEP and Brasilian agencies as well as the British Council.

The workshop was attended by 39 people from major biodiversity and biological information centres. A simultaneous computer conference and list-server enabled participation in the workshop for over 200 people from around the world. The resulting Workshop Recommendations were the distilled views of a large biodiversity constituency. The Workshop Proceedings have been published by UNEP and were distributed widely.

An interim steering committee and working groups on different aspects of network development were set up and work is continuing on such matters as the establishment of a secretariat, funding and development of an global inventory to biodiversity information resources.

Billing

MSDN's billing system is now fully computerised. The MSDN Committee of Management decided to return this activity and all other financial activities in-house at the end of 1992, and this has been successfully accomplished.

Training

In March a UNEP funded workshop was held in Pushchino, Moscow Region in Russia as a follow-up to an earlier course. This was very successful in consolidating previous training. As a result Russian databases have been developed and translated into English for distribution over the MSDN network. A Memorandum of Understanding was signed between the Institute of Physiology and Biochemistry of Microorganisms and the MSDN.

Another UNEP funded training course was held in October in Beijing immediately following the WFCC's International Congress for Culture Collections. It was attended by 18 students from China, Thailand, Phillipines and Mexico.

Microbiologists from the UK, Australia and Slovenia received training at the MSDN Secretariat in different aspects of microbial information management and retrieval.

Meetings

A number of visits and meetings have been held throughout the reporting period. These have been with companies, biodiversity groups, database providers, research groups, culture collections and biotechnology organisations.

Publications/promotion

A newsletter was distributed to all MSDN subscribers. The MSDN instruction manual is continually up-dated as new databases and services are added to the network. Towards the end of 1992 the format of the manual was redesigned to accomodate the growing volume of material and improve its appearance. A demonstration disk of the network is similarly up-dated for distribution as the system changes. Both the manual and disks are available either at registration or subsequently as users wish.

Other projects

Work contracted by the US EPA and the NIDR has continued. Activities associated with the DGX111 DISNET project have primarily been concerned with development of a microbiology knowledge base and integration of MSDN databases within the DISNET environment.

Usage

Usage has fluctuated throughout the year on the BTNA system (mostly USA users), but has increased steadily, though slowly, on the Telecom Gold system (mostly European users). The overall number of issued mailboxes (representing an unknown number of users) remains at about 400, the population fluctuating as interests and needs change. It is anticipated that the availability of a number of important new databases and the connection to MSDN through the Internet will lead to substantially increased usage.

Summary

The major effort in 1992 has been towards 1) establishing an effective and relatively cheap database storage system on the BDT host computer, 2) arranging access to MSDN from the Internet, and 3) streamlining billing and accounting systems. These major and essential developments have taken priority over work on

the MSDN Directory of collaborating laboratories. The developments reported show a steady expansion of network activities as additional databases and user groups become linked to the system.

WIDER CONSIDERATIONS

The major efforts undertaken throughout 1992 were concerned with improvements to the MSDN network interface and increased distribution, and hence availability, of MSDN networked services to a wider audience. The Internet is a huge network of computers, with it's origins in research-based activities, but increasingly used by the commercial sector. A number of very useful networking and software tools have recently been developed for use within the Internet environment (WAIS, gopher, etc.) and these, together with the wide availability of the Internet world wide, resulted in the decision to transfer all MSDN networking activities from British Telecom to a new hosting system that will enable access to MSDN over the Internet as well as the X.25 networks. The model of MSDN foreseen in the future is that of a series of distributed nodes providing access to a basic range of MSDN services on a local or regional basis. Another important effect regards the transfer to a new host system is a move away from a billing system based on hours of network use (connect time). This has been necessary to date given British Telecom's billing algorithms are designed this way. The move to the new host will enable a new method of charging, i.e. one based on a fixed advanced subscription fee. This new charging policy should prove attractive (i.e. cheaper) to MSDN subscribers, enable 'freer' use of databases given time will no longer be a limiting factor, and significantly reduce MSDN administrative costs concerned with billing operations.

EUROPEAN DIMENSION

The benefits to international research through a global information and communications network are obvious. In 1992 there was considerable collaboration with colleagues from Eastern Europe, especially Russia. Such collaboration not only facilitates communications between scientists from East and West Europe, but also benefits all researchers by making available scientific data originating from Eastern Europe and previously unaccessible outside the region.

Information Centre for European Culture Collections (ICECC) (BIOT CT-900162)

COORDINATOR:
D. CLAUS, DSM, Braunschweig, D
PARTICIPANT:
DSM, Braunschweig, D

OBJECTIVES

Information on European culture collections; advice on availability of biological material, information on microbial data and databases and on pertinent regulations

RESULTS

General aspects

The Information Centre for European Culture Collections (ICECC) was founded in the beginning of 1989 with full financial support from the Commission of the European Communities under its Biotechnology Action Programme. Since 1991 the tasks of the Information Centre have been continued under the BRIDGE programme of the Commission and with main support coming from the German culture collection DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen.

From its beginning the project reflects the demand from academic and industrial institutions for a central contact point for information on European culture collections, their holdings and services and for a centre where related information is readily available.

Public relation activities

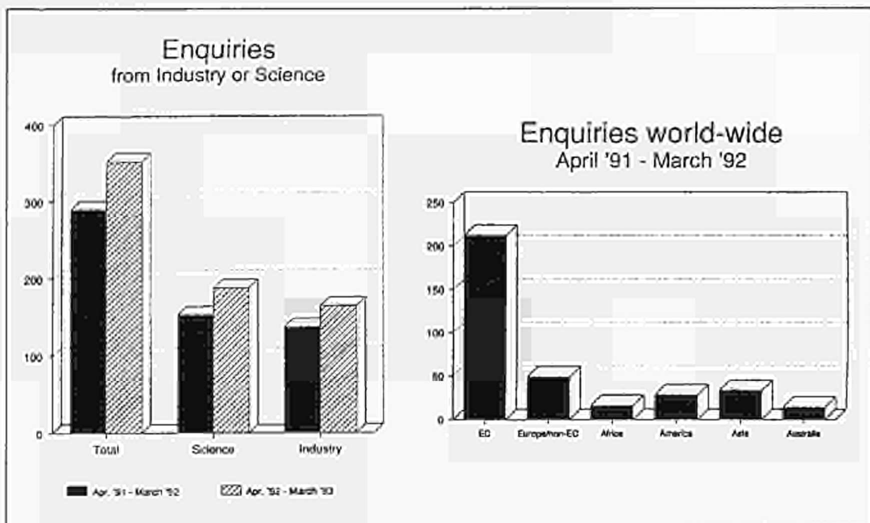
As the promotion centre for European culture collections the ICECC has published a series of leaflets on the activities and services of various collections. On the occasion of the VII. International Congress of Culture Collections, organized by the World Federation of Culture Collections under the title *Biodiversity and the Role of Culture Collections* in October 1992 in Beijing, China, the Information Centre has distributed the brochure *European culture collections: Microbial diversity in safe hands*. On 48 pages, information on all 43 member collections of the European Culture Collections' Organization and their holdings and services is given, together with a short introduction of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedures and on the European facilities for deposit of patent

strains. Information on the tasks of the ICECC, the Microbial Strain Data Network (MSDN) and the Microbial Information Network Europe (MINE) is also included in the brochure.

In 1992 the Information Centre has participated at different national or international meetings. At the annual meeting of the German Society of General and Applied Microbiology (Düsseldorf, March 1992) information on all activities of the European culture collections was distributed. On the occasion of the International Conference on Taxonomy and Automated Identification of Bacteria, held during July 1992 in Prague, Czechoslovakia, the Information Centre together with the Czechoslovak Collection of Microorganisms and the Czechoslovak National Collection of Type Cultures presented the services offered by the European culture collections.

Two numbers of the ICECC Newsletter (circulation copies 2500-3000) have been published within the reporting period. They were distributed at the meetings and to different institutions or individuals which have shown interest in the work of the Information Centre and the European culture collections. Since the last progress report an updated edition of the Instructions for Shipping Non-Infectious and Infectious Biological Substances has been prepared and distributed.

Advice and information on cultures and culture collections



Besides the information given during meetings the centre have received during 1992 requests for the availability of specific cultures from all over the world. In most cases strains in question were available at least in one European culture collection. Other requests were on microbial identification or chemotaxonomic studies performed in European collections or were of general nature. Inquiries received by the Information Centre are shown in the previous page.

New connections to culture collections from middle and eastern European countries have been established in 1992. Some of these collections were rather unknown in western Europe but may considerably contribute to the microbial diversity held in European culture collections.

The database of the UK culture collections, the Microbial Culture Information System (MiCIS), was still offered by the ICECC. Searching of information during 1992 was mainly done via the Microbial Strain Data Network.

WIDER CONSIDERATIONS

European culture collections play an important role in maintaining biological resources. Recent attempts to estimate the total number of microbial species have shown however, that the current inventory of the microbial diversity is very incomplete. It is generally agreed that only 10 % or less of the microbial species are known up to now.

There is growing concern about the conservation of biological diversity and the need to identify, record and preserve genetic resources of potential value for the future benefit of mankind. In the *Decade of Microbial Diversity*, European culture collections with their well known expertise will play a major role in developing new techniques for the isolation, culturing, description, identification and long-term preservation of hitherto unknown microbial species and will transfer appropriate data in public accessible databases: European culture collections, microbial diversity in safe hands.

COOPERATIVE ACTIVITIES

The Information Centre helds good connections to all European service culture collections and to the main collections outside Europe. The Microbial Information Network Europe (MINE) has been demonstrated during fairs to visitors. The centre is also actively engaged in problems of data integration within the MINE Responsible Committee for Bacteria. The sectretary of the Information Centre acts as the Secratary of the European Culture Collection's Organization (ECCO).

Together with the Culture Collection of the University of Göteborg the Information Centre was involved in the preparation of the annual meeting of the European Culture Collections' Organization (ECCO), which was held during June 1992 in Göteborg (Sweden). In collaboration with the DSM the Information Centre prepared a computerized version of the list *Bacterial Nomenclature Up-to-Date*. The database is distributed free of charge to all European culture collections and to members of the UNESCO sponsored MICROBIOLOGICAL RESOURCE CENTRES (MIRCEN's). To improve the use of bacterial names in publications a free subscription has been offered also to the editors of the main microbiological journals in Europe. To the scientific community the database is available for a fee.

In order to improve the shipping manual contacts to the Committee on Postal, Quarantine and Safety Regulations of the World Federation for Culture Collections have been established.

Within the scope of an International Training Programme held during September 1992 by the GBF in Braunschweig, the participants were introduced in the tasks of the ICECC and to the Microbial Information Network Europe.

The Information Centre is involved in the organization of the workshops on *Advanced Techniques in Animal Cell Cultures* and *Techniques in Plant Cell Cultures*, to be held during May, 1993 in Brno, Czech Republic, by staff members of the Department of Biology, University of Brno, the European Collection of Animal Cell Cultures, and the DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen.

PUBLICATIONS/PATENTS

Information Centre for European Culture Collections NEWS, No. 4, April 1992 and No. 5, February 1993
Instructions for Shipping Non-Infectious and Infectious Biological Substances. ICECC Publication No. 1, 1992 edition
Price List of the ECCO culture collections. ICECC Publication No. 2, June 1992
Bacterial Nomenclature Up-to-Date. ICECC Publication No. 3, 1993 (with quarterly updates)
Claus, D., 1992. A standardized Gram staining procedure. *World Journal of Microbiology and Biotechnology,* **8**, 451-452

Microbial Information Network Europe (MINE) (BIOT CT-910280)

COORDINATOR:
D. van der MEI, CBS, Baarn, NL

PARTICIPANTS:

J. de BRABANDERE, BCCM, Brussels, B
Belgian National Node
Participating collections:
LMG, Ghent (Bacteria)
MUC, Louvain-la-Neuve (Fungi/Yeasts)
IHEM, Brussels (Fungi/Yeasts)

M. CHAUVET, BRG, Paris, F
French National Node
Participating collections:
LCP, Paris (Fungi)
CFBP, Angers (Bacteria)
CNRZ, Jouy-en-Josas (Bacteria)
ADRIA, Villiers-Bocage (Bacteria)
CLIB, Thiverval-Grignon (Yeasts)

M. MARTINI, DVBPG, Perugia, I
Italian National Node

F. URUBURU, CECT, Burjasot-Valencia, E(Bacteria, Fungi, Yeasts)
Spanish National Node

M. KALANTZOPOULOS, ATHUM, Athens, GR
Greek National Node
Participating collections:
ATHU-M, Athens (Fungi)
ACA-DC, Athens (Bacteria)
BPIC, Kiphissia, Athens (Bacteria, Fungi)

D. van der MEI, CBS, Baarn, NL
Dutch National Node
Participating collections:
CBS, Baarn, Delft (Fungi, Yeasts)
LMD, Delft (Bacteria)
Phabagen, Utrecht (Plasmids, Bacterial Mutants)
CDI, Lelystad (Animal Pathogens, Mycoplasmas)
KIT, Amsterdam (Leptospira)
PD, Wageningen (Bacteria, Plant Pathogens)
LMAU, Wageningen (Bacteria)
RIVM, Bilthoven (Bacteria, Bacterial Serotypes)

D.L. HAWKSWORTH, IMI, Egham, UK
British National Node
Participating collections:
IMI, Egham (Fungi)
ECACC, Porton Down (Animal Cell Lines)
CCAP, Ambleside/Porton Down (Algae, Protozoa)
NCTC, Colindale, London (Bacteria)
NCIMB, Aberdeen (Bacteria)
NCFB, Reading (Bacteria)
NCPF, Colindale, London (Fungi)
NCWRF, Watford (Fungi)
NCYC, Norwich (Yeasts)

R.M.. KROPPENSTEDT, DSM, Braunschweig, D
German National Node
(Bacteria, Plasmids, Fungi, Yeasts, Plant, Viruses, Plant and Animal Cell Cultures)

H.-E. KURZWELLY, DIMDI, Köln, D
Central Data Node

I. SPENCER-MARTINS, IGC, Oeiras, P
Portugese National Nod
(Yeasts)

E. FALSEN, CCUG, Gothenburg, S
Swedish National Node
Participating collections:
CCUG, Gothenburg (Bacteria)
UPSC, Uppsala (Fungi, Yeasts)

M.-L. SUIHKO, VTT, Aspoo, SF
(Bacteria, Fungi, Yeasts)

CCM, Brno, Cz
(Fungi, Bacteria)

OBJECTIVES

Introduction

The European collections of microorganisms contain well over 100.000 strains of fungi, yeasts and bacteria. Information about these strains is of direct importance to a great variety of users in the fields of science, biotechnology, food conservation and processing, agriculture, medicine and environmental sciences.

The MINE project has two main objectives:
1. To integrate and harmonize the data in the participating collections.
2. Deposition of these data in one Central Data Bank, through which the data are made available to the users.

In the project collections from 12 European countries (9 EC-countries) participate.

The project, combining many different institutes in a great variety of countries, is coordinated by project managers, one from each of the participating EC countries. The data, made available through the Member Collections (MC's), are integrated by Data Integrating Nodes (DIN's), one for fungi and yeasts at CBS, Baarn, and one for bacteria at LMG, Ghent. Harmonization and correction of the data is

accomplished through Responsible Committees (RC's), one for fungi, one for yeasts and one for bacteria.
The Central Data Node (CDN) at DIMDI, Cologne, provides the infrastructure for the database and the menu's for the retrieval of data.

The objectives set for 1992 were:
1. Completion of correction and harmonization of the minimum data set by the RC's.
2. Establishment of data structure and menu systems by CDN.
3. To make a start with the operationalization of the CDN for the data on unique strains.

MAJOR PROBLEMS ENCOUNTERED

During the reporting period, two major problems were encountered:
1. The RC's, partly due to underestimation of their tasks, did not meet the deadline for correction and harmonization of the minimum data set.
2. The MINE-project, which is a complicated endeavour, needs a great coordinative support. During 1992 it became clear that the coordinative input did not meet the requirements and challenges set by the formulated goals.

The integrated databases, supplied by the DIN's contain data on many strains of the same origin present in more than one collection. These data need to be harmonized so that the database will only contain one set of data for each of these strains. Also the data must be corrected for misintegration due to errors in collection numbers. Formulation of the guidelines for harmonization and the checking of the reports of the DIN's (1300 pages for fungi, 400 pages for yeasts and 1500 pages for bacteria) proved to be a tremendous task to be performed by a very limited number of people. The reports on fungi and yeasts have been corrected; the RC on bacteria has not yet finished its work.

The first integration undoubtedly is the most time consuming one; subsequent integrations can be performed much faster. The results of the first integration will be stored by the DIN's and applied automatically during subsequent integrations. To gain time, the project managers decided to skip the second integration of the minimum data set and to proceed directly to integration of the full data set.
The unique strains, which are not subjected to the harmonization procedure by the RC's, will be entered after they have been checked by the MC's, directly to the CDN.
The problem in the coordination of the project will be discussed at a project managers meeting at Egham, UK, April 1993.

RESULTS

Notwithstanding the problems encountered, good progress was made in a number of fields.
The guidelines for the harmonization of the data were established. Species names were checked and corrected separately. For bacteria the list of valid names as maintained by DSM was used; for fungi and yeasts the names corrected by the RC are kept as a reference list for checking. The 'MINE list of fungal species names' was printed and handed out to all MC's.

The structure of the database and the menu system was established at the CDN in close cooperation with the DIN's. The first version of the menu system was demonstrated in May in a meeting with the MC's at the DIMDI headquarter in Cologne.

The MINE collections received passwords to access the test-database at DIMDI and to test the menu system.
Their comments are used to improve the system.

In September new guidelines for entering data on the Minimum Data Set were sent to the MC's together with the data on their own, unique, strains.
The data, corrected according to the guidelines, will be entered in the CDN as soon as they have been returned by the MC's.

During the meeting of ECCO (European Culture Collections Organization) in Gothenburg and of the WFCC (World Federation for Culture Collections) in Beijing, presentations about the MINE project were given to large audiences of culture collections.

The MINE project managers meetings were organized at Brussels (March 6) and Gothenburg (June 15-18) in conjunction with the ECCO meeting.

At the end of 1991 the CCM (Czechq Collection of Microorganisms) joined MINE, bringing the total of countries participating in MINE at 12.

WIDER CONSIDERATIONS

The goal of the MINE project is the creation of one databank through which workers in a great variety of fundamental and applied research fields can easily access the wealth of data and strains available within the European collections of microorganisms.
This databank can be seen as an important facility for the inventarization of microbial biodiversity (in functioning and conservation of natural habitats and for their potentials for biotechnological processes) within the wider perspective of the European and world wide programmes of biodiversity research.

COOPERATIVE ACTIVITIES

February 10-11: Visit of the Data Integrating Nodes (LMG and CBS) to DIMDI, to discuss the structure of the MINE database and the menu system.

March 6: Meeting of the members of the Responsible Committees, of representatives from the Data Integrating Node and the Central Database Node at Brussels to discuss the correction of the reports of duplicate strains.

May 25-26: Meeting of 20 people from MINE Member Collections in 11 countries at DIMDI (Cologne), to inform the Member Collections on the work done by the RCs and the DINs, and to present the MINE database created at DIMDI (including the menu system).

June 15-18: MINE project managers meeting and presentations of MINE in Gothenburg, during the ECCO meeting organized by the CCUG.

CBS sent an application developed with ORACLE to the coordinator of the FLAIR project, to enable the participants in this project to create and use a database for Bacteria according to the MINE format (database structure, data entry screens, etc.).
At several occasions, MINE cooperated with the Information Centre on European Culture Collections (ICECC), to disseminate its information, e.g. in the ICECC newsletter, and in an ECCO brochure printed by the ICECC.

SOME FIGURES ON THE INTEGRATION

Below, the number of strains received from each collection is given.

	Fungi/Yeasts:		Bacteria:	
Belgium	IHEM	1.896	LMG	7.211
	MUCL	6.108		
France	LCP	1.130	ANIE	21
			CFBP	1.455
			CNRZ	617
Germany	DSM	1.817	DSM	3.800
Greece	ATHUM	216	ACA-DC	78
	BPIC	332	BPIC	1.066
Italy	DBVPG	2.115		
Netherlands	CBS	27.454	CBS	937
			KIT	209
			LMAU	695
			LMD	1.024
			PC	2.790
			PD	1.155
Portugal	IGC	451		
Spain	CECT	874	CECT	1.113
Sweden			CCUG	1.518
UK	IMI	10.576	NCFB	2.119
	NCPF	823N	CIB	3.462
	NCYC	1.886	NCMB	816)
	NWRF	554	NCTC	2.663
Total		56.232		32.749)

For Fungi and Yeasts, data have been sent on 42.481 unique strains (present in only one collection) and on 13.751 duplicate strains, representing another 5.615 different isolates.

For Bacteria, the number of unique strains is 22.111, and 10.638 duplicate strains give another 3.824 isolates.

AREA B:

ENABLING TECHNOLOGIES

- **PROTEIN DESIGN/MOLECULAR MODELLING**
  (from page 51 to page 75)

- **BIOTRANSFORMATION**
  (from page 76 to page 106)

- **DNA SEQUENCING**
  (from page 107 to page 111)

Engineering of microbial peptide lantibiotics for use in agro-food and biomedical industry (BIOT CT-910265)

COORDINATOR:
C.W. HILBERS, KUN, Nijmegen, NL
PARTICIPANTS:
H.-G. SAHL, Fr.-Wilhelms-Universität Bonn, Bonn, D
R. SIEZEN, Ned. Inst. Zuivel Onderzoek, Ede, NL
G. JUNG, Eberhardt-Karls-Universität Tübingen, Tübingen, D

OBJECTIVES

1) Chemical synthesis of leader peptides, pro- and prenisin, chemically modified lantibiotics, and mutant/variant lantibiotics. Preparation of immunogens.

2) Characterization of processes involved in:
   a) biosynthesis and secretion: genes, prepeptides and enzymes involved,
   b) cell killing and immunity of producing strains.

3) Determination of the chemical and three dimensional structures, and of biological and physico-chemical properties of various wild type, variant-, mutant- and synthetic (pre-)lantibiotics.

MAJOR PROBLEMS ENCOUNTERED

1) Chemical modifications of (pre)nisin have not yet been started due to lack of manpower. In contrast to our expectations, no biosynthetic enzymes are encoded upstream of the immunity gene *pepI*. The putative protease gene downstream of the structural gene *pepA* is followed by a non-coding sequence too. Therefore, it is possible that the genes for Pep5 biosynthesis might not be arranged in an opereron. This greatly hampers the construction of an expression system for mutated Pep5. Alternatively we try to express mutant Pep5 in strain *S. epidermis 280* which produces a Pep5 variant (see below).

2) For the solubilization and isolation of membrane-bound Pep5 prepeptide new chromatographic methods in the presence of various detergents had to be established. Yields of such purified peptide are currently too low for further studies. Production of antibodies against prediceted epitopes of the immunity peptide and the putative protease failed when synthetic peptides were employed. In the case of PepI this problem was overcome by use of fusion proteins.

RESULTS

The phase of screening for new lactococcal and staphylococcal strains producing novel lantibiotics has now been finished. As reported before, this has led to the finding of seven novel lantibiotics and the natural nisin variant nisinZ.

ad 1)
The synthesis of leader and propeptide parts of prenisin has been completed. Leader and pronisin parts of prenisin could be prepared in pure form. Both segments have been purified and extensively analyzed by HPLC and eloctrospray mass spectrometry. The segments are under study by NMR (Nijmegen) and CD spectroscopy (Tübingen) in various solvent mixtures. The chemical total synthesis

of the prenisin itself containing 57 amino acid residues, including 5 cysteines, was successfully finished in Tübingen. The synthesis of such a long and difficult peptide in a high purity is still a major achievement even for experienced peptide chemists. Various approaches have been attempted until finally a segment condensation approach which included purified intermediates was successful. The 57-peptide is analytically pure according to HPLC and MS. Next it must be studied by 2D NMR for its 3D structure. This may provide insight with respect to models of the biosynthesis of nisin from this precursor protein. Most interesting was the finding of Zn^{2+}-complexing properties of prenisin and possibly also other prelantibiotics (see below).

Further syntheses of chromophore labeled substrates for the leader peptidase of pre-Pep5 were carried out. However, it seems that the leader peptidase cannot be 'fished' and characterized by these substrates. Similar results were obtained with substrates derived from preepidermin. The Tübingen group presently works on a enzymatic semisynthesis of fully modified pregallidermin in order to obtain the natural substrate in high amounts.

Various immunogens for the preparation of antibodies against pre-lantibiotics have been synthesized, and several more are being synthesized.

The total synthesis of the immunity peptide of Pep5 has been carried out in amounts and purity sufficient for biological studies of its potential activity.

A new lantibiotic, Pep280 from strain *S. epidermis* 280 was produced on a large scale in Bonn and sequenced by means of a novel method (see 3) in Tübingen. The amino acid sequence showed that it is closely related to Pep5. The producer strain is cross immune against Pep5 and will therefore be used for expression of mutant Pep5. In contrast to Pep5, the structural gene is located on chromosomal DNA. A 3.5 kb fragment was identified by Southern hybridization and is currently being subcloned and sequenced.

Staphylococcin 1580 is a bacteriocin that is produced by *S. epidermis* 1580 and was described as early as 1972 by Jetten and Vogels as a 400 kD carbohydrate-lipid-protein complex. The bacteriocin was purified in Bonn and identified as epidermin.

In collaboration with I. Nes and H. Stoffels carnocin U149 was produced in large scale and sent to Tübingen for sequence determination.

Several new mutants of nisin Z have been produced in Ede. N27K and H31K showed similar activity as wild-type nisin Z, but had improved solubility at pH $>$ 6. M17W-nisin Z has 2-5 fold lower activity than wild-type, and is currently being used for time-resolved fluorescence measurements with micelles and vesicles of varying phospholipid composition.

ad 2)
In Bonn 4.1 kb of DNA upstream of *pepA* have been sequenced. In contrast to expectations no open reading frame homologous to a biosynthetic gene could be detected. The only open reading frame orfX' shows no homology to any published amino acid sequence. The Bonn group is currently sequencing a 3.9 kb fragment that is located downstream of the putative protease gene and pepA.

A method for site-direced mutagenesis of *pepA* has been established and the first mutated gene (exchange of lysine for proline in position 18) has been obtained.

The membrane bound fraction of the Pep5 prepeptide was isolated in Bonn from *S. epidermis* Pep5, harbouring the cloned structural gene. Its status of modification is currently under investigation in Tübingen.

A genetically engineered fusion protein (orf*I*-maltose binding protein) was successfully employed for antibody production against the immunity peptide of Pep5. With these antibodies the immunity peptide was detected in *S. epidermis* 5. The peptide is partly associated with the cytoplasmic membrane but also found in the cytoplasm. Moreover, it could be shown that all phenotypically immune clones express the immunity peptide. All clones that are not immune (although the gene is present) show no signal in Western blots. The Bonn group is currently preparing the fusion protein between the maltose binding protein and the putative protease orf*P*.

More than 10 mutations in the leader sequence of nisin have been made in Ede to study the role of the leader in biosynthesis, secretion and processing. Several precursors of nisin have been produced and characterized.

Analysis of the nisin operon on transposon Tn5276 has shown that six genes are located directly downstream of the structural nisin A gene. These genes (and encoded products) were named *nisB* (possibly modifying enzyme), *nisT* (translocator protein), *nisC* (possibly modifying enzyme), *nisI* (immunity protein), *nisP* (leader peptidase) and *nisR* (regulatory protein).

Nijmegen and Bonn collaborated to localize the gene for the novel lantibiotic K7 on the chromosome of *S. epidermis* K7 by Southern hybridization. Based on the tentative amino acid sequence, derived from NMR data, two DNA probes were designed. Hybridization of *S. epidermis* K7 chromosomal DNA with these probes gave specific signals which were similar for both probes. These results indicate a chromosomal location of the gene, and support the purported sequence. Experiments to clone a gene-harbouring DNA fragment are in progress.

ad 3)
The elucidation of the chemical structure of many more isolated intermediates of pre-Pep5 has been successfully finished. The exchange of products and spectroscopic know-how between Ede, Nijmegen, Bonn and Tübingen steadily increased. A number of mass spectrometric analysis and protein sequencing studies were carried out in Tübingen. There novel derivatization procedures, prior to Edman degradation and Mass spectrometry, were developped which allowed for the sequencing through α,β-didehydro amino acids. Even N-terminally blocked lantibiotics like Pep5 (N-terminal oxybutyryl) can now be degraded. Various novel lantibiotics from the Bonn group have thus been fully or partially elucidated.

The chemically syntethized prenisin 57-peptide has been characterized by iospray-MS, CD and 2D NMR. The NMR results indicate that when free in aqueous solution the molecule is essentially unstructured. It is anticipated that the Zn^{2+}-prenisin complex is more likely to possess a well defined 3D structure, which may be relevant with respect to the process of post-translational modification of this molecule. E.g. this complex could be involved as an intermediate in the biosynthetic pathway for stabilization of the prenisin molecule against oxidation of cysteines and enzymatic degradation. The stoichiometry of the complex is 2 Zn^{2+} per molecule of prenisin.

Analysis of the structure of purified mutant nisins and mutants containing the leader peptide by 2D NMR is continued.

Mutants with resolved structures are being used in monolayer experiments with different phospholipids.

The structure of lacticin 481 was studied by 2D NMR (collaboration with Dr. J.C. Piard, INRA, Jouy-en-Josas, France). Only a partial characterization was achieved:

the configuration of two thioether bridges could not be determined, probably due to conformational averaging.

The solubility of nisin A, nisin Z and some mutants was determined as a function of pH and ionic strength. The mutants N27K and H31K nisin Z showed an improved solubility at neutral pH compared to nisin Z.

NMR studies have been conducted in Nijmegen to unravel the structure of nisin in a membrane-like environment. The results indicate that nisin complexes to micelles of various detergents and assumes a structure similar to the one in aqueous solution. The most prominent differences in the NMR spectrum are in the vicinity of Dehydroalanine 5, which is known to be essential for activity. The relative orientations of nisine and micelles have been characterized using micelles containing electron spin probes.

Sufficient material of K7 is now available to conduct ^{13}C NMR spectroscopy in order to obtain confirmation of the model proposed previously on the basis of 1H NMR. Good quality spectra have been obtained and are being analysed.

HIGHLIGHTS

— Total chemical synthesis of prenisin.
— Finding of Zn^{2+} complex formation of prenisin.
— Total chemical synthesis of Pep5 immunity peptide.
— The structural genes for the new lantibiotics K7 and Pep280 were localized on the chromosome.
— The Pep5 immunity peptide was identified.
— Sequencing of genes of the nisin operon.
— Functional assignment of *nisI, nisP* and *nisR.*
— Characterization of the new lantibiotic lacticin 481.
— Production, isolation and characterization of precursor nisin species.

WIDER CONSIDERATIONS

Good progress has been made towards achieving our original goal. The first novel lantibiotics with altered properties have been developped.

COOPERATIVE ACTIVITIES

Meetings:
— Workshop in Ede (organized by the Ede group): 31-3 / 1-4-1992.
— Workshop in Oberjoch (organized by the Tübingen group), 18-1 / 21-1-1993.

Materials exchanged:
Bonn-Tübingen: further intermediates of pre-Pep5, isolates of novel lantibiotics, Pep280, carnocin U149
Bonn-Nijmegen: 25 mg of purified K7
Nijmegen-Bonn: strain S. epidermis 1580
Ede-Nijmegen: purified nisin A
Bonn-Ede: DNA sequences, purified Pep5
Ede-Tübingen: polypeptides for MS studies, nisin and prenisin analogs
Tübingen-Bonn: synthetic immunity peptide, substrates for leader peptidase of Pep 5, immunogens
Tübingen-Ede: prenisin-Zn^{2+} complex, immunogens, chromogenic leader peptide analogs as substrates for leader peptidase
Tübingen-Nijmegen: synthetic prenisin, prenisin-Zn^{2+} complex

Staff exchanges:
Tübingen-Bonn: Coworkers and head of the group for experimental work and meetings.
Bonn-Tübingen: Various visits of the head of the group for consults on on-going work programme, discussions, and preparing publications.
Nijmegen-Bonn: Visit of Dr. M. v.d. Kamp for the localization of the K7 structural gene.
Ede-Nijmegen: Visits by Dr. H.S. Rollema on a regular basis for NMR analysis of nisin mutants and degradation products.
Nijmegen-Ede: Visits by Drs. H.v.d.Hooven to NIZO for purification of nisin A.

Interaction with other BRIDGE projects:
BRIDGE T-project Lactic Acid Bacteria:
— Studies on the structure of lacticin 481 (Dr. J.C. Piard, INRA, France).
— PCR primers for nisA and nisZ genes (Dr. J. Suarez, Sevilla, Spain).

EUROPEAN DIMENSION

The project benefits from a combination of expertises that will be hard to establish in any of the two countries alone. It enhances the international mobility of the coworkers.

JOINT PUBLICATIONS

For the period until April 1991 we refer to the book on 'Nisin and novel lantibiotics' (G. Jung, H.-G Sahl, eds) ESCOM, Leiden (1991). This book collects the state of the art in lantibiotic research based on the First International Workshop on this topic held in Bad Honnef (FRG), 14.-18 April 1991.
Following our workshop international activities increased in the general field as can be recognized from two further books:
a) 'Bacteriocins, Microcins and Lantibiotics' (R.James, C.Lazdunsky and F.Pattus, eds), Springer, Berlin 1992);
b) 'Food Biopreservatives of Microbial Origin' (B.Ray and M.Daeschel, eds), CRC, London 1992.

Recent joint publications are:
T. Kupke, S. Stevanovic, H.-G Sahl and F.Götz (1992) 'Purification and characterization of EpiD, a flavoprotein involved in the biosynthesis of the lantibiotic epidermin' in: J. Bacteriol. 174, 5354-5361
H.-G Sahl, G. Jung and R. Benz (submitted to Eur.J.Biochem) 'Mode of action of lantibiotics: Membrane depolarization by epidermin and gallidermin in comparison to the type B lantibiotic cinnamycin'.
H.W. van den Hooven, F. Fogolari, H.S. Rollema, R.N.H. Konings, C.W. Hilbers and F.J.M. van de Ven (1993) 'NMR and circular dichroism studies of the lantibiotic nisin in non-aqueous environments' in: FEBS Lett. 319, 189-194.

OTHER PUBLICATIONS
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S.Freund, N.Zimmermann, A.Fredenhagen and G.Jung (1993): 'NMR-Based Solution Structure of the Lantibiotic Duramycin B' in: C.Schneider and A.Eberle (eds), Peptides 1992, Proc. 22nd Eur.Pept.Symp., Escom, Leiden.
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C. Kaletta, K.-D Entian and G. Jung (1991) 'Prepeptide Sequence of Cinnamycin (Ro09-0198): The First Structural Gene of a Duramycin-Type Lantibiotic' in: Eur.J.Biochem. **199**, 411-415.

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S. Freund, G. Jung, O. Gutbrod, G. Folkers, W.A. Gibbons, H. Allgaier and R. Werner (1991) 'The Solution Structure of the Lantibiotic Gallidermin' in: Biopolymers **31**, 803-811.

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M. van de Kamp, H.W. van de Hooven, R.N.H. Konings, C.W. Hilbers and F.J.M. van de Ven (1992) 'NMR studies of lantibiotics - structure determination of epilancin K7, a new lantibiotic' in: Proceedings of the XVth ICMRBS, Jerusalem, p. 192

H.-G Sahl (submitted, Antimic. Agents Chemother.) 'Staphylococcin 1580 is identical to the lantibiotic epidermin. Implications for the nature of bacteriocins of gram-positive bacteria'.

G. Bierbaum and H.-G Sahl (1993) 'Lantibiotics - unusually modified bacteriocin-like peptides from Gram-positive bacteria' in: Zbl. Bakt. Hyg. **278**, 1-22

G. Bierbaum, J. Süling, C. Szekat and H.-G. Sahl (submitted, FEMS Lett.) 'Lysis of *Staphylococcus epidermis* with the lantibiotic nisin for isolation of DNA and RNA'.

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H.-G. Sahl (1992) 'Biosynthesis of the lantibiotic Pep5 and mode of action of type A lantibiotics' in: R.James, C.Lazdunski, F.Pattus (eds) 'Bacterial plasimid-coded toxins: bacteriocins, microcins and lantibiotics' Springer, Heidelberg, pp. 93-106.

O.P. Kuipers, H.S. Rollema, W.M.G.J. Yap, H.J. Boot, R.J. Siezen and W.M. de Vos (1992) 'Engineering of dehydrated amino acid residues in the antimicrobial peptide nisin' in: J. Biol. Chem. **267**, 24340-24346.

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W.M. de Vos, J.W.M. Mulders, R.J. Siezen, J. Hugenholtz and O.P. Kuipers (1993 'Properties of nisin Z and distribution of its gene *nisZ* in *Lactococcus lactis*' ιν: Αππλιεδ ανδ Ενωιρονμενταλ Μιψροβιολογυ **59**, 213-218.

P.J.G. Rauch, O.P. Kuipers, R.J. Siezen and W.M. de Vos (in press) 'Genetics and protein engineering of nisin' in: L.de Vuyst, E.J. Vandamme (eds) 'Bacteriocins of Lactic Acid Bacteria: Microbiology, Genetics and Applications', Elsevier, London.

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W.M. de Vos (in press) 'Future prospects for research and applications of nisin and other bacteriocins' in: D.Hoover, L.Steenson (eds) 'Bacteriocins of Lactic Acid Bacteria', Academic Press, New York.

Multidisciplinary approach to the analysis of enzyme catalysis, protein stability and folding (BIOT CT-910270)

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OBJECTIVES

Further development of the research tool box and studies focussing at the mechanism of folding and unfolding, as well as enzyme kinetics.

RESULTS

1. **Development of the reserarch tool box**

1.1 ***Structure determinations*** — The Cambridge group has succeeded in determining the X-ray structures of the following Barnase mutants: S91A, I96V, I76A, I88A, I96A and I76A + I88A + I96A.

1.2 ***Thermal analysis*** — A new (Russian) scanning calorimeter was installed in Granada and subsequently used for all the work summarized in this progress report. Furthermore, the reconstruction of the multi-frequency calorimeter is finished. Finally, the software necessary for the analysis of scanning calorimetry records in order to calculate ligand binding parameters, was further developed.

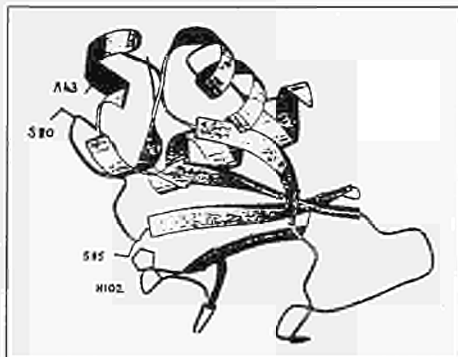
1.3 ***Modelling tools*** — D. van Belle (Brussels) implemented into the software package Brugel, a new Molecular Dynamics technique called *Multiple Copy Simultaneous Dynamics* which allows the efficient simulations of interactions between an enzyme and its substrate (or inhibitor).

2. **Results aiming at the understanding of the mechanism of folding and stability**

2.1 ***Folding of Barnase fragments*** — Fragments of Barnase were (chemically) synthesized in order to study the formation of the tertiary structure of barnase, via the secondary structure of these fragments. It was found that the major α-helix is formed even as short sequence and does not aggregate in solution. The central β-sheet, however, does tend to form large aggregates.

2.2 ***Engineering of disulphide bonds into Barnase*** — J. Clark and S. Clement (Cambridge) have introduced disulphide bridges in barnase in order to act as probes of the folding pathway of this protein (Figure 1). One disulphide, between residue 85 and 102, links two loops known to pack together early in the folding pathway. A second one, between residue 43 and 80, links two elements of secondary struc-

Figure 1 →



ture known to pack together only after the rate determining step of folding. Both disulphide bridges confer stability, as measured by equilibrium denaturation (Figure 2A and 2B). The kinetics of unfolding and refolding of the mutant proteins have been measured, and a comparison of the disulphide proteins and their corresponding dithiol forms has been made by use of thermodynamic cycles. These data have been used to construct folding profiles of the disulphide proteins. The disulphide bond engineering into the part of the protein that folds early confers stability upon the intermediate and transition states of folding. The protein with a disulphide bond connecting parts of the protein that fold late is not stabilized until the protein reaches its final folded form.

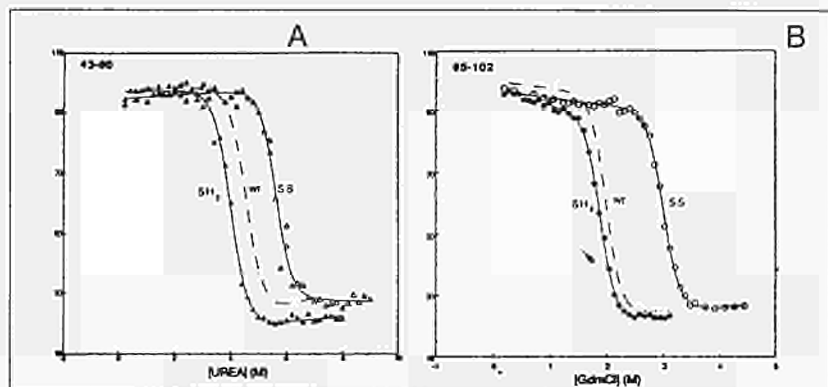


Figure 2

This study demonstrates that engineering disulphide bonds may be used to probe the pathway of protein folding.
A disulphide bond, as well as increasing the thermodynamic stability of a protein, may well increase the kinetic stability, by **decreasing** the rate of unfolding of a protein. Both disulphide mutants unfold more slowly than wild type barnase and more slowly than their corresponding dithiol form. The effect of placing a disulphide bond to cross-link parts of a protein which normally unfold early is dramatic. Bridge 43-80 unfolds 19 times more slowly than wild type barnase and 170 times more slowly than its dithiol form.

2.3 *Unfolding of Barstar and its complex with Barnase as studied by thermal analysis*

Initial thermal analysis experiments with Barstar, carried out by the Granada group, indicated that the protein sample was heterogeneous with respect to the oxidation state of the two cysteins at position 40 and 82. Nevertheless, the following preliminary results were obtained:

— Below pH 5 Barstar unfolds already at room temperature
— Its heat denaturation (most likely a two-state process) at pH > 6 is relatively reversible
— At neutral pH Barstar shows a high stability compared with Barnase. Its melting temperature (75°C) is 20°C higher than that of Barnase (55°C).
— The specific heat of unfolding is rather low in comparison with that of globular proteins of the same size.

The equimolar complex between Barstar and Barnase appears to be more stable than each of the partners alone! The complex melts around 80°C at

neutral pH (see above for the values of the individual partners). However, the unfolding of the complex did not proceed according to a two-state process. This can be explained by the fact that the heterogeneous Barstar forms complexes with Barnase of different stabilities. Another observation was that the complex was insoluble between pH 3.5 and 6 which required the measurements to be carried out at either very low pH (2.5) or above pH = 6. At pH 2.5, dissociation of the complex was observed with instanteneous unfolding of Barstar and only a calorimetric record of Barnase alone.
In order to overcome the problems described, the Cambridge group made mutants of Barstar lacking one or both cysteins (i.e. C40A, C82A and C40A + C82A).
All mutants, as expected, behaved much better in the scanning calorimeter. As was found for the wild type Barstar, also the mutants unfolded rapidly below pH 5. Furthermore, they showed after melting a strong tendency for aggregation which, however, was a slow process. During a second heating — following a quick cooling after the first melting — gave about 70-80% of the main peak recovery which is good enough for a practical determination of the thermodynamic parameters.
From the presence of a pretransition peak in the case of the mutants C40A and C40A + C82A (at pH 9.5 and 10.3), it was presumed that at concentrations used for the scanning calorimetry, these native (i.e. not unfolded) mutants have the tendency to form aggregates which dissociate before unfolding.
At the pH range 6-10.5, no differences in melting enthalpy between wild type and mutants was observed (Figure 3). Yet there were remarkable differences between the melting temperatures of the 4 proteins (Figure 4).

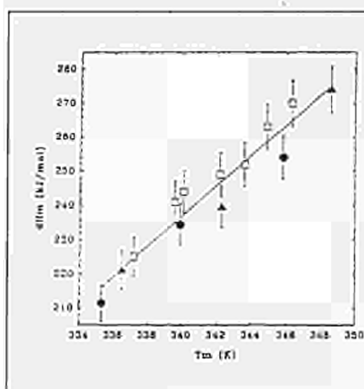


Figure 3

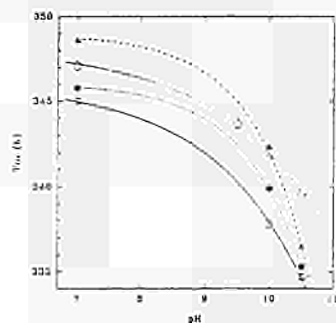


Figure 4

2.4 *Analysis of a 250 ps room temperature molecular dynamics trajectory of barnase.*

A 250 picosecond trajectory of solvated barnase has been generated by molecular dynamics simulations. The system included all the protein heavy atoms, as well as all hydrogen positions generated using standard bond distances and angles, 94 crystallographic positioned water molecules and 2265 generated water molecules.
The simulated protein displayed very modest deviations relative to the crystal structure (1.22 and 1.65 Å rms of the backbone and all atoms respectively). The accessible surface and corresponding accessible volume increased 17%

and 8% respectively over the 250 ps time scale, consistent with a slight expansion of the protein (0.5Å increase in gyration radius). A detailed monitoring of the H-bonds formed during the simulations indicated that a large majority of those identified crystallographically are maintained during the simulations. It also revealed frequent exchange between water and protein H-bonds, mostly in loop regions.

The structural and dynamic properties of the water molecules near the protein surface were analyzed by computing radial distribution functions for the water oxygens and hydrogens. The results were in excellent agreement with expected H-bonding distances and with distances between water and non-polar groups obtained in simulations of small water molecules. Computed self diffusion coefficients of water molecules around amide and AlaCß protons are very low compared to the water. Computed residence times of water molecules around certain atom types were very close to those determined experimentally for water surrounding BPTI using NMR techniques by others.

3. Results aiming at the understanding of the mechanism of enzyme kinetics

3.1 ***The interaction between Barnase and Barstar*** - The Cambridge group determined the dissociation constant ($K_D < 10^{-13}$ M) for the Barnase:Barstar complex from experiments measuring the on-rate ($\approx 5*10^8$ $M^{-1}.s^{-1}$ by fluoresence change of barnase) and the off-rate ($10^{-5}s^{-1}$ using tritiated barstar).

3.2 ***The binding of 3'-GMP to Barnase*** - The binding of 3'-GMP to wild type Barnase was measured by the Granada group using several methods such as Differential Scanning Calorimetry (DSC), Isothermal Calorimetry (IC) and Potentiometric Titration Calorimetry (PTC). Using these techniques, a full set of thermodynamic information about the interaction of Barnase with 3'-GMP was obtained at pH values ranging from 3.5 to 8.5.

A joint publication from the Granada and the Cambridge group about these results is currently in preparation.

3.3 ***Interaction of Barnase with methylphosphate: Molecular Dynamic Simulations*** — In a collaboration between Delft and Brussels, the work already mentioned in the previous progress report, was extended and finally published during the Protein Engineering Conference in Korpilampi (Finland). The major observation from this simulation is that 2 of the 5 PAME-molecules (Phosphoric Acid Mono Methyl Ester) move for more than 4 Å in the direction of the positively charged Arg83 and Arg87 in the active site cleft of Barnase (see Figure 1). This led to the conclusions that electrostatic interactions constitute an early driving force during substrate approach, and that the partially buried positive charges of Arg83 and Arg87 are more attractive for the negatively charged ions than solvent exposed positive charges.

With the new Molecular Dynamics technique *Multiple Copy Simultaneous Dynamics*, by which it is possible to simulate interactions between an enzyme and its substrate, the following preliminary results were obtained:

— In a 75 ps simulation of Barnase with 615 randomly placed PAME-molecules, only 15 were found to diffuse into the active site of the enzyme during the simulation. A larger number migrated towards the surface lysines.

— A second, 100 ps simulation of Barnase with 100 PAME-molecules placed in the active site, revealed that none left the active site at 300 K but did so at 500 K.

3.4 ***Use of free energy perturbation methods to analyse the origins of the pK_a shift of His18 in Barnase*** — The approach to the problem involved computing the free energy changes associated with the (de)protonation of His18 in the native protein (ΔG_P), and with a histidine dipeptide unit in water (ΔG_W). The pK_a-shift was calculated from the equation $\Delta pK_a = (\Delta G_P - \Delta G_W)/(2.3RT)$. The calculations were all performed with the Stochastic Boundery procedure (CHARMM — version 20). The result was $\Delta pK_a = 4.0 \pm 1.0$. Although qualitatively in good agreement with the experimental value ($\Delta pK_a = 1.6$ units), it is still a factor 2 too high, probably due to errors in treating electrostatic interactions, caused by applying a distance cut-off in computing non-bonded interactions. This is now being verified.
Also the from fluorescence experiments determined ΔpK_a of 0.6 pH-units, caused by the aromatic-histidine interaction between Trp94 and His18, could reasonably be substantiated by calculations: 0.40 pH-units.

WIDER CONSIDERATIONS

Proteins used in biotechnology become degraded during the course of reaction or on recycling. In some cases, it is the rate of unfolding that is important. The present results with the disulphide bridges point to a way of stabilizing such proteins. The structure should be examined to determine which regions unfold early, using either the kinetic procedures developed on barnase or other biophysical or computational methods. These early-unfolding elements of structure may then be stabilized by disulphide crosslinks.

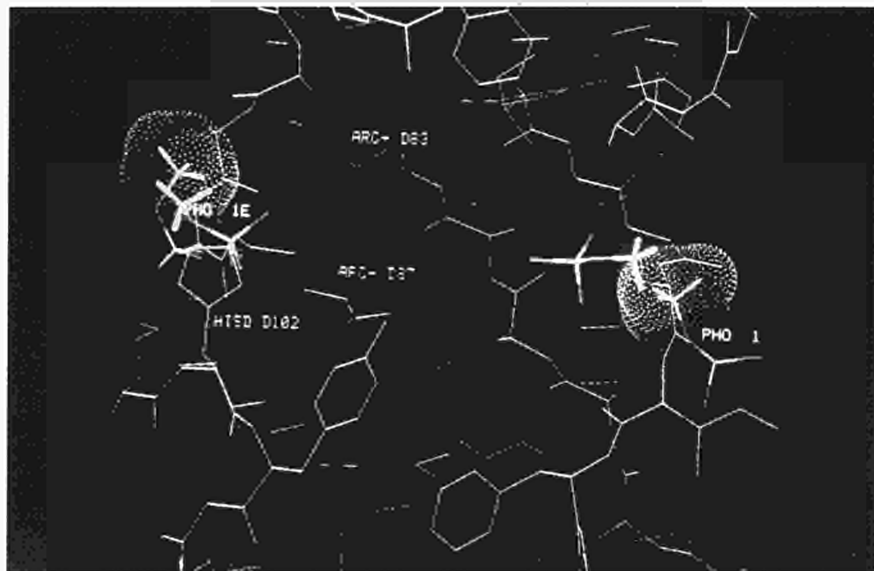


Figure 5: The initial (surface representation) and final (thick lines) positio of the two PAME molecules at the entrance of the active site of Barnase.

COOPERATIVE ACTIVITIES

Project meetings: 08/05/1992 — Granada, 11/09/1992 — Brussels, 05/02/1993 — Delft
Visits: D. van Belle (Brussels) to W. Aehle (Delft): 06/07, 30/09, 09/11-1992; van Belle and Aehle to M. Bycroft (Cambridge): 10/11/92; W. Aehle to D. van Belle: 11/08/92

LIST OF JOINT PUBLICATIONS WITH TRANS-NATIONAL AUTHORSHIP

Aehle, W., Misset, O. (1992), 3rd Nordic Conference on Protein Engineering, August 12-16, 1992 *'Molecular Dynamics Simulation of Barnase in water in presence of a substrate analogue?'*

OTHER PUBLICATIONS/PATENTS

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Stability studies and protein design studies with triosephosphate isomerases (BIOT CT-900182)

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H. KESSLER, Technical University of Munich, Munich, D
A. RENARD, Eurogentec, Liège, B

OBJECTIVES

After the first year of research, the main objectives have been:
- the design of a stable monomeric TIM by mutation of the wild type trypanosomal TIM.
- further characterisation of supercold (psychrophilic) TIM's and superhot (thermostable) TIM's.
- further characterisation of class-I cyclohexapeptides (loop-3 analogues) and class II cyclohexapeptides (inhibitory compounds).

MAJOR PROBLEMS ENCOUNTERED

The group of Dr. Hol has moved (September 1992) from Groningen (The Netherlands) to Seattle (USA). With the help of the EC officials Dr. Nieuwenhuis and Dr. de Taxis du Poët, an administrative procedure has been implemented to ensure that the crystallographic studies (on human TIM, hot TIM and cold TIM) can be continued.

RESULTS

The results are presented in the following sections. The major results are summarised in the section 'highlights/milestones'.

I. ICP (Opperdoes, Callens)

In collaboration with the EMBL group, class II cyclohexapeptides were further investigated. It turned out that the reported inhibitory properties of the class II cyclohexapeptides were not due to the specific binding between TIM and soluble cyclohexapeptides, but due to coprecipitation of TIM with precipitating cyclohexapeptides (manuscript in preparation). Because of this new knowledge, the study of the interactions between these cyclohexapeptides and trypanosomal TIM has been discontinued.

The kinetic properties of various interface point mutations (C14G, V46G, H47N) were investigated. The catalytic activity of these variants is concentration dependent. The apparent affinities for both substrates, GAP and DHAP, were somewhat lower with the highest increase in Km for the mutant C14G. Small differences in the pH stability and pH activity profiles have also been observed for these mutants.

The characterisation of Leishmania TIM has been initiated. In Leishmania TIM one of the conserved interface residues has changed (Gln65 in trypanosomal TIM and other TIM's is a glutamic acid).

II. EMBL (Wierenga, Borchert, Kishan, Noble, Siegert, Zeelen)

In a collaboration with Prof. Jaenicke (University of Regensburg) it could convincingly be shown by ultracentrifugation studies that under certain conditions the point mutations V46G, C14G, H47N behave as monomers.

A rather drastic deletion mutation was also carried out: residues 69-79 were deleted and replaced by four other residues (three additional point mutations in the residues flanking this 7-residue deletion were also included). This deletion mutant is a monomeric protein which is almost as stable as wild type TIM (interestingly, the point mutation variants are significantly less stable). Crystals have been obtained of the deletion mutant and of C14G, the data collected from these crystals are currently being evaluated.

The crystal structure of E. coli TIM has now been refined at 2.6Å resolution. The most surprising result is the structural differences at the dimer interface. In addition, the crystal structure of an E. coli TIM variant could be solved at 2.8Å resolution. In this variant, ETIM8CHI, the (βα)-unit 8 has been replaced by its chicken homologue, which involves 10 amino acid replacements. Although some significant structural adaptations are observed, ETIM8CHI has a stability similar to that of wild type E. coli TIM.

III. University of Liège (Martial, Goraj, Mainfroid, Rentier-Delrue, Houbrechts, Lion)

Human TIM

Interface residue Met14 has been mutated into a glutamine. This variant has been successfully produced in E. coli and purified. First enzymatic and structural analyses indicate that this variant is less active and less stable than the native enzyme.

E. coli TIM

An E. coli TIM variant has been constructed in which the eighth structural 'β/α'-unit has been replaced by its chicken homologue. This mutant enzyme can be routinely overexpressed in *E. coli* and purified. It is a stable protein and its properties have been characterised and documented in a joint Liège-EMBL paper, which has been submitted for publication.

Hot and cold TIM's

Large amounts of *B. stearothermophilus* and *Moraxella spp. TA137* TIM's have been purified for crystallisation experiments by the Hol group.
In order to extend this study to a wider range of extremophilic enzymes, other TIM genes are being isolated from the hyperthermophilic eubacterial strain *Thermatoga maritima* and from archebacteria such as the hyperpsychrophylic *Vibrio marinus* and the hyperthermophilic *Sulfolobus solfataricus*. The respective genomic libraries have been constructed and they are currently being screened.

Octarellins

A new octarellin sequence has been constructed and overexpressed. Its properties are currently being characterised; it seems to be more stable and more soluble than Octarellin I.

IV. University of Groningen/Seattle (Hol, Delboni, Mande)

Experiments have been performed with various TIMs provided by the Martial group:

Human TIM
The wild type structure has now been refined to 2.8Å resolution. Current R-factor is 16.7%, with good geometry. There is one dimer per asymmetric subunit. One subunit is in the open, unliganded conformation, the other subunit is in the closed, liganded (2-phosphoglycollate) conformation.

B. stearothermophilus TIM
Crystals have been obtained at pH 5.2 using 20% PEG4000 and 10% 2-propanol. There is one dimer per asymmetric unit. A 2.8Å dataset has been collected. The rotation and translation function have been solved, using trypanosomal TIM as a search model.

Moraxella TIM
Small crystals have been seen at pH 5.5 using 30% PEG4000 as precipitating agent.

Modelling:
For mutagenesis work with human TIM two different kinds of mutations have been proposed to the Martial group:
— interface point mutations to destabilise the dimer,
— point mutations to stabilise the human TIM dimer.

V. Technical University of Munich (Kessler, Guba, Schmitt)

Due to the inactivity of soluble class II cyclohexapeptides, the attempt will now be made to find cyclopeptides with affinity for trypanosomal TIM, using a receptor mapping program YAK (A. Vedani, SIAT, Basel) by interchanging the roles of ligand and receptor. Trypanosomal TIM is treated as ligand and a vector cluster analysis is performed to identify hydrophobic and hydrophilic anchorpoints situated in the selectivity region and the neighbouring cleft. Amino acid sidechains (the 'receptor') are linked to these anchor points and the resulting amino acid sequences will be synthesised and tested. Eventually, active sequences will be transformed into peptidomimetics to increase metabolic stability.

VI. Eurogentec (Renard, Dommes)

One task allocated to Eurogentec is to provide facilities for mass production of recombinant protein. Several fermentation runs (5 to 25 liters) of octarellin- and TIM-producing E. coli strains were performed for the laboratory of Prof. Martial, University of Liège. Lab scale and pilot scale purification facilities were also provided.

HIGHLIGHTS/MILESTONES

(1) A loop-deletion variant of trypanosomal TIM is a monomeric, stable protein.
(2) The crystal structure of E. coli TIM has been refined at 2.6Å resolution. A manuscript has been accepted for publication.
(3) A hybrid E. coli TIM, ETIM8CHI, has been constructed. This ETIM8CHI is a stable dimeric protein and its crystal structure could be solved.
(4) The crystal structure of human TIM has been refined at 2.8Å resolution.
(5) The sequences of cold (moraxella) and hot (B. stearothermophilus) have been compared.

COOPERATIVE ACTIVITIES

One major joint meeting was organised in Groningen, October 29, 1992 (host: Hol). In addition, meetings and visits between individual groups have taken place. Protein samples, sequences and experimental data have been exchanged.

LIST OF JOINT PUBLICATIONS / PATENTS WITH TRANSNATIONAL AUTHORSHIP

Rentier-Delrue, F., Mande, S.C., Moyens, S., Terpstra, P., Mainfroid, V., Goraj, K., Lion, M., Hol, W.G.J. and Martial, J.A. (1993), *J. Mol. Biol.* **229**, 85-93. 'Cloning and overexpression of the triosephosphate isomerase genes from psychrophilic and thermophilic bacteria: structural comparison of the predicted protein sequences'.

Borchert, T.V., Pratt, K., Zeelen, J.Ph., Callens, M., Noble, M.E.M., Opperdoes, F.R., Michels, P.A.M. and Wierenga, R.K. (1993), *Eur. J. Biochem.* **211**, 703-710. 'Overexpression of trypanosomal triosephosphate isomerase in Escherichia coli and characterisation of a dimer-interface mutant'.

Design and engineering of alpha-helical bundle proteins: modified structures and novel functions (BIOT CT-910262)

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OBJECTIVES

The goal is to understand, by a combination of theory and experiment, the structural principles of the 4-α-helix bundle proteins *rop* and *ferritin.* as a basis for engineering novel functions.

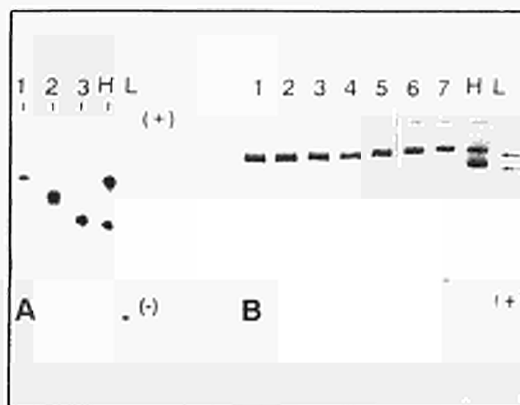
RESULTS

Ferritin as an engineered carrier protein — Milano

Ferritin renaturation
Ferritin subunits have an α-helical bundle structure and assemble to form highly stable 24-mer proteins. We now report the studies on ferritin refolding and reassembly. Far ultraviolet circular dichroism and fluorescence analyses showed that most ferritins and variants refold in similar conditions, irrespective of their large differences of denaturing conditions. The exceptions were the mutants produced to construct a ferroxidase centre in the L ferritin variant: the introduction of negative charges within the helical bundle had strong negative effects on the *in vitro* refolding. The other mutants readily formed the helical bundle, but reassembly was affected by the alterations of inter-chain interactions (del 1-13 and Leu169- > Arg) with the formation of subunit dimers, and by the alteration of the hydrophobic interactions along the two fold axes where two subunits come together, with the formation of folded subunit monomers. H and L subunit variants were renatured together thus obtaining heteropolymers with restricted heterogeneity and the expected subunit composition (see figure, page 68). These findings indicate that dimer formation is the first and essential step for ferritin assembly, and that the conservation of the interactions along the two fold axes permit the construction of polyfunctional molecules.

Ferritin as a carrier protein.

The C-terminal sequence of H ferritin was genetically fused with a 10 Kdal allergen. The construct was efficiently expressed by *E. coli* as a soluble and stable protein which retained most of structural and biochemical properties of ferritin and the allergenic properties of the allergen. It is suggested that fusion to ferritin overcomes the problems of solubility and high levels of expression often found for the production of peptides in *E. coli.*



Ferritin renaturation.

Formation of H and L chain heteropolymers of human ferritin. The homopolymers of H and L chains were denatured in 6 M guanidine HCl, pH 3.5 and renatured by dilution in buffer at pH 7.4. Panel A: isoelectric focusing of the renatured samples: H-homopolymer (lane 1), L-homopolymer (lane 3), mixture of the two homopolymers (lane H/L), heteropolymer made by equimolecular amounts of H and L chains. Panel B: non-denaturing electrophoresis of heteropolymers with increasing proportion of H chain (lane 1 to 7), and of the two homopolymers (lane H/L).

Selection systems for core mutants and epitope screening — Roma

During the second year of the contract, we have continued to provide support to the other groups and to develop and exploit genetic tools to study the folding of four-helix bundles and helix-helix packing.

Analysis of packing constrains in the hydrophobic core of Rop.

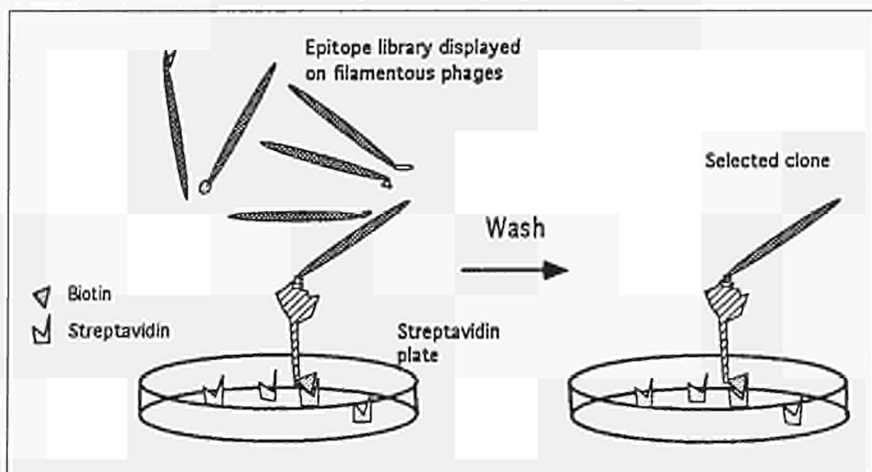
We have constructed and characterized (in collaboration with Münster and Iraklion) a series of Rop mutants with hydrophobic cavities in the core of the protein. Recently, we have exploited a genetic test (see first report), to ask whether the Rop core could accomodate larger residues. To this end we have constructed a large number of Rop mutants in which residues at positions 15(Ile), 19(Thr), 41(Leu) and 45(Ala) have been substituted by a random combination of five amino acids with a relatively large hydrophobic side chain (Leu, Ile, Met Phe, Val). We have characterized 20 random mutants for their ability to dimerize at 37 and at 42°C. Sequence analysis has confirmed that the vast majority of mutants do not show any longer the characteristic pattern of small and large side chains that was observed in the original description of the Rop structure (Banner et al.). Furthermore, simple calculations suggest that all mutants tested, compared to wild type, have an increase in the size of core side chains ranging from 20 % to approximately 60% Contrary to our expectation most of the random side chain combinations are tolerated by the Rop structure. Taken together with previous results, these results indicate that the Rop fold is mainly determined by the specific pattern of hydrophobic and hydrophilic side chains along its amino acid sequence, while precise and efficient packing of hydrophobic side chains in the core plays only a quantitative role in determining thermodynamic stability.

Filamentous bacteriophages as models for helix packing and vectors for peptide libraries

One of the aims of our project was that of exploiting a-helical bundles as scaffolds for the insertion of peptides and for the construction of peptide libraries. This was achieved with Rop and ferritin. More recently, we wanted to extend the scope of the technology by searching for protein vectors with the extra property of being able to bind their coding sequences. With this class of vectors, a selection proce-

dure would enrich a specific property of the hybrid protein and at the same time would permit the cloning of its gene and the characterization of the modification that has caused the change of property. For this purpose, we have chosen the a-helical major coat protein of bacteriophage M13 (gene VIII product). We have assembled a collection of 10^7 gene VIII, each of which synthesizes a different hybrid product formed by the native pVIII with an amino-terminal extension of nine amino acids. By probing this phage epitope library with a monoclonal antibody raised against a nonapeptide of human interleukin 1β, it was possible to select phages whose recombinant pVIII protein is recognized by the monoclonal antibody. Their characterization revealed a consensus sequence (SND) that is present in all the selected peptides. This consensus tripeptide is also found in the interleukin 1b peptide that was used to raise the antibody. This technology permits the mapping of immunogenic epitopes and the discovery of new ligands. Application in the diagnostic and pharmaceutical fields can be easily foreseen.

Selection system for epitope binding.



Selection from an epitope library of a filamentous phage that displays a peptide that binds a Fab fragment of immunoglobulin. The Fab fragment is linked to a plastic Petri dish via a biotin-streptavidin bridge.

Crystal structures of ferritin — Sheffield

Our work has concentrated on the X-ray analysis of the 4-helix bundle iron-storage protein ferritin. Our strategy has been to use site-directed mutagenesis in collaboration with Milano and Roma to alter internal residues and to observe the resulting structural effects. These results are being compared with similar experiments being conducted on Rop in Roma, Iraklion, Braunschweig and Heidelberg. We have characterised and begun to analyse species variants of ferritin in which much more extensive amino acid substitutions have been indtroduced.

Site-directed mutants of human H ferritin.

(i) We were able to extend from 3.1 to 1.9 Å the resolution of the A222 mutant of human H ferritin. In this mutant the ferroxidase site residues are converted into their inactive L equivalents (E62K, H65G) and in addition various metal-binding carboxyl residues are converted to alanine (E61A, E64A, E67A).

(ii) We have collected 2.5 Å resolution data on a Y34F mutation: Tyr 34 is close to the ferroxidase site and spectroscopic data indicate that it may be important in forming an Fe(III)-tyrosinate complex during iron uptake, but it will also be interesting to observe the structural effects of this mutation.

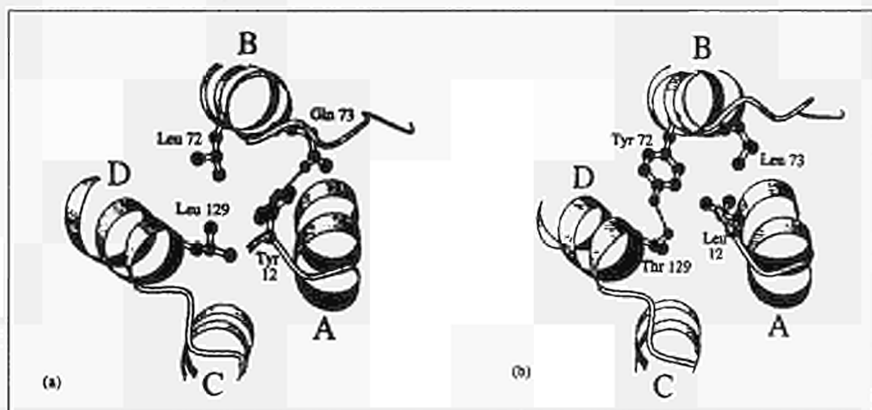
Species variants.

(i) We have extended the resolution of recombinant horse spleen L ferritin from its present 2.6 (wild-type) to 1.95 Å. Least-squares refinement is in progress. This protein has a small number of amino acid substitutions compared with rat L ferritin which we have also solved. Mammalian H and L ferritins have 55% sequence identity and some of the most interesting substitutions lie at the centre of the 4-helix bundle and involve the replacement of the metal-binding H chain ferroxidase centre residues with salt bridges.

(ii) We have extended the resolution of Schistosoma ferritin from 3.0 to 2.6 Å, and refined the structure. This protein has 40% identity with the mammalian H and L ferritins and consequently there are extensive changes in sidechains within the structure.

(iii) In recent months we also solved the structure of the ferritin from Escherichia coli at 2.5 Å by molecular replacement (I4, a = b = 130, c = 170). This protein is quite distinct from the haem-containing bacterioferritin, or cytochrome b1, which is also under investigation in Sheffield. E.coli ferritin has only 20%

Coupled amino acid changes in the ferritin core.



An example of coupled amino acid changes within the ferritin 4-helix bundle found when comparing mammalian ferritins (a), and E.coli ferritin (b). Tyr 12, Leu 72, Gln 73, Leu 129 in human H ferritin become respectively Leu, Tyr, Leu and Thr; the Tyr12-Gln73 hydrogen bond linking helices A and B disappears but a new Tyr72-Thr129 hydrogen bond appears between helices B and D.

sequence identity with the mammalian and Schistosoma ferritins whose structures we have previously solved. There are therefore many major amino acid substitutions, not only on the surface of the molecule, but also within the 4-helix bundle. These include a number of coupled amino acid substitutions within the bundle, one example being shown shown in the figure (page 70). Also, E.coli ferritin lacks a normally strongly conserved salt-bridge (which occurs in mammalian H and L, and in Schistosoma ferritins) which links the end of the B helix with the beginnings of the A and D helices. It will be interesting to determine the effect these and other changes have on the stability of the molecule.

X-ray crystallography, folding, and stability of Rop mutants — Iraklion

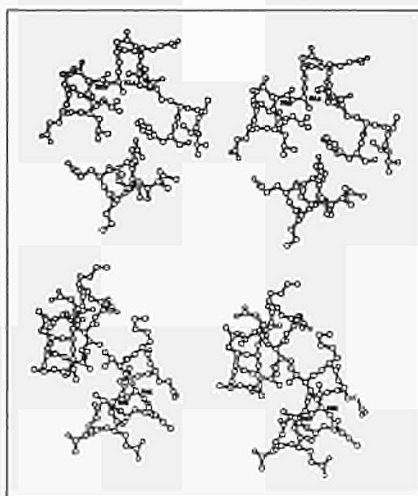
The crystallographic work in this part of the project has concentrated on ROP mutants with altered loop regions. Discontinuities in heptad periodicity of 4-α-helical bundles are frequently associated with loop formation. In order to test the sequence requirements for loops, a mutant (<2aa>) of ROP has been designed in which an uninterrupted heptad pattern is established with the insertion of two Ala residues in the loop. The structure of the dimeric mutant protein has been determined at 1.4 Å resolution, refined to an R-value of 18.9% and shows a larger part of the disordered C-terminus compared to wild-type ROP. Despite the removal of the break in heptad periodicity from the loop, the mutant protein folds again as a 4-α-helical bundle, with a bend of unsual conformation formed in the same position as in the wild-type protein. The conformation of the new bend is unique among the loop geometries of 4-α-helical bundles. The helical part of the structure shows very small changes relative to wild-type ROP. This confirms that folding in 4-α-helical bundles is dominated by helical packing interactions of hydrophobic nature and is tolerant to changes in the loop; loops adjust to the structural framework of the bundle and can even accomodate residues in unfavourable conformations, as it is the case of mutant <2aa>. Removal of breaks in sequence periodicity does not necessarily alter the overall folding of the bundle. X-ray diffraction data of <2aa> have been now collected to a resolution of 1.0 Å and will allow us to refine its structure at the highest resolution ever reached for 4-α-helical bundles. A further loop mutant, A31P, is very unstable and yields very small crystals. We are in the process of determining its structure by a combination of techniques.

An interesting correlation between protein stability and crystal properties has been derived from the ROP variants so far studied: For all mutants crystal size, sensitivity to X-irradiation, and diffraction limit are correlated to their stability (as determined by calorimetry) in a consistent manner, which is not understood in detail, but may be related to the structural flexibility of the protein. In practical terms, our results (which are confirmed by crystallizations of proteins from thermophile microorganisms) suggest that attempts to crystallize a protein are likely to be more successful if they are concentrated on the most stable species of the protein.

We have continued our analysis of ROP mutants with 'designed' cavities in the core. Our results suggest that the reduction of protein stability due to the cavity is correlated a) with the size of the cavity and b) with the change in the packing efficiency of the core. Sequence vs. structure relationships for loops and helical termini of 4-α-helical bundles as a basis for rational protein design were analyzed. Position-specific amino acid preferences for the N-terminal / C-terminal and central parts of the a-helices in bundles have been determined by a statistical analysis of structural and sequence data derived from seven families of aligned protein

sequences. In addition, we have analysed the correlations between loop conformations, loop length and topology in 4-α-helical bundles

Examples of the very conserved conformation of short loops.



Examples of the very conserved short loop conformations in 4-α-helical bundles: ROP (top) and cytochrome b562 (bottom). The loop residues are highlighted.

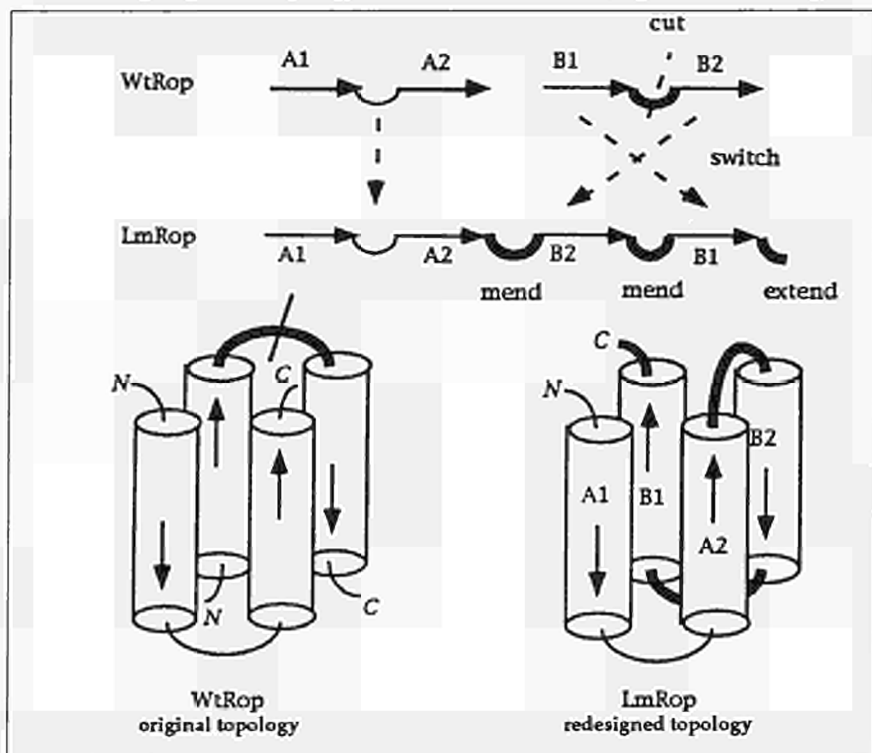
Thermodynamic stability and folding of Rop variants — Münster

Detailed thermodynamic and spectroscopic studies were carried out on Rop wildtype and mutated proteins to establish a quantitative basis for the contribution of noncovalent interactions to the stability of 4-helix-bundle proteins. The energetics of both heat- and GdnHCl-induced denaturation were measured by differential scanning microcalorimetry (DSC) and/or by following the change in circular dichroism in the far UV-range. Sedimentation equilibrium analyses were performed to characterize the state of aggregation of the protein. No intermediate species could be detected during thermal unfolding of the dimer in the absence of GdnHCl. Under these conditions ROP unfolding exhibits a strict two-state behaviour. The thermodynamic parameters for the reaction $N_2 = 2D$ are $\Delta HD = 580 \pm 20$ kJ. (mole of dimer)$^{-1}$, $\Delta C_p = 10.3 \pm 1.3$ kJ. (mole of dimer)$^{-1}$ K-1, and $T_m = 71.0 \pm 0.5$°C. The corresponding Gibbs energy change of unfolding is $\Delta G_D{}^0 = +71.7$ kJ. (mole of dimer)$^{-1}$ at 25°C and pH 6. In the presence of 2.5 M GdnHCl, however, ROP dissociates into monomers at elevated temperatures, as the loss of the concentration dependence of T_m and the decreased molecular weight demonstrate. The corresponding transition parameters are DH_D (2.5 M GdnHCl) $= 130 \pm 10$ kJ. (mole of monomer)$^{-1}$ and $T_m = 51.6 \pm 0.3$°C. Isothermal unfolding studies at 19°C using GdnHCl as denaturant yielded a Gibbs energy change of unfolding of 22.4 kJ (mole of monomer)$^{-1}$. This extrapolated value is 38% lower than the corresponding $\Delta G_D{}^0$-value of 35.85 kJ. (mole of monomer)$^{-1}$ calculated from thermal unfolding for the monomer in the absence of GdnHCl where the protein is known to be a dimer. The result suggests that subunit interactions are an important source of stabilization of the native four-helix-bundle structure of ROP.

Reengineering topology of loop connections

Regineering steps from wild type dimer (WtRop) to left handed monomer (LmRop). Top: at the gene level. Bottom: in three dimensions.

Redesigning ROP topology and core — Braunschweig and Heidelberg



The Rop protein has been redesigned by altering the topology of loop connections and turning a dimer into a monomer. For the left handed monomer (LmRop), X-ray data of crystals and results of NMR spectroscopy have now confirmed the stability of the reengineered protein and that it folds as originally designed. Right handed topology variants (RmRop) have now been designed by computer methods, the gene syntesized and expressed and the protein crystallized.

Work on mutations in the core is in progress (CmRop), building on the work of Iraklion, Rome and Münster. The exhaustive exploration of core mutations present a difficult computational problem that was approached by an efficient Monte Carlo method. Compensating changes in the hydrophobic core relative to the L41A and L48A mutants of Cesareni et al. have now been designed and are being expressed.

A third type of redesign aims at functional properties: inserts into the engineered Rop monomer of functional loops imported from other proteins (LmRop-Achr/Gloop/Hghr).

Table of selected engineered mutants of Rop protein

protein(s)	*description*	*status*	*remark*
LmRop1/ 2	left-handed monomers	NMR, Xtals, Xray data	first successful non-cyclic reengineering of topology
LmRop2 T21C/ K25C/ H44C	designed for crystal heavy atom derivatives	purified on mg-scale	stable proteins
RmRop1/ 2/3	right-handed monomers	expressed and purified, one Xtal	CD shows helical fold for RmRop1
CmRop1-10	compensationg core mutants	several proteins expressed	designed to fill holes left in L41A and L48A Rops
LmRop-Achr	insert of loop from acetylcholine receptor	purified on mg-scale	stable protein CD shows helical fold
LmRop-Gloop	insert of antigentic loop	expressed	unstable (proteolysis)
LmRop-Hghr	linsert of loop from growth hormone receptor	purified on mg-scale	stable protein CD shows helical fold

HIGHLIGHTS

Allergen carrier: Ferritin was engineered as a carrier for allergens.
Protein topology: Folded left- and right-handed Rop monomers demonstrate the protein engineer's ability to alter loop connections on a core framework.
Selection system for peptide libraries: A phage display selection system for specific epitopes was developed.
Guideline for crystallization: Protein crystallizability correlates with protein stability.
Bundle stability: Thermodynamic measurements determined the contribution of subunit interactions to the stability of the 4-helix Rop bundle.
High quality crystal structures: Higher resolution data and/or structures for ferritin (1.9 Å) and Rop (1.0 Å).

WIDER CONSIDERATIONS

A Bridge collaborative grouping of seven European universities and research institutes has concentrated on developing basic protein engineering techniques. As a research vehicle they use a particularly simple type of protein structure that occurs many times in nature as a basic protein building block and is made of four cylindrical substructures. Using a variety of techniques ranging from genetic engineering to biochemical and physical methods and design by computer software, the researchers have explored several new engineering principles for protein

molecules. Basic contributions have been made to the emerging discipline of biomolecular engineering - engineering at a scale 100 times smaller than the smallest elements of microchips - that will have both medical and technological impact in the years to come.

COOPERATIVE ACTIVITIES

Milano, Rome and Sheffield collaborate closely on ferritin, including exchange of material and results. Rome, Iraklion, Heidelberg, and Braunschweig do likewise for Rop protein. All groups exchanged information and results and had an intensive discussion and planning meeting in Heidelberg.

JOINT PUBLICATIONS WITH TRANS-NATIONAL AUTHORSHIP

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New ways of biotransformation in non-aqueous systems for the synthesis of pharmaceuticals. Application of supercritical gases, organic solvents, liquid membranes and microemulsions (BIOT CT-900176)

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OBJECTIVES

Our objectives for the reporting period are:
(1) Production and development of purification processes for new enzymes;
(2) Characterization of new enzymes;
(3) Modelization of enzymic reactions in Supercritical CO_2;
(4) Post reactional separations potentiality of supercritical fluids;
(5) Enzyme behavior in supercritical fluids;
(6) Role of microemulsions on enzyme structure function relationship;
(7) Modelization of enzymic reactions in microemulsions and supercritical CO_2;
(8) Comparative studies on chiral resolution catalyzed by lipases in organic solvents and supercritical CO_2.

RESULTS

Braunschweig lab.
Lipases from various sources were produced, purified and characterized. Lipase from *Penicillium expansum* DSM 1994: This strain contained the highest lipase activity observed during a screening of 40 isolated microorganisms and several commercial strains. It has been purified 219 fold resulting in a final specific activity of 560 U/mg. A molecular weight of 25 kDa was determined by SDS-Page electrophoresis, in absence of detergents active dimmers and large aggregates are formed. This lipase showed no positional specificity in the hydrolysis of different triglycerides and a maximum activity at 45° C and alkaline pH. The N-terminal sequence show an indication for a new type of lipase. Lipase from *Pseudomonas cepacia:* The protein-chemical characterization of this thermostable lipase has finished. The one-step purification procedure proposed enables an easy access to an homogeneous lipase, which are not commercially available. Lipase from *Penicillium simplicissimum*: This lipase has been distributed to other partners for studies on esterification reactions. Although it has been found very interested it can not be reproduced in solubilized form since the commercial inducer used is not longer available. Different substitutes failed to produce free lipase. From three new fermentation broths this lipase can only be solubilized by addition of 1% Triton-X100. All experiments for separation of the detergent from the enzyme resulted in high losses of activity.

The studies have been extended on lipase behavior in supercritical CO_2. For this reason a suitable multi-reactor system with an option for an automized switching between different reactors or other system components has been established. Also, in view of the strong influence of the method used for the enzyme withdrawal from the supercritical phase on its activity, a new on-line colorimetric assay for lipase activity was developed. Various factors effecting lipase activity were explored such, as water content of the systems and the influence of the support material on enzyme activity in the cases when immobilized lipase was tested.

Toulouse lab.
In the previous report a work has been described on the stability, influence of the water content and kinetics for the enzyme reaction in supecritical carbon dioxide in an attempt to compare this medium with organic solvents for enzymatic reactions. During this reporting period the work concerns the extension of the studies to continuous reaction in a packed bed of enzymes, coupled with separation process and recycling of supercritical CO_2. This recycled operation is the most likely to be extended to a large scale level.

Studies on the determination of the kinetic parameters on the esterification reaction of oleic acid with ethanol catalyzed by lipase in supercritical CO_2 were carried out for modelization of a continuous reactor and for obtaining further information about some additional kinetic parameters. Also, a pilot plant for continuous reaction and post reactional separation has been built up, using four separators and the same reaction catalyzed by lipase was studied. A 92.1% of the ester was recovered with a concentration of 851 g/L and purity about 93.3 in mass. When the same reaction was carried out in n-hexane, an ester concentration of only 409 g/L and purity of 49% in mass was obtained. The results of this work emphasize the interest of using the fractionation potential of supercritical CO_2. Another important observation is that the water content must be controlled through out the all process because it influences greatly the activity of the biocatalyst. It is very likely that this problem must be treated by numerical modeling. Another aspect, that has now to be developed, is to consider reactions that are limited by thermodynamic equilibrium. In this case such systems may be operated in the extractive reaction model, i.e. by condensing selectively one product in order to reduce the reverse reaction and to overcome thermodynamic limitation.

Madrid lab.
Transesterification between ethyl butyrate and glycerol using native, chemical modified and immobilized lipase in a two phase system was investigated. Optimal conversion was obtained in the presence of 5% water. Also, the selective hydrolysis of peracylated sucrose by serveal lipases in water, two-phases system and AOT-microemulsion system was extensively studied.

Hannover lab.
Chiral resolutions of racemic 3-hydroxy esters were performed in organic phases with lipases from *Pseudomonas cepacia, Chromobacterium viscosum* and Porcine pancreas. Also supercritical CO_2 was tested as non-aqueous media. The reaction conditions have been optimized with 3-hydroxy octanoic acid methyl ester. The reactions were performed in batch and in continuous process. In a solid bed reactor the continuous enantioselective separation of the substrate (99%eeS) was possible after a short time. Reactions performed in supercritical CO_2 showed larger reaction times with a decrease of the residual activity of the enzymes. The obtained enantioselectivities are similar to those obtained in non-polar organic media. Different organic solvents have been tested showing a tedious correlation with the hydrophobicity of the solvents expressed as log P. The reaction time was

shortened six fold by using irreversible acylating agents. It has been found solvent type, lipase type and acylating agent acting as tools for changing the enantioselectivity. The influence of water was also tested. Lipase from *P. cepacia* was lyophilized at different pH and the influence of the amount of water added was investigated. The water activity was measured, during the reaction on-line, with a humidity sensor. Water activities greater than 0.4 led to a decrease in enantioselectivity and reaction rate. In the optimized system the resolutions of other 3-hydroxy esters were tested. Aliphatic compounds reacted with lower enantioselectivity, only the substrates could be isolated in high enantiomeric purity. In contrast, aromatic 3-hydroxy esters were acylated by lipases with high stereoselectivity.

The lipase catalyzed reaction of racemic 3-hydroxy octanoic acid methyl ester (R,S-1), using vinyl acetate as acylating agent, in dodecane was chosen for kinetic studies. The apparent kinetic constants Vmax and Km were determined. From the data derived an ordered bi bi mechanism with substrate inhibition, due to vinyl acetate action at high concentrations, is suggested. No negative effects of the products on the reaction rate could be observed.

Athens lab.
The activity of lipases from *Rhizopus delemar, Rhizopus arrhizus* and *Penicillium simplicissimum* entrapped in microemulsions formulated by bis-(2-ethylhexyl)sulfosuccinate sodium salt (AOT) in isooctane has been studied in esterification reactions of various aliphatic alcohols with fatty acids. There are several important information derived from this studies concerning the activity of the three lipases in anionic microemulsion systems. The results on esterification reactions of various fatty acids with alcohols catalyzed by these lipases showed a remarkable selectivity regarding the chain length and the structure of the substrates. Fluorescence quenching measurements suggest that there are not any significant changes of the shape and size of the microemulsions, caused by the substrates, which could have been related to the enzyme selectivity. Extensive studies using a large number of substrates with different degree of polarity, as well as spectroscopic techniques, showed that this selectivity appears to be related to the localization of the enzyme molecule within the micellar microstructure, due to the hydrophobic/hydrophilic character of the protein, and not to a specificity of the enzyme itself. This hypothesis can further be supported if one takes into consideration the information concerning the structural differences of these lipases. In conclusion, we could point out that this work illustrates the importance of the enzyme structural properties, as far as the hydrophobic/hydrophilic character of the protein molecule is concerned, in addition to the effect of the micellar microenvironment on the lipase selectivity in esterification reactions taking place in microemulsions.

The kinetics of the esterification of lauric acid by (-)menthol, catalyzed by *Penicillium simplicissimum* lipase, were, also, determined in water/AOT/isooctane microemulsions. Due to their low water content, microemulsionns assist in reversing the direction of lipase activity, favoring synthetic reactions. The kinetics of this synthesis follow a Ping-Pong Bi-Bi mechanism. The values of all apparent kinetic parameters were determined. The theoretical model for the expression of enzymic activity in reverse micelles, proposed by Verhaert et al. (Verhaert, R., Hilhorst, R., Vermue, M., Schaafsma, T.J. and Veeger, C. (1990) Eur. J. Biochem. 187, 59-72.) was extended to express lipase activity in an esterification reaction involving two hydrophobic substrates in microemulsion systems. The model takes into account the partitioning of the substrates between the various phases and allows the calculation of the intrinsic kinetic constants. The experimental results showing the dependence of the initial velocity on the hydration ratio w_0 of the reverse micelles,

were in accordance with the theoretically predicted pattern. The determination of the kinetic parameters of the esterification reaction of (-)menthol by lauric acid was performed by initial velocity analysis. (-)Menthol concentration was varied at various fixed concentrations of lauric acid.

HIGHLIGHTS/MILESTONES

- Influence of reaction phase composition (additives, cosolvents) on enantioselectivity.
- Influence of reaction conditions (temperature, stirrer speed, reactor design) in batch and continuous processes.
- Enantiomeric separation strategies for a GC process monitoring.
- Development of optodes for pH measurements in organic solvents.
- Potentiality of supercritical CO_2 for post reactional separations.- Development of a pilot plant for continuous reaction in supercritical CO_2 and post reactional separation.
- Enzyme stability in supercritical CO_2.
- Influence of water on enzyme activity in supercritical CO_2.
- Influence of surfactant nature and organic solvent hydrophobicity on lipase specificity.
- Enzyme reaction mechanism in microemulsions.
- Production, purification and characterization of new lipases.
- Development of a colorimetric on-line assay for lipase activity in supercritical CO_2.

WIDER CONSIDERATIONS

Participation in the COMETT-program. Four workshops (each one day) were performed in Athens in October. About 12 other workshops will be organized in France, Germany and Greece in 1993.
The Italian Firm Recordati SPA was informed about this project from the Project Report of 1992 and showed an interest for cooperation.

COOPERATIVE ACTIVITIES

Exchange of materials:
Purified microbial lipases have been send from GBF lab to Athens lab and to Hannover lab. Merck Darmstad supported the project by supplyingfine chemicals (total value 8000 ECU for 1992). Modified enzymes has been send from SCIC lab to Toulouse lab and Hannover lab for studies of various esterification reactions.

Exchange of staff:
Three members of Toulouse lab visited Athens lab for 2 weeks for establishing a Supercritical CO_2 reaction unit in the latter lab.

Joint experiments:
One member of Hannover, Braunschweig and Toulouse lab visited Athens lab for 2 weeks participating in common experiments and exchange of know-how.

Joint meetings:
Meeting of all partners in Noordwijkerhout, The Netherlands, 26 April1992, during the International Symposium on 'Biocatalysis in non-conventional media'.

JOINT PUBLICATIONS

H. Stamatis, A. Xenakis, S. Sztajer, U. Megne and F.N. Kolisis (1992). Studies on the specificity of *penicillium simplicissimum* lipase catalyzed esterification reactions in microemulsions. *Elsevier Science Publishers., Ed. J. Tramper.* Progress in Biotechnology vol. **8**, 733-738.

T. Scheper, U. Bornscheuer, A. Capewell, A. Herar, H. G. Hundeck, H. Meyer, F. Schubert and F.N. Kolisis (1992). A comparison of enzymatic reactions in aqueous, organic and multiphase systems. *Elsevier Science Publishers., Ed. J. Tramper.* Progress in Biotechnology vol. **8**, 37-44.

U. Bornscheuer, A. Herar, L. Kreye, V. Wendel, A. Capewell, H.H. Meyer, T. Scheper and F.N. Kolisis (1993). Factors affecting the lipase catalyzed transesterification reactions of 3-Hydroxyesters in organic solvents. *Tetrahedron Lett.,* **5(4)** in press

H. Stamatis, A. Xenakis, U. Menge and F.N. Kolisis (1992). Kinetic study of lipase catalyzed esterification reactions in w/o microemulsions. *Biotechnology and Bioengineering* (accepted).

A. Capewell, U. Bornscheuer, A. Herar, T. Scheper, H. Meyer, and F.N. Kolisis (1992). A comparison of enzymatic reactions in aqueous, organic and supercritical phases, *DECHEMA* Biotechnol. Conf., VCH, Weincheim **5**, Part A, 57-60.

Enzyme catalysis in non-conventional media. Review article by all partners, under preparation.

OTHER PUBLICATIONS:

Marty, A., D. Combes and J.S. Condoret (1993), Continuous reaction separation process for enzymatic esterification in supercritical CO_2. *Biotechnol. Bioeng.* in press

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A. Xenakis, H. Stamatis, A. Maliaris and F.N. Kolisis (1993). Effects of alcohols on the structure of AOT reverse micelles with respect to different enzyme activity. *Progr. Colloid Polym. Sci.* , accepted.

U. Bornscheuer (1993) Reaktiostechnische Untersuchungen zur enzymatischen Racematspaltung verschiendener 3-hydroxyester in unkonventionellen Lösungsmittelsystemen, VDI Fortschrittsberichte, Reihe 17, Heft 87.

Characterization and surface properties of semi-synthetic redox enzymes for application in biosensor devices (BIOT CT-910279)

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OBJECTIVES

1. Simplification of the synthesis of N^6-(2-aminoethyl)-NAD.
2. Synthesis of N^6-(2-aminoethyl)-FAD on preparative scale.
3. Development of a synthesis of N^6-(carboxyalkyl)-FAD from FMN (Riboflavin-5'-phosphate) and N^6-(carboxyalkyl)-AMP (alkyl: $(CH_2)_n$, n: 1-11).
4. Development of a synthesis of C(8)-functionalized FAD and functionalized FMN by modification of the 5'-phosphate moiety.
5. Preparation, characterization and electron transfer studies of FAD dependent oxidase-relay derivatives with glucose oxidase as model redox enzyme.
6. Isolation, characterization and electrochemistry of NADH oxidase from *Thermus aquaticus.*
7. Screening of thermophilic bacteria exhibiting activities of thermostable oxidases of potential importance for the design of biosensors.

MAJOR PROBLEMS ENCOUNTERED

Concerning N(1)-(2-aminoethyl)-FAD, N^6-(2-aminoethyl)-FAD and the byproduct 1.N^6-(ethanoadenine)-FAD, their purification from reaction mixtures on a preparative scale turned out to be difficult and could not satisfactory solved yet.

For the coupling of FMN and N^6-(carboxyalkylated)-AMP by diphosphate coupling after the obligatory activation of the 5'-phosphate moiety of the latter compound reaction conditions where the simultaneously activated carboxyl group not severely interfered could not be found.

Initially, the major difficulty was the choice of the chromogen for a test system with maximal selectivity to detect the formation of H_2O_2 by oxidase activities screened for in colonies of thermophilic strains.

RESULTS

NAD analogues

Work was finished concerning the development of a simplified procedure for the synthesis on preparative scale of N^6-(2-aminoethyl)-NAD (up to 10 g.) of importance for the synthesis of covalent dehydrogenase-NAD adducts (e.g. for L-phenylalanine dehydrogenase) and the new NAD analogue tricyclic 1.N^6-

(ethanoadenine)-NAD (1). Both NAD analogues have been used as structural probes with respect to the adenine binding site of lactate dehydrogenase isoenzyme H_4 from porcine heart (2).

FAD analogues

The aim is the development of chemistry to introduce in the adenine moiety of FAD functional groups ($-NH_2$ or -COOH) at variable distance to the C(6)- or C(8)-position determined by the length of the spacer inbetween. Final aim is to couple such FAD analogues covalently to the apoenzyme form of FAD dependent oxidases of interest for biosensor development in such a way that the enzymic activity is maintained (e.g. for glucose oxidase, D-amino acid oxidase, glutamate oxidase or NADH oxidase).

Three different strategies of synthesis for the functionalization of FAD are currently under investigation:

I) Synthesis of functionalized FAD from riboflavin-5'-phosphate (FMN) and functionalized AMP.

II) Synthesis of functionalized FAD from riboflavin and functionalized ATP using the bifunctional enzyme flavokinase-FAD synthetase.

III) Functionalization of FAD by chemical modification of intact FAD.

I) The preparation of the starting compounds FMN and N^6-(carboxyalkylated)-AMP with different chain length has been elaborated successfully. For this, a 1-step procedure based on silica gel chromatography has been developed for purifying FMN to approximately 100% on preparative scale (up to 1 g, yield 40%) from commercially available FMN containing around 6% riboflavin-3'-phosphate, 10% riboflavin-4'-phosphate and 10% riboflavin as was checked by HPLC. For synthesizing N^6-(carboxyalkylated)-AMP (up to 1 g, approximately 100% pure, yield 60%) by successively phosphorylation of 6-chloropurine-riboside to 6-chloropurine-riboside-5'-phosphate and N^6-carboxyalkylation of the latter compound procedures given in the literature were adopted without significant changes. For the diphosphate coupling one of the phosphate moieties either of FMN or of N^6-(carboxyalkylated)-AMP had to be activated. Two activation procedures were investigated, with carbonyldiimidazole or with dipohenylphosphorylchloride.

The phosphate moiety of the functionalized AMP was choosen for activation, since FMN easily cyclizes to riboflavin-4',5'-cyclic-phosphate under activation conditions.

Presumably, due to a strongly competitive simultaneous activation of the carboxyl group the diphosphate coupling could not succeed. It should be emphasized that the synthesis of FAD from FMN and AMP under equal conditions gave no problems. Due to this negative result another strategy is currently under investigation: synthesizing first the 6-chloro-derivative of FAD by diphosphate coupling of FMN and 6-chloropurine-riboside-5'-phosphate and final carboxyalkylation with aminoalkylcarboxylic acids by substitution of the C6 bound Cl.

A second strategy might be a combined chemical/enzymical synthesis by diphosphate coupling of FMN and N^6-(nitrilealkyl)-AMP to N^6-(nitrilealkyl)-FAD and subsequent transformation of the nitrile group to a carboxyl group by nitrilase or the combination nitrile hydratase/amidase under the premise that the nitrilated FAD will be a good substrate for the enzymes.

II) Functionalization at the C(8) position of the adenine moiety of coenzymes like AMP, ADP, ATP, NAD, NADP can be achieved by intermediate bromination of the C(8) position with Br_2 under aqueous conditions and subsequent substitution reaction with functionalized aminoalkyl compounds. This approach is not known for FAD, presumably due to reactivity of the isoalloxazine moiety with respect to Br_2.

The 8-Br-FAD analogue has been synthesized enzymically using the bifunctional enzyme flavokinase-FAD synthetase from *Brevibacterium ammoniagenes*. The microorganism was cultured and the enzyme purified from cell extracts according to published procedures.

The enzyme is known to use a variety of modified flavins as substrates, but its activity with ATP derivatives has not previously been examined in detail. For the first time it was found that when 8-Br-ATP replaces ATP in the enzyme reaction with riboflavin or FMN, 8-Br-FAD is synthesized (Fig. 1). Potentially, this enzymic method might be useful to synthesize both N^6- and C(8)- functionalized FAD analogues from the corresponding ATP analogues, if these are accepted as substrate by the enzyme and the enzymic reaction can be scaled up. If this scale-up is without perspective, the pure chemical approach by coupling 8-Br-AMP to FMN followed by the substitution reaction as previously indicated may turn out the method of choice.

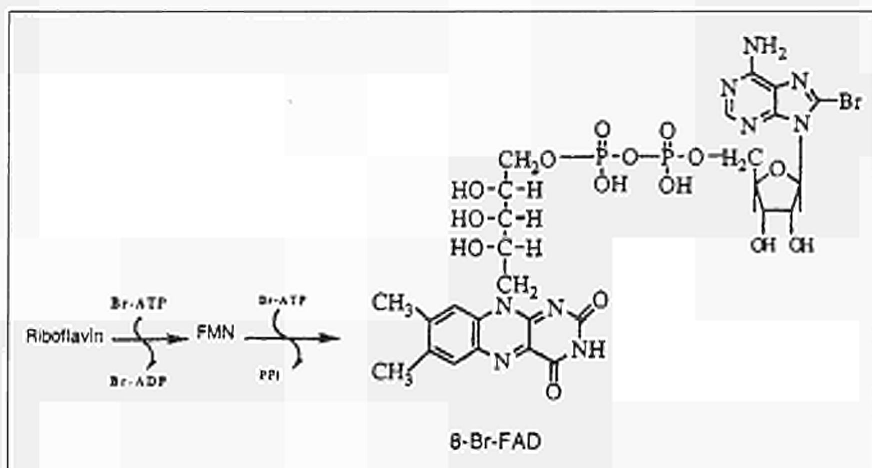

Fig. 1. — Enzymic synthesis of 8-Br-FAD with flavokinase-FAD synthetase from Brevibacterium ammoniagenes.

III) The introduction of functional groups in the adenine moiety of FAD has been achieved by an in 1991 patented method: alkylation with ethyleneimine to introduce a 2-aminoethyl group at the N(1)-position followed by Dimroth rearrangement under unusual mild aqueous conditions (Fig. 2, see next page). Starting out from 11 g FAD (12.7 mmol) reaction conditions have been found leading to reaction mixtures, containing unreacted FAD (2.5 mmol, 19.7%), N(1)-(2-aminoethyl)-FAD (8.5 mmol, 67%) and byproducts, supposedly, mainly N(1)-(2-aminoethylaminoethyl)-FAD (1.68 mmol, 13.3%) after alkylation and, after Dimroth rearrangement, unreacted FAD (2.28 mmol, 18.8%), N^6-(2-aminoethyl)-

FAD (6.69 mmol, 55%), 1.N^6-(ethanoadenine)-FAD (1.82 mmol, 15%) and byproducts, presumably, mainly N^6-(2-amino-ethylaminoethyl)-FAD after rearrangement of N(1)-(2-aminoethylaminoethyl)-FAD (1.37 mmol, 11.3%). Finding methods for the purification of modified FAD from both reaction mixtures on a preparative scale turned out to be difficult. Currently, the development of procedures is under investigation based on ionexchange and adsorption using HPLC and middle pressure chromatography. Nevertheless, sufficient N(1)-(2-aminoethyl)-FAD, N^6-(2-aminoethyl)-FAD and tricyclic 1.N^6-(ethanoladenine)-FAD have been obtained to assign their structure by ^{1}H-NMR and positive ion FAB-MS. It should be stressed that the products N(1)-(2-aminoethylaminoethyl)-FAD and N^6-(2-aminoethylaminoethyl)-FAD with elongated spacer may become the main products, if the pH and the ethyleneimine concentration are increased for the alkylation reaction. These latter FAD analogues have not yet been obtained in pure form.

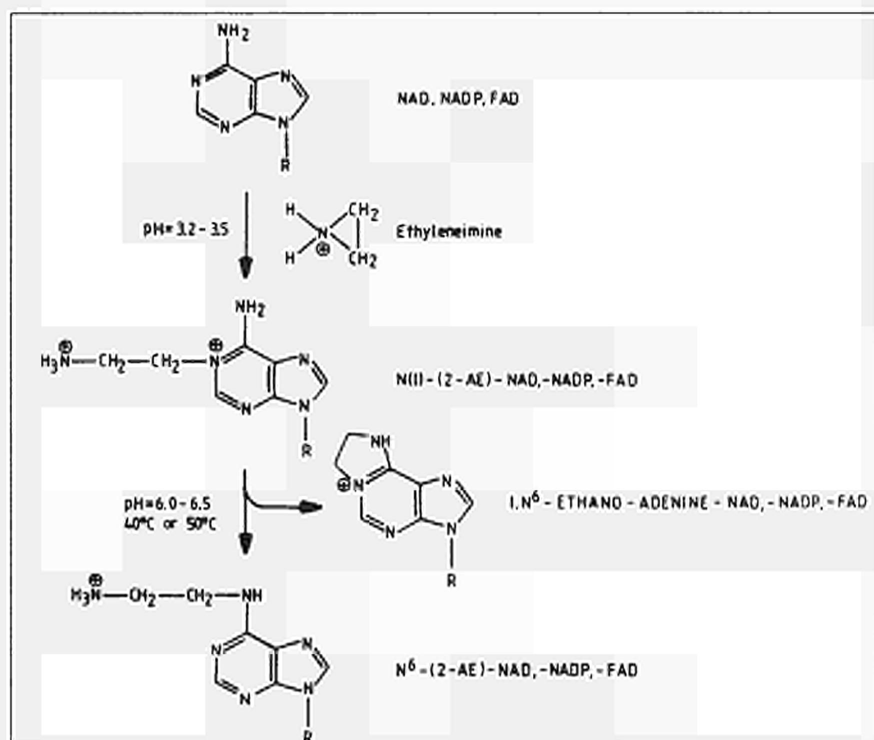

Fig. 2. — Reaction pathway for the simplified synthesis of N^6-(2-aminoethyl)-NAD, -NADP and -FAD

NB: The reaction mechanism of the unexpectedly fast simultaneous conversion of N(1)-(2-aminoethyl)-NAD, -NADP and -FAD to N^6-(2-aminoethyl)-NAD, -NADP and -FAD and tricyclic 1.N^6-(ethanoadenine)-NAD, -NADP and -FAD under mild aqueous conditions has been elucidated using 6-$^{15}NH_2$-N(1)-(2-aminoethyl)-adenosine as model compound based on ^{15}N NMR and positive ion FAB-MS (3).

The tricyclization of the N(1)-(2-aminoethyl)-adenine moiety to a $1.N^6$-ethanoadenine moiety in aqueous solution is a new reaction in heterocyclic organic chemistry.

FMN analogues

The aim is to synthesize functionalized FMN analogues by modification of the 5'-phosphate moiety. No results are available yet, but ideas on a promising procedure exist. The final aim is to couple covalently functionalized FMN to the apoform of FMN dependent oxidases of interest for the development of biosensors (e.g. cytochrom b_{12} having NAD independent L-lactate dehydrogenase activity or NADH oxidase that accepts both FMN and FAD as coenzyme).

Redox studies concerning electron acceptors interacting with glucose oxidase

One of the first objectives in the field of research concerning redox mediators in amperometric biosensor systems is the synthesis and characterization of some functionalized redox compounds. These should, covalently bound to redox enzymes, mediate as electron relays intra-molecular coenzyme oxidation and facilitate direct electron transport from the enzyme to the detecting device of amperometric biosensors. As a model redox enzyme glucose oxidase (GOD) from *Aspergillus niger* was choosen due to its high catalytic activity and its multiple application in the field of enzyme technology and clinical and analytical chemistry. As artificial electron acceptors, organic compounds with quinoid structure were studied, including pyrrolo-quinoline quinone (PQQ). PQQ was investigated as a possible electron acceptor for GOD in the pH range 2.4-8.0 for enzyme concentrations ranging from 18 nM to 1 μM, When PQQ was added under anaerobic conditions to a mixture of glucose and GOD in the pH range 6.0-8.0, usual for analytic reaction conditions, no reaction could be detected despite the fact that PQQ is known as an oxidizing agent with a redox potential for the couple $PQQ/PQQH_2$ at pH 7.0 of + 90 mV. By gradually decreasing the pH from 6.0 to 3.0 the rate of the reaction smoothly increased. From the recorded UV-VIS spectra it could be seen that together with the consumption of PQQ (decrease of A_{249}) and formation of $PQQH_2$ (increase of A_{314} and A_{369}) a new peak at 214 nm develops in time due to glucose oxidation to finally gluconic acid. A 1:1 stoichiometry with respect to gluconic acid and $PQQH_2$ was clearly demonstrated by analyzing the gluconic acid formation by HPLC. Kinetic studies on the reduction of PQQ with reduced GOD at different pH values show that PQQ acts as oxidant for the enzyme only at acidic pH values. An increase of the reaction rate with at least three orders of magnitude was observed when the pH decreased from 5.0 to 3.0.

The second-order rate constant for the reaction of PQQ with GOD at the pH optimum 3.0 (k_{cat}:5.6 sec^{-1}) is of the same order of magnitude as determined for ferrocene and its derivatives as artificial electron acceptor reacting with GOD at neutral pH values.

This result together with the fact that PQQ and ferrocene including its derivatives function respectively as $n=2$ and $n=1$ electron acceptor suggests a different electron transfer mechanism.

In order to obtain a better insight in the mechanism of the reaction of PQQ with reduced GOD, the reaction of the enzyme with organic compounds with a quinoid structure was examined [PQQ, 2,6-dichlorophenol indophenol (2,6-DCIP) and p-benzoquinone (pBQ)]. The results indicate for 2,6-DCIP a similar pH dependent reaction behaviour with respect to GOD as PQQ. Again the value of the reaction

rate increases when the pH decreases with a maximum at pH 3.0. At acidic pH pBQ reacts at a rate about one order of magnitude higher than the rate determined for 2,6-DCIP and PQQ. Actually, pBQ reacts with high rates with respect to reduced GOD over the entire pH range 2.6-8.0, suggesting that pBQ can react with both the protonated and unprotonated form of the reduced FAD within the enzyme. From the pH dependency of the rate constant for the oxidation of GOD by pBQ it can be assumed that the principal mechanism of the reduction of pBQ involves the transfer of 2 electrons and 2 protons at low pH values, while at higher pH values the reaction proceeds by 2 successive steps of one electron transfer with the intermediate formation of both a flavin and quinoid semiquinone.

Obviously, the basic mechanism of the reduction of PQQ and 2,6-DCIP by reduced GOD is similar despite their difference in structure, PQQ being an ortho-quinone and 2,6-DCIP a para-quinoimine. The explanation for this similarity may be that the reactive form of PQQ has a para-quinoid structure due to internal tautomerization. In contrast to PQQ, 2,6-DCIP can serve as electron acceptor above pH 5.0. Beside the para-quinoid structure its charge affects the interaction with the protein environment and influences the pH dependency of the reaction. In addition, the carboxylic groups of PQQ with pK_a values ranging from 5.0 to 2.0 may affect the onset and rate of the reaction. In other words, the carboxylic groups of PQQ with a pK_a of around 5.0 must be protonated before charge transfer-complex formation and catalysis can take place.

The difference between the reactivities of PQQ and 2,6-DCIP was also confirmed by their behaviour with respect to FMN. The iso-alloxazine moiety of the flavin in the oxidized form is an electron-deficient molecule. This property allows interaction with any other electronricher molecule. In aqueous solution, the iso-alloxazine ring is strongly polarized, involving the carbon atoms in positions 8, 6, 5a, 10a and 2. The resulting mesomeric structure is stabilized by a strong hydrogen bonding to the carbonyl oxygen in position 2. When PQQ is added to an aqueous solution of FMN an interaction between both molecules occurs through the carboxylic acid groups, quinone moiety and the pyrrol ring of PQQ, the pyrimidynoid ring of FMN and the benzenoid rings of both compounds. The charge transfer complex formation between PQQ and FMN resulting from these interactions could be confirmed by UV-VIS spectrophotometriy, fluorescence spectra and ^1H NMR. Their complex is stable in time and its formation is favoured by changes in pH from neutral to acidic values. By fluorescence studies the dissociation constant between PQQ and free flavin at acidic pH values has been estimated and expressed as pK_d with value 13 $\pm$ 1. The observation that PQQ is a strong complexer with respect to flavin leads to the suggestion that this association might be of importance for the reaction with FAD containing GOD.

Using fluorescence measurements to study the interaction between PQQ and GOD, the phenomenon of a strong association at acidic pH values was observed. There is a quenching of quinone fluorescence and also the appearing of a new fluorescence, that may result from certain non-covalent contacts of PQQ with several amino acid side chains of the particular environment of the FAD binding site and complexation with the flavin moiety of FAD. The observation that the association between PQQ and GOD is favoured under acidic conditions emphasizes the protonation of the carboxylic groups of PQQ as condition for appropriate structural interaction resulting in electron transfer from the reduced iso-alloxazine moiety of FAD to PQQ. A paper related to the study of electron transfer from reduced GOD to PQQ is submitted (4).

Another class of artificial electron acceptors for GOD are polymers based on 4.4'-dipyridyl (polyviologens). Polyviologens were synthesized by a reaction between equimolar amounts of 4,4'-dipyridyl and α, α'-dibromo-o- or p-xylene in dry acetonitrile at room temperature for 24 h. The polymeric products precipitated in the form of yellow or orange crystals. They were characterized by UV, IR and ^{1}H NMR spectroscopy.

The reaction of polyviologens with GOD could be easily followed spectrophotometrically over the pH range 6.0-8.0. The pH optimum for this reaction is 8.0. Due to the folded structure of the polymer a dimer-formation side reaction occurs. Due to this dimerization, the polymer does not mediate the electron transfer between the enzyme and the electrode in an electrochemical cell when the electrical solution contains the natural enzyme, solubilized mediator and glucose. To eliminate this side reaction, we synthesized a polymer with a terminal amino group that might be incorporated within the enzyme. This polymer could be coupled to GOD by covalent attachment to the protein part of the enzyme by amide bonding between the polymer amino group and acid residues of the enzyme. Thus modified GOD directly transfers electrons to a glassy carbon electrode. As a result, a glucose-concentration dependent current flows in an electrochemical cell when the electrolytic solution contains the polymer-modified GOD.

It is reasonable to conclude that the reaction of an electron acceptor with reduced GOD depends strongly on the intrinsic properties of reduced FAD, apo-GOD, and the electron acceptor related to their structural conformation and/or electric charge.

Enzymological studies

I) Purification and characterization of a clone of the flavin domain of cytochrome b_2 (L-lactate dehydrogenase) from *Saccharomyces cerevisiae.*

II) Isolation, characterization, immobilization and electrochemistry of NADH oxidase from *Thermus aquaticus.*

I) A clone of the gene encoding the flavin domain of cytochrome b_2 (NAD independent L-lactate dehydrogenase) from *Saccharomyces cerevisiae* was obtained as an insert in a plasmid in *Escherichia coli* (supplied by Drs S.K. Chapman and C. Brunt, University of Edinburgh). After expression (900-1600 U/g cell paste), lysing the cells with lysozyme and purification by ion-exchange chromatography on DEAE cellulose and gel filtration on Sephadex G-150 in the presence of EDTA, DL-lactate and PMSF, enzyme preparations with approximately 160 U/mg were obtained in about 20% overall yield. By PAGE and SDS-PAGE it was shown that no more than 10% impurity was present (pure cytochrome b_2, with intact haem regions: 208 U/mg). The enzyme is stable for long periods at +4 or −80°C provided that excess lactate is present and it is protected from air. By analogy with the native enzyme, it appears that the major source of instability is loss of oxidized FMN on oxidation of the enzyme; reduced FMN is evidently bound more tightly than oxidized FMN. The enzyme has been subjected to several treatments known to remove flavin from other flavoproteins. The FMN is removed readily by treatment with KBr at pH 5.0, but with complete loss of activity. Conditions must be found to obtain a regenerable apo-enzyme.

II) A new purification procedure has been devised giving a higher yield of enzyme by replacing the affinity step with AMP-Sepharose between the ion exchange and the gel filtration step by a hydrophobic chromatography step with phenyl-Sepharose. The physicochemical characteristics and the effects of temperature and pH, substrate specificity and kinetic parameters were all identical to the previously purified enzyme.

The purified NADH oxidase was immobilized on various modified solid support. (Table 1)

Table 1 — Yields of immobilisation of βNADH oxidase on various solid supports with different coupling methods

Supports (methods)	*mg enzyme/ g support*	*%*	*Enzyme units/ g support*	*%*	*Specific activity*
A	3.3	66	21.0	30	6.4
B	3.2	64	21.0	30	6.6
C	3.7	74	25.3	36	6.9
D	3.3	66	14.6	21	4.3
E	3.3	66	16.1	23	4.9
F	1.8	36	1.0	1	0.5
G	4.0	80	2.4	4	0.6
H	0.7	14	4.0	6	5.8
I	2.9	58	19.7	28	6.8
J	3.2	64	26.7	38	8.3
K	2.9	58	23.0	33	7.9

A = Sepharose 4B (CNBr); B = Sephacryl S-200 HR (CNBr); C = Sephacryl S-300 (CNBr); D = Cross-linked PVA (CNBr); E = Sephacryl S-200 HR (TCT); F = Sephacryl S-200 HR (p-benzoquinone); G = Aminopropyl cross-linked PVA (glutaraldehyde); H = Acrylonitrile-based resin (N-hydroxy succinimide); I = Succinylated cross-linked PVA (N-hydroxy succinimide); J = Glutarylated cross-linked PVA (N-hydroxy succinimide); K = Affi-gel 10 (N-hydroxy succinimide).

The best results were achieved with carboxylated supports following N-hydroxysuccinimide activation. For the immobilized NADH oxidase preparations no significant change concerning the K_m and V_{max} were observed compared to the soluble enzyme (Table 2; see page 89). The thermostability of immobilized and soluble NADH oxidase is currently under investigation. The results of the effect of organic solvents on the activity of soluble and immobilized NADH oxidase are summarized in Table 3A (see page 89) and 3B (see page 90). Also under these conditions, the general behaviour of the immobilized enzyme was not very different from its free counterpart.

Investigations on the development of an amperometric biosensor system with NADH oxidase for NADH detection or for the amplification of by NAD dependent dehydrogenases catalysed reactions of analytical interest were carried out. Methods involving both direct and mediated electron transfer have been examined. A variety of metal complexes were investigated for possible use as mediators for NADH oxidase. Two compounds were found to mediate with the enzyme. These were ammonium hexachlororuthenate and ruthenium red. This is the first reported use of ammonium hexachlororuthenate as a mediator. The electrochemistry of the mediators was examined using glassy carbon, carbon paste, graphite foil and

Table 2 — Kinetic parameters of βNADH oxidase immobilised on various solid supports with different coupling methods

Support (methods)	K_m (μM β-NADH)	V_{max} *μMOL O_2/mg/min)*
A	36	11.2
B	40	10.8
C	38	11.0
D	38	8.0
E	42	7.2
F	84	0.9
G	160	1.0
H	27	9.8
I	21	11.3
J	26	12.8
K	27	12.2

A = Sepharose 4B (CNBr); B = Sephacryl S-200 HR (CNBr); C = Sephacryl S-300 (CNBr); D = Cross-linked PVA (CNBr); E = Sephacryl S-200 HR (TCT); F = Sephacryl S-200 HR (p-benzoquinone); G = Aminopropyl cross-linked PVA (glutaraldehyde); H = Acrylonitrile-based resin (N-hydroxy succinimide); I = Succinylated cross-linked PVA (N-hydroxy succinimide); J = Glutarylated cross-linked PVA (N-hydroxy succinimide); K = Affi-gel 10 (N-hydroxy succinimide).

Table 3A — Activity of β-NADH oxidase in the presence of organic solvents

Concentration (v/v %) of the solvent	*MeOH*	*EtOH*	*PrOH*	*Dioxane*	*DMF*	*DMSO*
4	77	62	62	106	74	94
8	58	58	58	102	68	72
17	50	46	28	75	46	51
25	31	27	7	48	37	28
42	14	5	—	12	14	5
58	4	—	—	3	0	4
83	0	—	—	—	—	0

Enzyme in 20mM Tris/Hcl buffer. pH 7.2 was incubated for 30 minutes at 20°C with the indicated concentrations of the solvents. The activity was measured photometrically.

platinum disc working electrodes. Glassy carbon gave the lowest oxidation potential for hexachlororuthenate (+75 mV), while ruthenium red could be oxidated at below 0 mV at glassy carbon (−131 mV), carbon paste (−146 mV) or platinum (−136 mV). In the absence of oxygen both mediators could give an increase in the current response but both fail to fully replace oxygen. Investigations are proceeding on the determination of the mechanism of action of the NADH oxidase activity. Preliminary investigations have been carried out to determine the role of active site amino acids and modified flavins in the catalytic activity. Arginine was modified with phenylglyoxal, butanedione and 1,2 cyclohexanedione. The first two reagents caused immediate precipitation and inactivation of the enzyme. However, for 1,2 cyclohexanedione a linear relationship between incubation time and residual activity was seen for each concentration tested. Arginine modification was

found to take place, and further experiments are currently underway to explain this modification.

Table 3B — Activity of immobilised β-NADH oxidase in the presence of various organic solvents

Solvent concentration (v/v %)	*4*	*8*	*17*	*25*
Relative activity in the presence of:				
Dioxane	88	80	68	50
Dimethyl sulfoxide	92	74	52	30
Dimethyl formamide	68	60	43	22
Methanol	100	96	80	68

The enzyme was immobilised on glutarylated cross-linked PVA and incubated for 30 minutes with the appropriate solvent at 20°C. Activity measurements were carried out polarographically.

In spectrophotometric determinations of activity, N^6-(2-aminoethyl)-FAD was found to give 68% of the NADH oxidase activity found with native FAD. This result points to positive perspectives for the preparation of enzymically active covalent NADH oxidase-FAD-analogue adducts to overcome the disadvantage of a very weak bonding of FAD to ntive NADH oxidase. For the next period, further activity studies with respect to NADH oxidase with other FAD analogues are planned.

Screening of thermophilic bacteria exhibiting thermostable oxidase activities of interest for the development of amperometric biosensors

The search for the presence of thermostable oxidases in thermophilic microorganisms is a rather neglected field in applied microbiology.

During the first year the major effort was to enrich and purify thermophilic bacteria from natural environment (combust, bark and straw) or industrial plants (hot production waters of a local dairy and sugar factory) for the screening of thermophilic bacteria for oxidase activity during growth on different carbon sources or amino acids.

With respect to a potential application in biosensors a screening was started for oxidases converting glucose, lactate, glycerol or amino acids, for example, L-phenylalanine, L-arginine and L-asparagine.

Enrichment cultures, supplemented with the appropriate substrates, were incubated at 60°C on solid and in liquid complex media.

The development of a reliable qualitative test system to detect H_2O_2 formed by the oxidase screened for based on conversion of a chromogen and excluding disturbing side reactions was a major problem to solve. After a time intensive study, the test system with the chromogen ABTS to be converted by reaction with H_2O_2 catalysed by added peroxidase in the presence of a catalase inhibitor appears to be the best choice.

Since lactate oxidase is an enzyme with comparable potential for the development of biosensors as glucose oxidase, the initial effort was concentrated on the screen-

ing for its activity. The mesophilic lactic acid converting bacterium *Pediococcus acidilactici* with known lactate oxidase activity was choosen as reference strain for the developpment of a specific text system.

So far, in liquid cultures from 4 ABTS positive colonies the presence of lactate oxidase in 2 cultures and of glucose- and glycerol oxidase, each in 1 culture, could be verified.

HIGHLIGHTS / MILESTONES

1. Development of a 1-step procedure for the purification of FMN to approximately 100% from commercially available FMN that is usually only 75-80% pure.

2. First observation that the bifunctional enzyme flavokinase-FAD synthetase accepts adenine modified ATP for the enzymatic synthesis of adenine modified FAD.

3. Phenomenon that PQQ functions only in the acidic pH range 2.6-5.0 as electron acceptor with respect to reduced GOD (maximal transfer rate at pH 3.0) due to an only under these conditions for electron transfer appropriate structural interaction between PQQ and reduced FAD by charge-transfer complex formation and the apo-enzyme part of GOD.

4. First reported use of ammonium hexachlororuthenate as relay for the electron transfer from an oxidase to an electrode as exemplified for NADH oxidase.

5. Coenzyme for N^6-(2-aminoethyl)-FAD with respect to apo-NADH oxidase comparable with that of FAD, which points to favourable perspectives for the preparation of enzymically active covalent NADH oxidase-FAD analogue adducts.

WIDER CONSIDERATIONS

The started research may lead to a better understanding of the structural aspects of electron transfer to electrodes from FAD or FMN dependent oxidases, of interest for the development of amperometric biosensors, by mediators functioning as electron transfer relays. From the results, ideas may be obtained how to modify such oxidases, chemically or by site-directed mutagenesis, to improve their properties as element of biosensor devices.

COOPERATIVE ACTIVITIES

Second meeting for participants of the project at the ETH Zürich, Department of Chemical Technology, September 15, 1992.

Supply of N^6-(2-aminoethyl)-FAD by the GBF to the Cranfield Institute of Technology for enzymological studies concerning NADH oxidase.

Contacts with NOVO NORDISK, Bagsværd, Denmark, and the Institute of Genetics and Selection of Industrial Microbiology, Moscow, Russia, for the supply of nitrilase or the combination nitrile hydratase/amidase for the transformation of nitrilated FMN or FAD analogues to carboxylated derivatives.

JOINT PUBLICATIONS / PATENTS WITH TRANSNATIONAL AUTHORSHIP

Foreseen in the coming months.

OTHER PUBLICATIONS

A.F. Bückmann and V. Wray: A simplified procedure for the synthesis and purification of N^6-(2-aminoethyl)-NAD and tricyclic 1, N^6-(ethanoadenine)-NAD, Biotechnol. Appl. Biochem. **15**: 303-310 (1992).

J. Hendle, A.F. Bückmann, W. Aehle, D. Schomburg and R.D. Schmid: Structure/activity relationship of adenine-modified NAD derivatives with respect to porcine heart lactate dehydrogenase isoenzyme H_4 simulated with molecular mechanics, Eur. J. Biochem., in press (1993).

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New types of redox enzymes for the production of chiral synthons: Basic research, functionalisation and application (BIOT CT-900157)

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OBJECTIVES

1. Synthesis of commercially not available mediators, TUM
2. Enzymology of enzyme relay systems, TUM
3. Chemical stability test of new mediators, TUM
4. Supply of new viologen dependent enzymes and/or cells, TUM
5. Large scale production of anaerobes, TUM
6. Purification of viologen accepting oxidoreductases, TUM
7. Screen for new PQQ enzymes, TUD
8. PQQ enzyme purification, TUD
9. PQQ modification, TUD
10. Enzymology of PQQ holoenzymes, TUD
12. Electroactivity tests of modified redox proteins, TNO/ATO
14. Quantit. electrochem. measurements of modified redox proteins, TNO/ATO
15. Immobilisation of electroactive systems on carbon, TNO
16. Heterogeneous electrocatalysis, TNO
17. EPR spectroscopy, CTQB
18. NMR, EPR spectroscopy of modified PQQ enzymes, CTQB
19. EPR spectroscopy redox titrations, CTQB
20. NMR spectroscopy, CTQB
21. Moessbauer studies of PQQ holoenzymes + viologen acc. oxidored, CTQB
23. Mathematical modelling, TNO/ATO

RESULTS

The enzymic production of chiral synthons at bearable costs by redox reactions becomes feasible if involved pyridine nucleotides can be regenerated or pyridine nucleotide independent redox enzymes, which accept cheap and eassily regenerable artificial mediators are available.

The groups in Munich and Delft detected several enzymes of the latter type, which exchange electrons with viologens and/or other redox mediators. Among other sources the group in Munich found in the crude extracts of *C. thermoaceticum* reversible viologen accepting pyridine nucleotide oxidoreductase (VAPOR) activities of 5-10 U/mg protein for NADPH and of 1-3 U/mg protein for NADH formation using reduced methylviologen as electron donor. The pH optimum for the reduction of NADP with methylviologen in 0.1 M potassium phosphate buffer was found to be at 6.0 whereas the optimal pH for dehydrogenations of NADH with carboxamidomethylviologen (CAV^{++}) was 9.0. In a partially purified enzyme preparation the ratio of the rates for NADP and NAD reduction increased to 700:

1 starting from 3.6: 1 in crude extracts. On gel electrophoresis crude extracts of *C. thermoaceticum* resolved to several protein spots which exhibited VAPOR activity. Different VAPOR containing protein fractions during enrichment had specific activities from 1000 to 1500 U/mg protein for NADPH regeneration. According to gel chromatography highly enriched VAPOR showed a molecular mass of 480 kD with 40 kD subunits. Kinetic parameters of this fraction are: $K_mMV^{+\cdot}$ = 0.17 mM, $KmNADP^+$ = 0.22 mM, K_iMV^{++} = 20 mM and K_iNADP^+ = 3 mM.

Up to now NADPH regeneration remained difficult and costly. Crude extracts of *C. thermoaceticum* are therefore especially suitable for NADPH regeneration. Using enzyme relay systems with VAPOR of crude extracts or cell suspensions of *C. thermoaceticum* and pyridine nucleotide dependent oxido reductases in electrochemical cells we optimised a number of preparative syntheses of chiral compounds:

(a) the reductive carboxylation of 2-oxoglutarate to D-isocitrate catalysed by isocitrate dehydrogenase and increased the yield of tentative experiments from 14% to 54% (value adding factor product/educt 50).

(b) starting from racemic isocitrate oxidative decarboxylation of the D- enantiomer catalysed also by isocitrate dehydrogenase but using CAV or anthraquinone-2-sulfonate as mediators yielding the opposite enantiomer L-isocitrate with productivity numbers of 1.3 or $1.4*10^4$, respectively.

The productivity number is defined as product[mmol]/(dry catalyst [kg]*time[h].

(c) the complete dehydrogenation of up to 300 mM solutions of glucose-6-phosphate to 6-phosphogluconate by glucose-6-phosphate dehydrogenase and

(d) the preparation of ribulose-5-phosphate by the decarboxylating dehydrogenation of 6-phosphogluconate catalysed by 6-phosphogluconate dehydrogenase. With a substrate concentration of 20 mM complete conversion to Ru-6-P resulted only with enriched VAPOR from *C. thermoaceticum*. A 300 mM solution yielded 45% only.

Since anaerobic extracts of *C. thermoaceticum* also contain an NADP dependent formate dehydrogenase the preparative NADPH regeneration can be achieved at significantly lower apparative expenses using formate as electron donor instead of the cathode of an electrochemical cell. With formate as electron donor D-isocitrate was synthesised with a yield of 37%. Ethyl S-3-hydroxybutyrate, ethyl S-3-hydroxyvalerate and ethyl S-3-hydroxycaproate all with ee >98% were prepared by this method with 95% and 100% yields respectively. Here ß-ketoester reductase from *C. kluyveri* was applied.

Studies on three β-ketoester reductases and a β-ketoacid reductase in *C. thermoaceticum* were continued. For the newly detected ß-ketoacid reductase a specific activity of 1400 U/mg protein was found. The reversible enzyme does not contain FAD or FMN and has a molecular mass of 40-43 kD. To our knowledge it seems to be the first microbial S-specific ß-ketoacid reductase. The four enzymes were partially sequenced starting at the amino end.

Screening for quinoproteins: The enzymology section of TUD continued the search for quinoproteins and the investigation of their applicability for enantioselective conversions. A major breakthrough has occurred in the field of alcohol- and aldehyde dehydrogenases of Gram positive bacteria. Upon characterisation of these enzymes they turned out to be so-called nicotinoproteins, enzymes containing

firmly bound nicotinamide cofactors. Strong indications for the role of a nicotinoprotein alcohol dehydrogenase as the highly enantioselective catalyst of a particular bacterium, patented for bulk kinetic resolutions of racemic solketal, have also been obtained. Several interesting dye-linked dehydrogenases were discovered. In addition, dismutases catalysing the conversion of aldehydes into alcohols and carboxylic acids have been detected and described. The enantioselectivity of these enzymes remains to be established.

Quinohemoprotein alcohol dehydrogenase from *Comamonas testosteroni:*
The group in Delft characterised the kinetic behaviour of this enzyme pertinent to its application to the enantioselective oxidation of several commercially interesting chiral alcohols. Enantiomeric ratios (E) of the intermediate steps involving the formation of intermediate aldehyde and the carboxylic acid from chiral alcohols have been determined. A remarkable change in the chiral preference for alcohol (solketal, $E=50$) and aldehyde ($E=4$, for the mirror configuration) was noted. Purification problems have been resolved. Fundamental studies of the internal and external electron transfer pathways connecting the heme- and PQQ redox cofactors are performed. Crystallisation for X-ray structure determination is conducted in collaboration with Prof. Hol (Univ. Groningen).

'Wired' QH-EDH: Also in Delft synthetic efforts have resulted in the preparation of a number of PQQ derivatives that were specially designed to allow covalent linking of PQQ to polypyrrole-coated electrodes. Low but significant activity has been measured upon addition of spacer-equipped PQQ to the QH-EDH apoprotein. Collaboration with TNO-Zeist will start in the next future. The expertise of this group with respect to the preparation of polypyrrole-coated electrodes will be used to prepare QH-EDH that is 'wired' to the conducting polymers. Attempts to devise an electrochemical production cell for enantioselective kinetic resolutions based on this principle will be explored next.

Molybdoprotein Aldehyde Dehydrogenase from *C. testosteroni*:
Further on efficient methods for the cultivation of this microorganism and the purification of this enzyme have been established in Delft. Initial problems regarding its stability could be solved. Studies designed to incorporate ^{57}Fe suitable for Mössbauer spectroscopy are under way. Preliminary investigations of the enantioselectivity towards oxidation of chiral aromatic aldehydes suggest (complete) absence of selectivity. The implications of this finding will be further persued.

The group of CTQB has received enzymes purified from the groups in Munich (enoate reductase (ER) and carboxylic acid reductase (CAR)) and Delft (aldehyde dehydrogenase (QH-EDH) and alcohol dehydrogenase).

The nature of the iron center of ER could previously not be unambiguously indentified. Therefore we received pure ER enriched in ^{57}Fe from the group in Munich in order to perform Mössbauer, ENDOR and ESEEM studies.

Mössbauer results: Samples of oxidised and reduced ER were studied at low temperature at high magnetic fields and high temperature at zero field. The enzyme contains one 4Fe center. The oxidised form shows one quadrupole doublet with the parameters $\delta = 0.43$ mm/s and $E_Q = 1.32$ mm/s. In the reduced form the spectrum shows two quadrupole doublets, which extrapolated to low temperature have the values: $E_Q = 2.7$ mm/s and $\delta = 0.63$ mm/s (for the $2Fe^{2+}$-Fe^{2+} pair) and $E_Q = 1.23$ mm/s and $\delta = 0.52$ mm/s (for the $2Fe^{3+}$-Fe^{2+} pair). The parameters for the ferrous pair are very unusual compared to those of $[4Fe\text{-}4S]^+$ centers where the four ligands are cysteines. The analysis of the high field data reveals that one site has a hyperfine coupling constant $A_{av} = +140$ KG which is

higher than the one found for a normal $[4Fe\text{-}4S]^+$ center. The Mössbauer analysis is indicative of a none sulfur ligation at the cluster of enoate reductase.

ENDOR and ESEEM results: Samples of ^{56}Fe and ^{57}Fe reduced ER were prepared both in H_2O and D_2O. Very well resolved proton ENDOR lines were observed at different magnetic fields. These β-CH^2 and α-CH^2 protons from the cysteines show a very anisotropic behaviour and large hyperfine splittings. ^{57}Fe lines were also observed corresponding to a hyperfine coupling constant of 39 MHz. Preliminary ESEEM seem to indicate two nitrogens ligated with two different coupling constants.

With CAR from *C. thermoaceticum* preliminary EPR-studies were performed. The purified CAR shows some EPR signals with g-values lower than 2.002 which can be attributed to tungsten. In the dithionite reduced form an EPR signal typical of a $[4Fe\text{-}4S]^+$ cluster is observed. A redox titration will also be performed.

In continued studies on QH-EDH we measured NMR spectra of Holo EDH prepared in Delft and confirmed the finding that a large effect/control on the heme is observed upon binding of PQQ.

EPR studies on aldehyde dehydrogenase from *P. testosteroni* were performed on different reacting conditions. In the native form Mo(V) EPR signals are observed related to 'resting' form and correspond to 'inactive species'. The 'rapid signal' and 'slow signal' of Mo(V) was observed by different times of reduction of the enzyme by dithionite. Two different iron-sulfur centers that are most certain to be of [2Fe-2S] type are observed when the protein is reduced. Center I has a g_{max} of 2.020 and for center II g_{max} = 2.080. The reduction using benzaldehyde also reveals the reduction of the iron-sulfur centers and a Mo(V) type 'rapid signal'.

Aim of the research at TNO is the development of a preparative bioelectrochemical cell. The research at TNO focused in the past year on a PQQ-dependent alcohol dehydrogenase (PQQ-EDH) and the interaction with conducting polymers in electrochemical cells. Conducting membranes were prepared by chemical deposition of pyrrole in the pores of track-etch and microporous membranes. PQQ-EDH from *Cocomomas testosteroni* was immobilised on the pyrrole matrix. The membranes were compared with respect to their immobilisation capacities for the enzyme. On the track-etch membranes 0.017-0.24 $U{*}cm^{-2}$ membrane, on the microporous memebranes 0.11 $U{*}cm^{-2}$ membrane was immobilised. This reflects the difference in surface available for binding in these two membranes which is approximately a factor of twenty. A binding constant (K_s) of 60 μg/ml and a maximum amount adsorbed of 26 $\mu g{*}cm^{-2}$ was found on basis of protein determinations. The specific activity of the immobilised enzyme was calculated to be 4.1 U/mg.

Until now direct electron transport from the enzyme to the conducting polymer, as observed before with the system glucose oxidase/polypyrrole, was not observed with PQQ-EDH. In addition the track-etch membrane was used in electrochemical studies using $K_3Fe(CN)_6$ as the electron mediator. The membranes were incorporated in a three electrode cell with a working potential of +0.35 V (vs. Ag/AgCl). An overall catalytic current was reached with 1- pentanol as the substrate. Other components like benzoquinone and several ferrocen derivatives can also serve as electron acceptor in this system.

The pH optimum of the immobilsed PQQ-EDH, obtained by electrochemical measurement, was found to be pH 8.0 which is comparable with the pH optimum for the free enzyme. The kinetic parameters (K_m) of the immobilised enzyme are

similar to those found for the free enzyme. K_m-values of 0.025 mM and 0.12 mM were obtained for 1-pentanol and $K_3Fe(CN)_6$ respectively. For 1-butanol and 1-propanol K_m-values of 0.005 mM and 0.06 mM were observed.

Long term stabiliy experiments with the immobilised PQQ-EDH yielded a slow decrease in enzyme activity in time. A half life time of approxymately three days can be calculated operating the system at room temperature.

In order to study the interaction of galactose oxidase (from *Dactylium dendroides*) and an aryl oxidase (from *Pleurotis eryngii*) with the conducting polymer electrode purification and characterisation of these enzyme is now performed.

A simulation model for diffusion controlled electrochemical reactions was set up. This model was validated by comparison with the analytical solution given by the Cottrell equation and the semianalytical solution for an electrochemical reaction followed by an irreversible homogeneous chemical reaction. This strategy was chosen to analyse the problems which can be encountered in i) the simulation of the Cottrell equation and ii) replacing a homogeneous chemical reaction by an enzymatic reaction.

COOPERATIVE ACTIVITIES

Meeting of all groups at the University of Delft organized by the 'Delft' group in March 1992.

Supply of ^{56}Fe and ^{57}Fe enoate reductase (electrophoretically pure) and a tungsten containing aldehyde reductase by the group in Munich to C.T.Q.B. in Portugal.

Supply of aldehyde reductase by the group in Delft to C.T.Q.B. in Portugal.

Kinetics of carboxylic acid reductase were modelled in Delft in collaboration with TUM.

Cooperation between the group in Delft and TNO-Zeist started, designing a student project on covalent linking of PQQ to polypyrrole-coated electrodes.

Enantioselective biotechnological resolution of racemic epoxides in the production of optically pure epoxides (BIOT CT-910269)

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OBJECTIVES

A stereoselective enzyme has been discovered in a Xanthobacter species which resolves cheap racemic epoxide mixtures to very valuable optically pure epoxides which can be used in subsequent industrial chemical synthesis of optically pure bioactive compounds. The objective of the present proposal is to study epoxide-degrading enzymes with an emphasis on the enzyme from Xanthobacter Py2. The goal of the work is to arrive at an understanding of stereoselective epoxide degradation on basis of which the industrial partner or others can decide on possible industrial applications of the method.

For the reporting period, specific objectives set in the main were as described in the previous progress report.

MAJOR PROBLEMS ENCOUNTERED

No major, unexpected problems have been encountered. The research strategy remains as described in the previous progress report.

RESULTS

The results are described in 11 sections. Sections 1-5 have been dealt with in Wageningen, sections 6-8 in London, and sections 9-11 in Lisbon.

(1) Optimization of Xanthobacter Py2 in chemostat culture.
The expression of the epoxide-degrading enzyme in Xanthobacter Py2 was studied in chemostat culture. As reported in the previous progress report, the organism expressed the enzyme at similar specific activities when grown at varying growth rates under propene-limiting conditions. Similar results have been obtained for other growth limitations as for instance oxygen limitation (Fig. 1, next page).

(2) Artificial cofactor replacing the low molecular weight factor.
Degradation of epoxides in Xanthobacter Py2 apart from a protein also requires a low molecular weight factor (LMF).

A great deal of effort has been given to the purification and identification of this factor both in London (see section 7) and in Wageningen. Recently, it was observed the artificial compound dithiothreitol may replace the unknown cofactor in the assay for enzyme activity in dialyzed extracts (Fig. 2, next page).

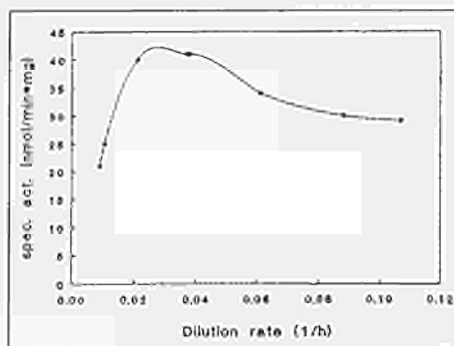

Fig. 1: Specific epoxide-degrading activity in whole cells of Xanthobacter Py2 grown in chemostat culture under oxygen limitation.

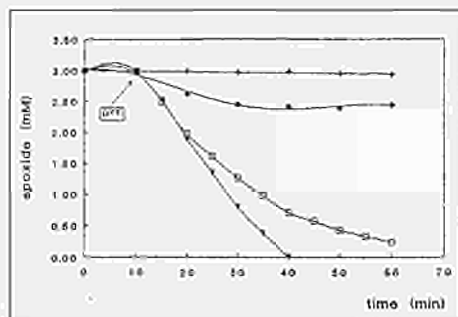

Fig. 2: Effect of varying concentrations of dithiotreitol (DTT) on the degradation of 1,2-epoxypropane by dialyzed cell-free extracts of Xanthobacter Py2. (+) no DTT; (◆) 1 mM DTT; (□) 3 mM DTT; (▼) 6 mM DTT. The incubation system also contained NAD^+.

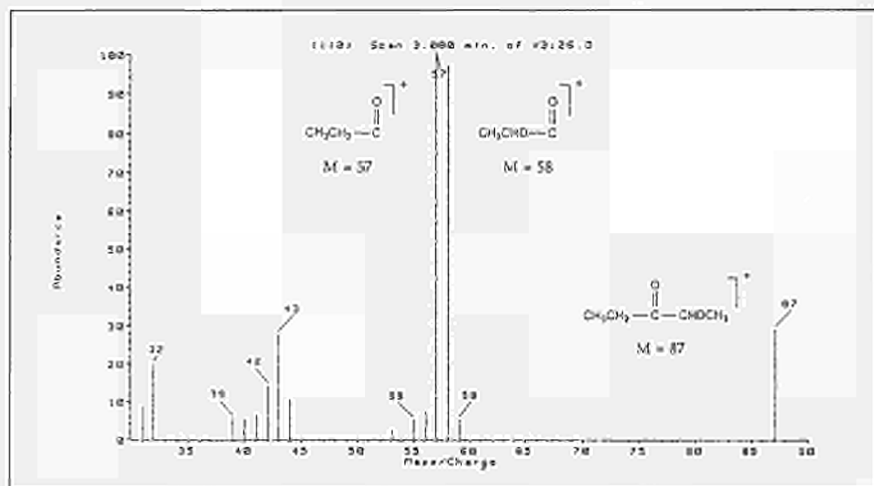

Fig. 3: Mass spectrum obtained from 3-pentanone produced by Xanthobacter Py2 from 2,3-epoxypentane labelled at positions 2 and 3.

(3) Possible mechanism of epoxide degradation as studied by deuterated epoxide.
The mechanism of epoxide degradation in Xanthobacter Py2 is very puzzling. One approach employed to obtain information on the mechanism was by making use of deuterated epoxide and by analyzing by GC/MS the ketone product formed. This approach was followed in the Department of Organic Chemistry of the Agricultural University by Dr. M.C.R. Franssen, H. Jongejan and Prof. Dr. Ae. de Groot. Epoxypentane D-labelled at the 2 and 3 position was converted by Xanthobacter Py2 and the resulting 3-pentanone was analyzed. Only one deuterium was detected in the product (Fig. 3) indicating an alcohol is an intermediate in the epoxide degradation.

(4) Isolation of mutants devoid of epoxidase activity.
The complementation method pursued for the isolation of the epoxidase gene from Xanthobacter Py2 (section 5) requires epoxidase-negative mutants. Such mutants were obtained by U.V.-radiation (Wageningen) or nitrosoguanidine treatment (London, section 7). Eventually, 3 mutants designated Py2M3, Py2M8 and Py2M10 were obtained which degraded acetone but did not grow on 1,2-epoxypropane or on propene. They still contained the unknown low molecular weight cofactor involved in the 1,2-epoxypropane degradation while the enzyme fractions of these mutants were inactive.

(5) Complementation of Xanthobacter mutant Py2M10.
The broad-host-range cosmid pLAFR5 was used as a cloning vector because of its ability to replicate in Xanthobacter species. Furthermore, the vector contains a double cos-site, therefore, relatively large insert DNA can be obtained. A gene bank was constructed and a total of approximately 7000 transduced E. coli LE392 were found. Twenty clones were analyzed for determining the efficiency of the gene bank preparation. The average insert size was 16.7kb $\pm$13.7kb; 25 per cent of the clones contained no insert.

For screening of the gene bank the recombinant cosmids were individually transferred from the transduced E. coli LE392 to the recipient Py2M10 using triparental mating. The conjugated recipients were selected for complementation on mineral medium/1,2-epoxypropane plates with tetracycline. Of the colonies tested nine clones were found to restore the epoxidase activity. The cosmids were isolated from the Py2M10 transformed to E. coli TG1 and then again conjugated to Py2M10 to rule out the chance of revertation of the mutation and to get rid of unstable plasmids. Six of the nine clones restored the epoxidase activity again in Py2M10.

These results are very encouraging because it appears the gene encoding for the epoxidase has now been obtained.

(6) Isolation of mutants of Xanthobacter Py2 for ^{13}C NMR studies.
As a guide to the mechanism of degradation of the epoxide and possibly the nature of functional groups in the low molecular cofactor, we wish to follow degradation of epoxide in whole cells by ^{13}CNMR. As ^{13}CNMR is not a particularly sensitive technique we have isolated mutants (F1654,F169,F2516) which degrade epoxide slowly. These will be ideal candidates for ^{13}CNMR studies as it is feasible that one or more will be mutated at a step downstream form the initial epoxide ring opening step and may therefore accumulate intermediates which are normally transient in the wild type.

(7) Studies on the epoxidase enzyme and cofactor from Xanthobacter Py2.
Procedures for growth of Py2 on propene on a 15L scale have been optimised with advice from the Wageningen group, particularly the growth of cells without slime (polysaccharide) production. Cells produced in this way have high specific activities

for epoxide degradation and can be readily disrupted in a French pressure cell (2 passages at 90mPa).

In a preliminary study an attempt was made to determine the optimum concentration of LMF by increasing the amount in the standard assay. However a maximum/optimum was not obtained as the specific activity increased to »500% before the experiment was discontinued through lack of LMF (a 15L fermentation yields about 5 ml of LMF). Subsequently a similar optimisation has been done by reducing the protein concentration in a stepwise manner.

Prior to attempting purification, the cofactor and protein were examined for evidence of the involvement of sulfhydryl groups. The cofactor appeared to be stable to aeration with O_2 at pH 8.5 and was not inhibited by 0.1M N-ethylmaleimide suggesting that it did not contain a functional sulfhydryl. However the combined assay of HMF and LMF was completely inhibited by 0.1M N-ethylmaleimide indicating that the protein component contained a functional sulfhydryl.

Although NADH is included in the assay procedure as supplied by Wageningen, removal from the assay did not appear to affect activity. However, the possibility that the LMF contains NADH has not been discounted, as yet.

(8) Isolation and characterization of cyclic epoxide-degrading organisms.
In the previous submission we reported the isolation of a Corynebacterium (strain C12) capable of degrading cyclic epoxides. This was isolated after an extensive screen of bacteria capable of growth on cyclohexene oxide.

Subsequently elaborate screens have not yielded further strains and screening has now been discontinued. Detailed studies of C12 are now under way including analysis of range, stereospecificity and enantioselectivity of the enzyme. Preliminary results confirmed that whole cells and cell extracts degrade cyclohexene oxide to the trans-diol. With a view to carrying out molecular genetic studies of this strain we have also demonstrated that cells grow in the presence of 1% glycine (to soften the cell walls) can be electroporated efficiently with the Corynebacterium-E.coli shuttle vector pSR1. However preliminary evidence suggests that the E.coli derived portion of the vector is unstable and readily deleted.

(9) Cell growth on 1-pentene.
In order to obtain cells that were able to use 1-pentene as the sole carbon source, cells from a culture were repeatedly transferred to fresh media containing only 1-pentene (yeast extract was added when no growth occurred) until the cells grew on the alkene alone.

The operational conditions which allow the maximum use of the flasks in terms of total biomass production under 1-pentene limiting conditions, were found by varying both the initial 1-pentene concentration and the medium volume. This resulted in a culture density of 0.60 g (dry weight)l-1 for a liquid volume of approximately 15% of the capacity of the sealed flask, at an initial 1-pentene concentration of 0.1% (v/v). Under these conditions 1-pentene is limiting, while oxygen is not.

This biomass yield on 1-pentene under C-source limiting conditions is approximately 0.52 (Ceq/Ceq), neglecting the substrate flow needed for maintenance.

(10) Epoxide-degrading activity.
The effects of the reactional conditions on the cell epoxide degrading activity were studied by incubating cell suspensions (app. 15 mg) at 30°C and 200rpm with an organic substrate reservoir (n-dodecane or iso-octane) or without an organic phase.

A moderate decline in activity was found in the latter case (activity decreased 30% during the first 5 days of incubation). The presence of iso-octane caused an almost complete inactivation within 2 days, whereas cells incubated in the presence of n-dodecane still showed 50% of their initial activity after 2 days.

(11) Immobilization of Xanthobacter Py2.
The in situ immobilization of Xanthobacter Py2 has been carried out by adding polyurethane cuboid foams to batch culture media. Two types of cuboids have been tested: type A from SCOTCHFOAM, and type B from RECTICEL. Type A has a smaller pore size than type B.
The first variable tested was medium composition. Medium 1 contained glucose, yeast extract, 1-pentene and Bis-(2-ethylhexyl)phtalate (BEHPh, the solvent for 1-pentene); medium 2 was composed of BEHPh and 1-pentene; medium 3 had only 1-pentene. The richer the initial medium was, the higher the final concentration of biomass was (either sum of free and immobilized or only immobilized expressed in mgcells/gfoam). The performance of the type A and B foams were depending on the medium used but in all cases immobilized cells gave higher specific activities than the free cells.

The influence of the cuboid size (8.5, 2.1 and 0.5 cm^3) was also tested using cells grown in medium 2. The best results were obtained for the in situ immobilization in type B foams of 8.5 cm^3 and corresponded to a load of 48.4 mgcells/gfoam.

HIGHLIGHTS/MILESTONES

The discovery that DTT may replace the unknown physiological cofactor will facilitate enzyme purification. Several types of epoxidase-negative mutants have been obtained allowing ^{13}CNMR studies as well as recombination experiments. Complementation with recombinant cosmids has been obtained opening the way for genetic analysis of the epoxidase gene. Whole cells were self-immobilizable in polyurethane supports and such cells showed a higher activity than free cells.

COOPERATIVE ACTIVITIES

Mutant strains have been exchanged between London and Wageningen. For the purpose of cofactor isolation, a researcher from Wageningen worked in London for one week. A joint meeting was held in Lisbon on December 11th,1992 with the participation of London (2 persons), Wageningen (3 persons) and Lisbon (3 persons).

PUBLICATIONS

Keulen, F. van; Santos, P.B. and Fonseca, M.M.R.: 'Enantioselective epoxide-degrading capacity of Xanthobacter Py2' Poster presented at the 9thInternational Biotechnology Symposium, Virginia, USA, August 1992.
Keulen, F. van; Santos, P.B. and Fonseca, M.M.R.: 'Degradation of epoxides by Xanthobacter Py2 grown in the presence of 1-pentene'. Poster presented at I Congreso Hispano-Luso de Biotecnologia, Santiago de Compostela, September 1992.
Swaving, J; Bont, J.A.M. de and Ooyen, A.J.J.: 'Complementation of an epoxidase negative mutant of Xanthobacter Py2' Poster presented at BION-meeting, Oud-Leusen, The Netherlands, April 1993.

Glycosyltransferases from *Streptomycetes* as tools in biotransformations (BIOT CT-900155)

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OBJECTIVES

— Identification and cloning of glycosyltransferase (GT) genes;

— Sequencing and expression of GTs from various sources and in various hosts;

— Testing GT specificity for aglycones and further enzyme characterization;

— Preparation of aglycones.

RESULTS

(1) The COORDINATORS group was working further on evaluation of the GT steps in streptomycin-producing *S. griseus* and *S. glaucescens*. Several further genes putatively involved in sugar activation, modification, and transfer were identified by cloning and sequencing: Gene products StrQ and StrP activate and modify by oxidoreduction/epimerization, respectively, the precursor hexose for the N-methyl-L-glucosamine moiety of (5'-hydroxy-)streptomycin. Two further StrS-related gene products were found to be encoded by *stsA* and *stsC* genes, and all three proteins have significant similarity to aminotransferases, and, therefore, could be members of a new class of specifically secondary metabolic transaminases including Prg1 and TylB (see below). As rate-limiting for progress achievement turned out to be the lack of substrates for the test of specific GT activities, e.g. the activated sugar derivatives dTDP-dihydrostreptose, dTDP-L-rhamnose, dTDP-mycosamine, dTDP-mycarose, and dTDP-mycaminose. Therefore, attempts have been started to prepare these substrates via enzymatic synthesis from commercially available precursors. For this purpose the gene products StrD, StrE, StrM, and StrL and/or their counterparts from gram-negative bacteria (*rfb* genes; LPS O-chain biosynthetic genes for L-rhamnose) were started to be overexpressed in *E. coli* (Fig. 1, see next page) — GT enzymes reported from cytoplasmic pathways including transfer of nucleotide-activated sugar (derivatives) were found to be distantly related and fell into two different enzyme families being significantly conserved in C-terminal motifs (for one group shown in Fig. 2, see page 105).

(2) In the group of PARTICIPANT (2) the genes/enzymes for the biogenesis of O-methyl-D-rhamnose in A201-producing *S. capreolus* were further searched for by use of the *strDELM,*, *strH,* and *strS* probes from *S. griseus* and *pur3,5,6* and *pgl* probes from puromycin-producing *S. alboniger* in hybridization experiments against the genomic DNA of this strain. Clear signals could only be detected for *strM* and *strH*, and for *pur6* in two groups of A201 resistance-mediating cosmid clones, each represented by two independent isolates (group 1: pCAR13/14; group 2: pCAR11/23), which therefore are good candidates for encoding both the A201 aglycone and sugar biosynthetic enzymes. Subcloning and analysis was started.

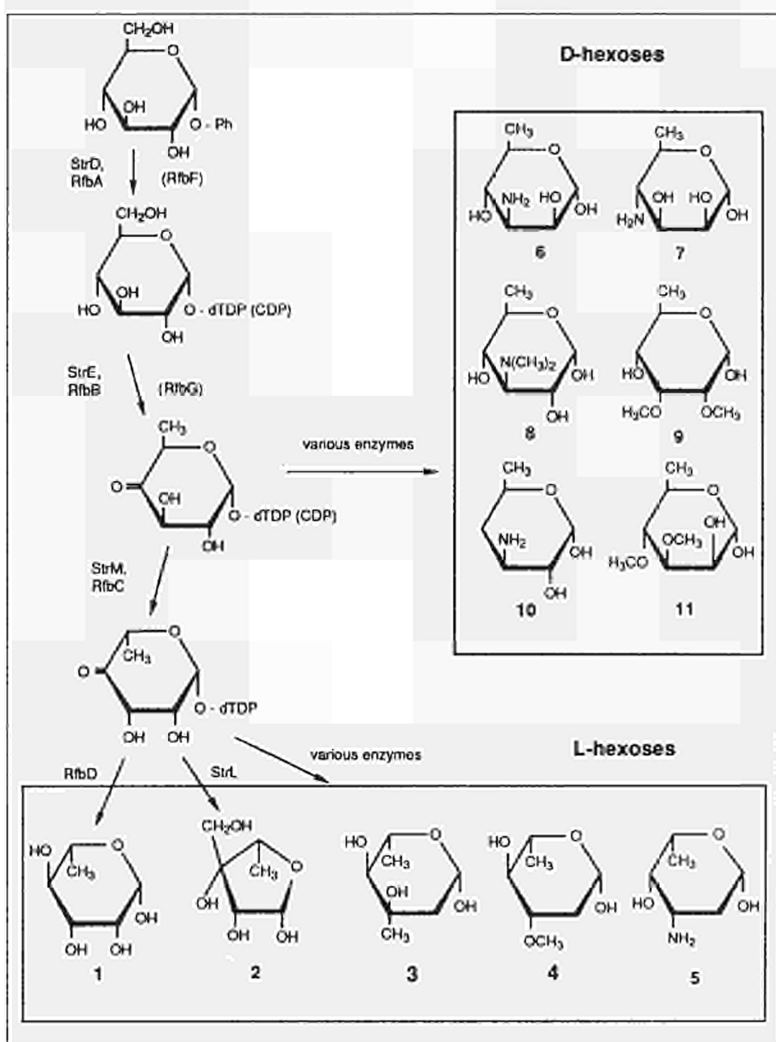

Fig. 1: Basic pathways for D- and L-6-deoxyhexose (6DOH) biosynthesis and some of their end products. End products are: (1) L-rhamnose; (2) L- dihydrostreptose; (3) L-mycarose; (4) L-oleandrose; (5) L-daunosamine; (6) D-mycosamine; (7) D-perosamine; (8) D-mycaminose; (9) D-mycinose; (10) D-desosamine; (11) O-methyl-D-rhamnose

(3) *By use of the strE* and *strS* probes from *S. griseus* in the group of PARTICIPANT (3) the *tylIBA1A2* genes from *S. fradiae* were detected and analysed by sequencing. They encoded a cytochrome P450 hydroxylase (TylI; active on tyllactone); a possible aminotransferase (TylB; similar to StrS); a dTDP-glucose synthetase (TylA1; StrD-like); a dTDP-glucose dehydratase (TylA2; StrE-like). Search for the further dTDP-mycaminose, dTDP-mycarose, and dTDP-mycinose

biosynthetic genes/enzymes and the relevant GTs is in progress. GT substrate specificity of the MGT enzyme was further testetd by using more macrolide aglycones (cf. 1992 report).

(4) At HOECHST AG further genes of the mycosamine pathway in the amphotericin B producer *S. nodosus* DSM40109 were analysed from a 2.6 kb DNA fragment hybridizing to the *strD* gene of *S. griseus*. The three genes identified, *snoD, snoM,* and *snoT*, encoded besides enzymes related to two enzymes from *S. griseus*, gene products StrD (equivalent to SnoD; dTDP-glucose synthetase) and StrM (high sequence similarity to SnoM, a putative dTDP-4-keto-6-deoxyglucose 3,4-isomerase), respectively, for a possible mycosaminyl-transferase (SnoT). The evidences for the assignment of the SnoT function were the similarity to the macrolide glucosyltransferase (MGT; see 1992 report) of other Streptomycetes and the similarity to other GT's in a carboxy-terminal peptide motif (Fig. 2).

```
(1)        WAPLDVVA--PTCDVLVHHGGGVSTLTGLNAGVPQLLIPRGAVL
(2)        WVPQLAIL--QQADLFVTHAGAGGSQEGLATATPMIAVPQAADQ
(3)        WAPQVAVLRHPSVGAFVTHAGWASVLEGVSSGVPMACRPFFGDQ
(4)        WLPQNDLLGHPKARAFITHSGSHGIYEGICNGVPMVMMPLFGDQ
(5)        WFNQRAVLRHKKMAAFITQGGLQSSDEALEAGIPMVCLPMMGDQ
-----------------------------------------------------
consensus  W-PQ--VL-H----AFVTH-G--S---GL---VPM---P--GDQ
```

Fig. 2: C-terminal GT-motif of several glycosyltransferases. The protein segments aligned are from: (1) amphoteronolide B dTDP-D-mycosaminyltransferase (SnoT; S. nodosus); (2) macrolide-2'-O-D-glucosyltransferase (*S. lividans*); (3) flavonol-O-(3)-D-glucosyltransferase (Zea mays); (4) 17-β-hydroxysteroid-UDP-D-glucuronosyltransferase (rat); (5) ecdysteroid-UDP-D-glucosyltransferase (baculovirus).

HIGHLIGHTS / MILESTONES

1. A putatively specific GT, SnoT (amphoteronolide B::mycosaminyltransferase), was identified as gene product of a gene in a 6DOH-pathway gene cluster from *S. nodosus*.

2. The basis for the preparation in vitro of nucleotide (dTDP)-activated 6DOH derivatives by expression of enzymes for the 2 initial steps of this pathway from genes derived from *S. griseus* or gram-negative bacteria. Further streptomycin-biosynthetic genes have been identified which are involved in sugar modification and — possibly — in glycosyltransfer.

3. A first set of 6DOH-pathway genes from tylosin-producing *S. fradiae* was identified and analysed.

4. GT sequence comparisons revealed the existence of two different enzyme families with distant relationships, especially conserved in C-terminal motifs.

WIDER CONSIDERATIONS

The recent developments in the field, now called 'glycobiology', the realization of importance of glycosylation of low-molecular weight bioactive molecules in pharmaceutical use, in cell signalling processes, and of macromolecular glycoconjugates in cell-cell recognition, and the need for advanced methods in modern medicine make the activities initiated in the running project an urgent one with possible broader influence on future developments. This before all since most of the substances available via enzymatic sugar activation, modification, and transfer are not accessible on merely synthetic routes.

COOPERATIVE ACTIVITIES

1. Two meetings for all contractors and coworkers were organized:
   - May 5., 1992, HOECHST AG, Frankfurt-Höchst, D
   - Oct. 2.-4., 1992, University of Leicester, Leicester, GB.
2. Exchange of material, informations, and ideas, also between coworkers, was achieved routinely several times in 1992.

PUBLICATIONS

Distler, J., K. Mansouri, G. Mayer, M. Stockmann, and W. Piepersberg. 1992. Streptomycin production and its regulation. *Gene* **115**:105-111.

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Retzlaff, L., G. Mayer, S. Beyer, J. Ahlert, S. Verseck, J. Distler and W. Piepersberg. 1993. Streptomycin Production in Streptomycetes: A progress report. In: Hegeman, G.D., R.H. Baltz, and P.L. Skatrud (Eds.) Genetics and Molecular Biology of Industrial Microorganisms, pp. 183-194. Proceedings of the 5th ASM Conference. American Society for Microbiology, Washington DC.

Tercero, A., R.A. Lacalle, and A. Jimenez. 1992. Cosmid pJAR4, a novel Streptomyces-Escherichia coli shuttle vector for the cloning of Streptomyces operons. FEMS Microbiol. *Lett.* **90**:203-206.

Alternative methods of DNA-sequencing (BIOT CT-900252)

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OBJECTIVES

High Energy Electron Beam Lithographic fabrication of test sub-micron apertures in opaque screens.
Design and construction of laser (HeNe and Ar^+) beam launch and scattered light detection (CCD) system.
Assessment of polycarbonate track etched membranes as suitable alternatives to electron beam patterned systems for DNA fragment detection.
Assess sensitivity of prototype system to fluorescently labelled model particles prior to employing labelled DNA fragments prepared by both CAMR and Biotechnology Institute.

Determination of optimum free solution electrophoretic conditions to enable 50Kb lambda phage DNA to be detected.
Initial selection, design and construction of model DNA fragments for calibration purposes for use in both subsequent non-laser based gel scanning system and in the CAMR aperture detection system.
Selection of applicable fluorophores for coupling to different components of sequencing systems.

Determine specification and classification of the functional hardware and software requirements both the proposed aperture/pore system.

MAJOR PROBLEMS ENCOUNTERED

1. Delay in initial payment resulted in 10 month extension to contract (i.e. project start date being postponed to December 1991)

2. A change in participant structure (withdrawal of Danish sub-contractor) has necessitated an alternative approach to fulfilling some of the original project aims. Accordingly, initial work on the development of a non-laser based gel scanner has been replaced with a comprehensive feasibility study on the use of the CAMR optical aperture detection system as a method to enable rapid, one-step mapping of DNA (both physical and genetic mapping of large (10sKb) DNA fragments).

RESULTS

Biotechnology Institute, Lyngby, Denmark — Reporting period objectives.

1. Review current knowledge of electrophoretic behaviour of DNA in solution and gels.

2. Investigate electrophoretic conditions for the electrophoresis of large DNA molecules through pores in polycarbonate track etched membranes.

3. Provide fluorophore labelled DNA fragments.

1. Review of electrophoretic behaviour of individual DNA molecules.

Given that DNA electrophoreses in a random coil configuration in solution, the use of a linearising gel film above the pore bearing membrane was proposed in this project and has required a review of the behaviour of individual DNA molecules in gels. Recently published data [Bustemente et al (1993) TIBTECH, 11, 23-30] on the electrophoresis of large DNA fragments through gels has shown the mobility to be more complex than suggested by the popular reptating model. The interaction of the DNA with the gel encompasses contraction, elongation and collision of the molecule with the gel fibre, all of which will need to be considered when interrogating individual molecules migrating through pores from a gel film. Variation in electrophoretic velocity will be important in determining the success with which DNA length can be correlated to residence time within the pore. Similarly, the strength of signal afforded by internal labelling of the DNA is expected to determine the ultimate sensitivity of the technique.

2.Electrophoresis of DNA through pore bearing membranes.

In order to initially investigate the properties of the membranes bromophenol blue (BPB) was used as an electrophoresis model. A small electrophoresis apparatus employing capillary tubes (1mm id) onto the end of which small sections of submicron pore bearing polycarbonate membranes were attached was constructed and BPB used to monitor electrophoretic mobilities as a function of pore diameter (50 and 100nm) and number density. Electrophoresis was linear with respect to time (over 5 mins) and voltage (up to 60V) though resistance did not vary with pore density which is unexpected. Current drift under constant voltage was 5% (100 to 95 μA) over 15 minutes though it is expected that constant current control will solve this problem.

Both 3.6kb and 48.5kb (lambda) DNA fragments were shown to electrophorese through 50nm pore systems though quantification of the small amounts is difficult from gels. Southern blotting showed no evidence of breakage of the lambda DNA molecules on electrophoresis through 50nm pores.
Dot blotting with ^{32}P proved too insensitive (limited to 10^5-10^6 copies) to quantify the amounts of DNA electrophoresing through the pore membrane. Instead, PCR is being used to effect this quantification using a new quantitative method developed by Biotechnology Institute [Holmstrom et al (1993) Anal Biochem, in press] and which uses an external control protocol.

Current work employs two primers to give a 700bp PCR product using lambda phage genomic DNA as template. A modified version of the PCR-product detection system using an internal capture probe to capture PCR products in a microtitre plate format is being developed in parallel. The system avoids the detection of so-called primer-dimer phenomena, which sometimes causes severe background problems with the original detection system.

Employing multi-pore polycarbonate membranes with a CCD detection system will allow an increase in the amount of DNA being passed through the system facilitating subsequent detection.

3. Labelling of DNA.

The proposed technique will employ labelled probes to identify the position of a specific sequence within the DNA molecule without requiring cutting of the DNA. Two approaches are possible to achieve this labelling goal. Either

(i) small oligonucleotide probes (4-8mers) fluorescently labelled and complementary to restriction sites could be used or

(ii) peptide nucleic acids (PNAs) containing fluorescent labels could be used. PNAs readily displace the complementary strand and therefore no enzymes are necessary and, again, mapping based on restriction sequences would be a valuable tool using this procedure.

Once the linearity of the DNA molecules on electrophoresis has been demonstrated and the detection limit established, work on this labelling aspect will commence.

CAMR, Porton Down, UK — Reporting Period Objectives.

1. High Energy Electron Beam Lithographic fabrication of test sub-micron apertures in opaque screens. Assessment of polycarbonate track etched membranes as suitable alternatives to electron beam patterned systems for DNA fragment detection.

2. Design and construction of laser (HeNe and Ar^+) beam launch and scattered light detection (APD and CCD) system.

3. Assess sensitivity of prototype device to unlabelled and fluorescently labelled model particles prior to employing labelled DNA fragments prepared by both CAMR and Biotechnology Institute.

1. Device Fabrication.

A variety of methods by which membranes and films containing sub-micron apertures can be fabricated or obtained have been investigated. High energy electron beam writing has been used to fabricate 50nm pores in thin (200nm) films of Al_2O_3 (supplied by Prof. Humphries, Cambridge University) but subsequent illumination of the surface failed to detect the apertures in sufficient detail. Devices fabricated by eximer laser photoablation of polyimide sheet and containing 5×5 arrays of 5μm pores were also assessed and proved suitable but supplies were unreliable. An alternative membrane type (polycarbonate track etched membrane) that is both commercially available and inexpensive was identified and proved adequate for our initial studies. These membranes, moreover, can be supplied with a wide range of sub-micron pore diameters, coated with a 200nm gold film or supplied pre-stained black for optical opacity and finally have excellent insulating properties.

Devices (100+) comprising metallised (60nm Cr) quartz substrates in which arrays of apertures of varying sizes have been fabricated using electron beam lithography and wet and plasma etching, have been prepared at CAMR. These devices have been used to assess the detectability of sub-micron particulates by the detector system described below.

2. Construction of optical illumination and detection system.

Both back-illumination and grazing incidence illumination have been considered. For model systems in which metallised optical substrates bearing sub-micron apertures are used to determine optical sensitivity to fluorophores the use of low powered HeNe and Ar beams at grazing incidence have proved most sensitive.

Employing chrome as the metal gives the advantage of durability and flexibility in accuracy of launch angle. Oblique back-illumination with low powered Ar is likely to be employed when track etched membranes are used in fluorescent studies.

Detector systems based on avalanche photodiodes (APD) and charge coupled devices (CCD) have both been designed and the latter is being used to follow intensity fluctuations in light emanating from sub-micron apertures in response to the presence of labelled and unlabelled sub-micron particles diffusing in their immediate vicinity. Output from the CCD detector array is monitored by either video line selector which allows the intensity of light from any given aperture to be monitored in real time or by a special data acquisition system (Brian Reece Scientific Ltd, UK) allowing digital processing of the image (e.g. averaging, expansion, background suppression etc..). This latter system also allows monitoring of individual apertures in real time though limited by the frame refresh rate of the CCD array (i.e. 50Hz). The CCD detection system has proved successful in our initial work and the image analysis system is now being modified in software (Optimum Ltd, Greece) to enable data collection and storage to be improved.

3. Model particulates detection.

A variety of model sub-micron particulates have been studied in order to define the sensitivity of the detection system and characterise the optical response of the apertures:

i) Calibration polystyrene microspheres.
ii) Au sol.
iii) Fluorescently labelled microspheres.

Polyimide membrane systems, back illuminated with a white light source, were assessed using unlabelled 2µm polystyrene microspheres prior to using polycarbonate track etched membrane containing pores of 200nm diameter. These membranes were stained with Ergolan black to enhance contrast and proved successful in detecting 400nm microspheres diffusing in aqueous conditions. Current work involves using similar membranes coated with 200nm Au and illuminated by grazing incidence Ar laser in order to achieve higher resolution.

Fluorescently labelled microspheres (410nm labelled with fluorescein) have been monitored using grazing incidence illumination (fibre-optic mediated 488nm Ar ion beam) of an Al coated glass substrate containing apertures of 200nm diameter.

Samples of 48.5kb lambda DNA are currently being stained with the newly developed intercalating DNA dyes TOTO and YOYO (Molecular Probes Inc.) and will be used to determine the correlation between fluorescence signal with DNA fragment size. Significantly, while this work was being commenced at CAMR, Keller and his coworkers [Goodwin et al (1993) Nucl Acid Res, 21(4), 803-806] have demonstrated that such a staining protocol can be used to accurately and rapidly size individual lambda DNA fragments over the 10-50kb size range with better than 2% accuracy using flow cytometry thus proving the feasibility of such an approach. CAMR efforts will concentrate on applying this protocol to the pore based detection system.

Optimum Ltd.

During this first period of work at Optimum, different data analysis scenarios were introduced and theoretically assessed with respect to the expected range of intensities and speeds of optical signals expected from the aperture device in order to best predict the hardware requirements for the proposed system.

In order to obtain preliminary data on which Optimum could base their future work, the relatively low cost and rapidity with which CCD detector array technology could be applied to the problem resulted in the CAMR procurement of a 1024 x 768 monochrome CCD system with PCX high resolution video line digitiser.

Following analysis of preliminary data, Optimum are now engaged in changing and enhancing the s/w capabilities of the data analysis system. Specifically, file handling routines were improved allowing recall and retrospective manipulation of previous data. The speed at which the data handling routines could operate were increased permitting faster analyses in CAMR studies.
A major advance in this part of the work is being achieved through data storage compression on disc. Currently extensive, the data collected in time from relatively short experiments has to be compacted for later analysis and limited mass storage restricts the number of experimental runs achievable at any given time. This part of the project necessitates continuous exchange of data files and s/w between CAMR and Optimum.

Data manipulation will become increasingly important as the requirement for peak height analysis and curve fitting becomes necessary. Variations in the duration of signals from DNA fragments of different lengths will need to be addressed as will determination of system noise levels and baselines. It is expected that the presence of DNA molecules in the scattering volume (pore) will be reported by the generation of fluorescence from the intercalating dyes (1 fluorophore per 4 bases) and the position of the oligo probe by different fluorophores attached to the probe resulting in the START-MARK-END sequence from which a mapping capability will obtained. The data analysis routines will need to be able to differentiate between single molecules and multiple occupancy events as well as non-linear migration and reversal of orientation of the electrophoresing molecule.
Solutions to these non-trivial problems are currently under active consideration.

HIGHLIGHTS / MILESTONES

Electrophoresis of 50kb DNA fragments through 50nm pores in polycarbonate membranes without observable degradation.

Identification of a suitable pore membrane (track etched polycarbonate) for optical studies.

Visualisation of sub-micron fluorescent model particles using the pore membrane system.

COOPERATIVE ACTIVITIES

Full meeting of all participants in Athens in February, 1993.

AREAC C:

CELLULAR BIOLOGY

- PHYSIOLOGY AND MOLECULAR GENETICS OF INDUSTRIAL MICROORGANISMS
(from page 115 tot page 163)

- BASIC BIOTECHNOLOGY OF PLANTS AND ASSOCIATED MICROORGANISMS
(from page 164 to page 249)

- BIOTECHNOLOGY OF ANIMAL CELLS
(from page 250 to page 300)

Integration of primary metabolism, secondary metabolism and differentiation in *Streptomyces coelicolor:* A biochemical, physiological and genetical approach (BIOT CT-910255)

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OBJECTIVES

The objectives are set both by our original scheme and in response to progress and results achieved during the first year of the project. Purify and partially sequence glucose metabolic enzymes, and use information and mutants to clone genes — Study properties of glucose metabolic enzymes relevant to their *in vivo* roles in flux control — Characterise catabolite repression-relieved mutants — Clone *cya* from *S. coelicolor* — Purify, characterise and partially sequence enzymes of glycogen and trehalose metabolism — Clone and sequence genes of glycogen and trehalose metabolism — Raise antibodies to selected ribosomal proteins and use to analyse post-translational modification — Clone and sequence genes for selected ribosomal proteins — Further develop software for 2D PAGE analysis — Identify proteins regulated by *act*II-orf4 using 2D PAGE, and analyse their abundance in different physiological conditions — Raise antibodies against ActII-ORF4 protein and use to study protein abundance and properties — Evaluate abundance and mode of action of ActII-ORF4 protein (especially DNA-binding properties) — Initiate studies of *act*II-ORF4 using site-directed mutagenesis — Characterise the means by which certain *S. antibioticus* and *S. fradiae* DNA fragments induce actinorhodin production in *S. lividans.*

MAJOR PROBLEMS ENCOUNTERED: Problems raised in previous report:

1. **Choice of strains and growth conditions:** This 'problem' constantly needs to be kept in mind but is not a current bottleneck; the new Norwich strain (see below) may well be very useful.

2. **Assay of actinorhodin:** Progress has been made in pin-pointing the existence (and some physical properties) of the non-actinorhodin blue pigment produced as the major product of the actinorhodin pathway in some *S. coelicolor* A3(2) strains under some conditions, but its detailed characterisation is still elusive (in spite of efforts in Bristol).

3. **Glycogen biosynthesis:** Following the visit to Norwich of E. Miguélez (Oviedo), the enzyme assays have been improved, and success at both Norwich and Oviedo in cloning relevant genes has overcome an earlier bottleneck.

RESULTS

1. Glucose metabolic pathways

University of Groningen (L.V. Bystrykh, A. Alves and L. Dijkhuizen). Variations in activity of glucose metabolic enzymes during the switch from primary to secondary metabolism were studied in strain MT1109 in Hobbs' minimal medium. Growth conditions resulting in methylenomycin synthesis (which can be measured quantitatively) were used to compare enzyme profiles with glucose radiorespirometry data of the Manchester group (T Obanye, S. Oliver). The latter suggest that, just before or at the point of methylenomycin production, glucose flux through the oxidative branch of the pentosephosphate pathway cycle increases relative to the Embden-Meyerhoff pathway and TCA cycle. Substantial increases in activities of the following enzymes were detected when cells entered idiophase: glucose kinase (GK), glyceraldehyde-3-phosphate dehydrogenase, transkctolasc, NADP- and NAD-dependent glucose-6-phosphate dehydrogenases, citrate synthases, PEP-carboxylase. In contrast, pyruvate kinase and malic enzyme activities were maximal in mid-exponential growth phase and decreased towards idiophase. Phosphofructokinase and malate dehydrogenase were most active in late-exponential growth. Similar experiments focussing on metabolic adaptations occurring during initiation of actinorhodin production will be carried out pending the development of suitable quantitative assays for this metabolite.

Phosphofructokinase, pyruvate kinase and citrate synthase were purified to homogeneity. All are under activity control by energy equivalents, such as ATP, ADP, NAD(H). This clearly suggests that primary glucose metabolism is regulated, contradicting earlier speculations about deregulated carbon fluxes as essential factors for initiation of secondary metabolism. Amino acid sequencing is being carried out for the N-termini and for several internal peptide fragments of phosphofructokinase and pyruvate kinase to allow cloning of the genes.

Growth kinetics, nutrient consumption and red/blue pigment production by strains MT1109 and M145 was studied in batch, fed-batch and continuous cultures. Pigments were produced on depletion of phosphate (accumulation of extracellular blue pigment) or ammonium (accumulation of actinorhodin as an intracellular red pigment) from the medium. A procedure for isolating these pigments and their subsequent analysis via liquid chromatography was developed. The prepared samples were sent to T J Simpson (Bristol) for structural analysis.

2. Glucose transport and catabolite repression

Glucose repression (a): John Innes Institute, Norwich (S.M. Angell and M.J. Bibb). A DNA fragment that complements GK mutants for glucose utilisation (Glc^+) and 2-deoxyglucose (2-DGlc) sensitivity was cloned on a low copy-number plasmid from one of the spontaneous Glc^+ derivatives of the ORF3 deletion mutant (*S. coelicolor* J1688). The level of GK activity conferred by the cloned DNA was about half that of the wild-type and of the spontaneous Glc^+ derivatives. Analysis of extracts from several of the spontaneous Glc^+ derivatives on GK activity gels revealed a new hexokinase which co-migrated with the activity in *S. limosus*. The ability of this new activity to restore glucose repression of *dagA* at the transcriptional level is being assessed and similar experiments are being done with the GK encoded by the *glk* gene of *Zymomonas mobilis* which shows little amino acid sequence similarity to the ORF3 product, and which has been expressed in *S. coelicolor.* Restoration of glucose repression would strongly suggest that the function of the ORF3 product is catalytic (implicating a function for glucose-6-phosphate or other glycolytic intermediates in glucose repression), rather than regulatory. The effects of over-expressing ORF2 and/or ORF3 on GK activity and

on glucose repression at the transcriptional level are also being studied. Interestingly, the spontaneous Glc^{+} phenotype, which arises at a frequency of 10^{-5}, is unstable, reverting between Glc^{+} 2-$DGlc^{S}$ and Glc^{-} 2-$DGlc^{R}$ at a frequency of 10^{-3}. The availability of the cloned DNA that presumably encodes this new activity will allow analysis of possible DNA re-arrangements that might account for the unstable phenotype.

Glucose repression (b): E.C. Slater Institute, University of Amsterdam (J. Kwakman and P Postma). To identify genes other than *glk* involved in catabolite repression, UV-generated mutants of strain M145 were sought which could synthesize agarase in the presence of glucose. These mutants appeared to have normal GK levels and all showed glucose repression of glycerol kinase. On the other hand, 18 mutants isolated by Tn*5096* mutagenesis of M145 after screening for agarase production in the presence of glucose lacked GK activity, and the transposons were all inserted into ORF3. The glucose-repressible agarase and glycerol kinase genes were used to study the role of GK in carbon catabolite repression in general. Surprisingly, absence of GK resulted in relief of catabolite repression of the two reporter genes by several carbon sources, including monosaccharides and TCA cycle intermediates, suggesting that GK also plays a role in catabolite repression by carbon sources other than glucose. This suggests that flux through GK does not determine catabolite repression, but that possibly GK serves as a regulatory factor in the cytoplasm. In contrast to the results obtained at low copy-number, when a *glk* mutant was transformed with ORF3 on a high copy-number vector (pIJ702), the transformants retained their mutant phenotype, suggesting that additional proteins are involved in catabolite repression. A candidate for one of these is the product of ORF2, which lies immediately upstream of ORF3. This idea is being tested by looking at the correlation between the copy number of both ORFs, GK activity and catabolite repression on several carbon sources. Recently, a conserved ATPase fold was reported from an analysis of the known crystal structures of different classes of ATP-hydrolyzing enzymes; this ATPase fold was found also in sugar kinases, including the product of ORF3. Oligonucleotides are being used to introduce specific point mutations in the conserved elements of ORF3. Such mutations have an effect on ATP-binding and phosphorylation in other hexokinases, and comparable amino acid changes affected glucose repression in yeast when introduced into hexokinase PII. To select mutants defective in glucose transport, 2-DGlc- and 3-*0*-methylglucose-resistant strains were isolated, but none was affected in glucose uptake, suggesting that there are two or more glucose transporters. The recently published sequences of four hexose transporters from yeast has revealed some small peptide segments whose sequence is absolutely conserved from mammalian to bacterial glucose transporters. We shall synthesize degenerate oligonucleotides in an attempt to identify homologous genes in *S. coelicolor* A3(2) by PCR.

Role of cAMP in the S. coelicolor life cycle: University of Warwick (F. Amini and D.A. Hodgson). We have continued to study the role of cAMP in the life cycle of *S. coelicolor* by analysing the properties of the enzyme (adenyl cyclase) responsible for cAMP production; analysing cAMP production during the growth cycle of cells on different substrates; and attempting to clone the adenyl cyclase gene. In collaboration with Glaxo Group Research Ltd we have attempted to develop a simpler, HPLC-based assay for adenyl cyclase activity as a prelude to enzyme purification. The assays proved either more time consuming and/or more complicated than the ^{3}H cAMP competition assay currently used, but they showed that there was little adenyl cyclase activity in the cells and that most of the ATP added to cell extracts is rapidly converted to AMP before adenyl cyclase has a chance to convert it to cAMP. In defined salts media developed by Glaxo for reproducible

induction of secondary metabolites, both intracellular and extracellular cAMP concentration was at its highest during lag phase and early exponential phase, fell throughout the exponential phase, and disappeared by stationary phase. Phosphate, nitrogen source and magnesium limitation did not affect the kinetics of cAMP production, nor did changing the carbon and energy source. We have obtained λ gt11 clones containing *S. coelicolor* A3(2) DNA with homology to the *Brevibacterium liquefaciens* adenyl cyclase gene (*cya*). These are being analysed to see if they represent the DNA that hybridised to the *B. liquefaciens* gene probe in Southern blot analysis of *S. coelicolor* DNA. An *E. coli* strain that is hypersensitive to cAMP is being used to screen for *cya* mutants of *S. coelicolor* and for the *S. coelicolor* cya gene.

3. Storage compound synthesis and degradation

(a) University of Oviedo (C. Hardisson, M.C. Martin, E. Miguelez and C.J. Villar). The amplicon generated from the ADP-glucose pyrophosphorylase gene was used as a probe with an *S. coelicolor* gene library, leading to isolation of two overlapping clones that are being sequenced. We are also using these cosmids for transformation of *S. lividans*, before transforming *S. coelicolor* for studies of their enzymatic activity.

Work has continued on the metabolic enzymes in liquid-grown cultures. All the activities, including glycogen synthase, were determined (some early problems with assays were overcome after a visit by E. Miguélez to Norwich). Specific activities were improved by purification by CsCl density gradient centrifugation, yielding a fraction containing most of the glycogen initially present in the crude extract. In this fraction the specific activities of both glycogen synthase and glycogen branching enzyme were increased significantly, implying a particulate enzyme complex that is now under study. In parallel, glycogen-rich hyphae were subjected to different stress treatments and the behaviour of glycogen, as well as the metabolism of trehalose, were investigated. After an osmotic shock (0.5-1.5 M NaCl or KCl), levels of trehalose increased almost immediately whereas accumulated glycogen was degraded. After a cold shock (4°C) there was an increase in trehalose levels and synthesis of glycogen remained constant. After a heat shock (45°C) there was an increase in the levels of glycogen but trehalose levels remain unchanged. On the other hand, shift-down experiments carried out at different times of hyphal development revealed that glycogen synthesis in liquid cultures starts only after about 3 h of preincubation from the end of the germination process.

(b) John Innes Institute (C.J. Bruton and K.F. Chater). Attempts to clone the genes for branching enzyme and ADP glucose pyrophosphorylase using oligonucleotides based on sequences conserved among homologues from other bacteria and plants led to the successful amplification of a putative branching enzyme gene (*glgB*) segment. This segment would encode an aminoacid sequence about 65% identical to the corresponding segments of *glgB* genes from *E. coli* and *Bacillus* spp. It has been used to identify larger clones from a genomic library. Further sequencing is in progress, with two main aims: to facilitate expression of the gene in *E. coli* for biochemical studies (in Oviedo); and to see if other glycogen metabolic genes are clustered with the cloned *glgB* gene. The PCR- amplified segment has also been used to disrupt *glgB*: initial examination showed no obvious changes in morphology or secondary metabolism. We are investigating the effect of the disruption on each of the two phases of glycogen synthesis (mycelium-associated and sporulation-associated), because Southern blots suggest that a second *glgB* gene may be present, raising the possibility that the two phases of glycogen synthesis involve different gene sets. A transcriptional fusion of *glgB* to the *xylE* reporter has been con-

structed to help in analysing the effects on *glgB* transcription of mutations in developmental genes (*bld* and *whi*) that affect glycogen deposition.

4. Regulatory cascades that lead to antibiotic production

(a) Centre for Biotechnology, Madrid (F. Malpartida, R.P. Mellado, M.A. Fernandez-Moreno). The ActII-ORF4 protein was overexpressed in E. coli using pAZ3ss as expression vector and antibodies were obtained against the purified protein. Although the *act*II-ORF4 transcript can easily be detected in wild type *S. coelicolor*, its translated product could not be seen on Western Blots. To overexpress the ActII-ORF4 protein in *Streptomyces*, the gene was cloned in a multicopy vector (from E. Takano, Norwich) downstream of the *tipA* promoter. The ActII-ORF4 protein could then be detected in Western Blots of cell-free extracts from *S. lividans* only after induction with thiostrepton (no growth was observed when the cultures were started in the presence of thiostrepton, suggesting that overexpression of the act genes might negatively affect growth rate). Attempts to measure the putative DNA-binding activity of the ActII-ORF4 protein, using the *act*III/*act*I intercistronic region as target, were unsuccessful, using cell-free material from either *Streptomyces* or *E. coli.*

From an *S. fradiae* library in *S. lividans*, a sporulating colony producing blue-pigment was selected. The plasmid contained an insert of approximately 3 kb which was further fractionated and the DNA subfragments recloned in *S. lividans*. The putative activator region was thus narrowed to a 476 bp fragment which weakly induced actinorhodin production and was highly stable on propagation. The sequence of the activator gene revealed no homology with any other known *S. coelicolor* genes concerned with actinorhodin biosynthesis or its pleiotropic regulation. The gene was named *micX* because it is transcribed in a 132 nt transcript, which could act as an antisense RNA.

By using heterologous activation of the *act* genes in *S. lividans* we identified a gene whose deduced product resembles other known transcriptional regulators, and contains a good helix-turn-helix motif at the N-terminus. The protein was expressed using the *E. coli* vector pAZ3ss and the partially purified protein was successfully used in gel retardation experiments. The gene is present in both *S. lividans* and *S. coelicolor* , with identical DNA sequence. A role as a regulator of the act genes is suggested by the phenotype caused by insertional inactivation of the *S. lividans* gene: the inactivated gene gives rise to a mutant phenotype in which actinorhodin is overproduced. This effect is stronger in *S. lividans* than in *S. coelicolor* because of the absence of actinorhodin production in *S. lividans* under the usual growth conditions. An interesting feature of this new regulator is that linked to and divergently transcribed from it is a gene whose deduced product is homologous to the *act*III (ketoreductase) gene. The role (if any) of this *act*III-like gene on actinorhodin biosynthesis or in any other biochemical process is being studied.

(b) John Innes Institute Norwich (H.C. Gramajo, E. Takano, J. White, A. Wietzorrek and M.J. Bibb). Growth phase-dependent production of both actinorhodin (Act) and undecylprodigiosin (Red) in strain M145 was found to be mediated by transcriptional activation of their pathway-specific regulatory genes, *act*II-ORF4 and *redD*. RNA polymerase from a transition-phase culture of strain M145, subjected to FPLC-Superose 6 chromatography, was used in *in vitro* run-off transcription experiments. The enzyme that recognises the *act*II-ORF4 and *redD* promoters clearly differs from the major holoenzyme; the nature of this difference (e.g. acquisition of an alternative σ factor, covalent modification of the holoenzyme), and its growth phase-dependence, will be studied. Transcriptional activation of *redD* does not occur in *bldA* mutants; this contrasts with *act*II-ORF4, whose trans-

cription is *bldA*-independent, and presumably reflects additional TTA-containing genes that are needed for *redD* expression.

Attempts were made to isolate ppGpp synthetase from *S. coelicolor* for N-terminal sequencing, with the aim of using reverse genetics to isolate the gene and the required null-mutant. Although activity was detected, its level was much lower than that in *S. antibioticus*. N-terminal amino acid sequence determined from the purified ppGpp synthetase of the latter strain will be used to generate oligonucleotide probes for the *relA*; attempts will also be made to regulate expression of the ppGpp synthetases of *E. coli* and *Streptomyces* (once cloned) in *S. coelicolor.*

Attempts are in progress to clone the presumptive pleiotropic regulatory gene *afsB* and to use transposon mutagenesis to isolate other genes that pleiotropically regulate antibiotic production.

A general comparison was made of different prototrophic SCP1$^-$ SCP2$^-$ derivatives of *S. coelicolor* A3(2), including M145, D132 and MT1109. The strains show very similar growth rates in our minimal medium; addition of 0.2% Casamino acids leads to a comparable reduction in doubling times for all three. However, M145 produces more undecylprodigiosin and actinorhodin (the purple pigment remains in the mycelium and has identical UV spectra to purified actinorhodin (kindly provided by T.J. Simpson) at several different pHs) than D132 on entry into stationary phase, and in our conditions MT1109 produces very little of either. Plate experiments suggest that antibiotic production in MT1109 is much more sensitive to ammonium inhibition or repression than in M145, D132 and two other *S. coelicolor* A3(2) SCP1$^-$ SCP2$^-$ auxotrophs (M124, M130) of independent origin. We have isolated a new SCP1- SCP2- derivative from the wild type strain 1147 by screening for loss of the plasmids (confirmed by PFGE and Southern analysis). This new isolate, M600, which has not been subjected to mutagenesis or protoplast regeneration, may be the most suitable standard plasmid-free derivative for future physiological studies.

5. Changes in ribosome structure during development

University of Oviedo (G. Blanco, M.R. Rodicio, C. Mendez and J.A. Salas). Following our studies on ribosomal protein operons in *S. coelicolor* , we sequenced a DNA fragment containing the L7/L12 and L10 ribosomal protein genes. The deduced amino acid sequences of these proteins were very similar to equivalent proteins from other microorganisms. Upstream of the L7/L12 and L10 genes another operon is found in most microorganisms that contains the L11 and L1 genes. In *S. coelicolor* , these genes do not lie immediately upstream of the L7/L12 gene where instead we found two ORFs with no similarity with other protein sequences in data bases. Fusion proteins between the *E. coli lacZ* gene product and the L7/L12 and L10 proteins were purified by affinity chromatography and used to raise polyclonal antibodies against the two ribosomal proteins for use to monitor expression of these two proteins through the growth cycle (see below).

Changes in expression of ribosomal proteins during growth of *S. coelicolor* in liquid medium were studied using ^{35}S-methionine pulse-labelled samples and further analysis by 2D-PAGE and autoradiography (partly during a 3-month stay by G. Blanco at the Institut Pasteur). The pattern of expression of individual ribosomal proteins was determined: while most of them are synthesized at all times, two proteins were synthesized only in early exponential growth. They were identified as the L7/L12 and L10 proteins by using the antibodies mentioned above. Study of changes in ribosomal proteins during development in solid medium have also

been initiated in collaboration with the Paris group. Comparisons of 2D gels from substrate and aerial mycelium revealed another 2 and possibly 3 ribosomal proteins present in the substrate mycelium but absent from the aerial mycelium. They are being identified and their corresponding genes will be cloned and sequenced.

6. Variations in proteins regulated by global metabolic switches

(a) Changes in S. coelicolor physiology and gene expression at the approach to stationary phase: Institut Pasteur, Paris (P. Kaiser, A.M. Puglia, J Vohradsky and C.J. Thompson). Our 2D gel analyses have revealed global changes in *S. coelicolor* gene expression coincident with a transitory hesitation in growth at the interface between exponential and stationary phases. At this time, the rate of specific incorporation of [^{35}S]. Met into proteins decreases abruptly over a 2 hour period to about 5% of its rate during growth; the cultures then resume growth for a short time, before entering the final stationary phase. Because our cultures were grown in a defined culture medium with maltose and glutamate as sole carbon and nitrogen sources, we could closely monitor changes in nutrient composition as a function of the growth curve. At the time of the transition phase, neither nitrogen nor carbon source had been significantly depleted and the pH of the cultures remained constant. Thus, under our conditions, it seems that accumulation of waste products, lack of O_2 availability, or other undefined metabolic imbalances triggers inhibition of cell division. Since these physiological conditions involve changes in expression of more than 30% of the genes observed on 2D gel, it is a very complex phenomenon for study and commercially available computerised systems had to be employed and refined. Autoradiograms of 2D gels were analysed using hardware and software from Protein Databases, Inc and SAS Institute. Some 1000 proteins are detected on a typical autoradiogram; the intensity (cpm [^{35}S]Met) of about half of them can be reliably quantified. A statistical package for cluster analysis on a SUN microcomputer was used to detect families of proteins with similar kinetic patterns of synthesis. Cluster analysis has been completed on a single experiment. Two other similar experiments have been carried out; all samples have been separated on 2D gels and are being analysed to confirm the reproducibility of the method.

We chose to focus on changes in expression of 3 stress-associated regulons as a function of growth phase: actinorhodin biosynthetic genes (*act*), heat shock genes, and ribosomal protein genes (in collaboration with the Oviedo growth). The first phase of these experiments, identification of the protein spots of each regulon, has been completed. Regulation of all three systems is affected by the physiological transition associated with the slow-down of protein synthesis and growth preceding entry into stationary phase. At this time, expression of the majority of the act genes is activated (*act* gene products were identified by their absence in an *act*II-ORF4 mutant provided by F. Malpartida). The physiological stress is also reflected by the fact that most heat shock genes are spontaneously expressed in the absence of thermal shock. Finally, the expression of several ribosomal proteins is dramatically altered (see report of the Oviedo group).

(b) Global changes in gene expression associated with genetic instability: Institut Pasteur (A. Dary, P. Kaiser, N. Bourget, J-M. Simonet, B. Decaris and C.J. Thompson). In collaboration with the University of Nancy, we also characterised gene expression within the 'silent' unstable region of the *S. ambofaciens* chromosome using 2D gels. The *S. ambofaciens* chromosome (as well that of other species including *S. coelicolor*) includes an extensive region of genetic instability which can be amplified or deleted without obvious effects on the strain's nutritional requirements under laboratory conditions and has therefore been loosely referred

to as the 'silent region'. Nevertheless, the fact that this region is present in independent isolates of the same species suggests that it may encode proteins playing important roles under natural conditions. Indeed, secondary metabolic functions such as pigment production and spiramycin biosynthesis (spi) are often affected by mutations in this part of the chromosome. We characterised spiramycin production in the wildtype and in two mutant strains: NSA229, spi^- having a large deletion and associated 89 kb amplification and NSA228, a spi^+ derivative of NSA229 having an extended deletion which removes the amplification. Our interpretation is that the amplification leads to overexpression of one or more gene products that have negative effects on spiramycin biosynthesis. The following major differences in gene expression of the three strains were observed during exponential growth as well as during stationary phase: (1) compared to the parental strain, many proteins were absent in both mutant strains suggesting that they are encoded by deleted DNA; (2) NSA228 lacked many gene products found in NSA229, suggesting that the second deletion event which removed the amplification, also eliminated additional active genes; (3) NSA229 over-expressed several proteins because of the gene amplification event (probably including at least one having a negative effect on spiramycin biosynthesis). Thus, the unstable region includes genes that are actively expressed during all phases of growth.

HIGHLIGHTS/MILESTONES

Methods successfully developed for the isolation and analysis of red/blue pigments. Purification to homogeneity of phosphofructokinase and pyruvate kinase proteins and demonstration of activity control by energy equivalents. Unexpected effect of *glk* transposon insertion mutations on catabolite repression by carbon sources other than glucose. Cloning of second *glk* gene from *S. coelicolor* , and expression of the *Zymomonas mobilis glk* in *S. coelicolor.* Sequencing of the L7/L12 and L10 ribosomal protein genes from *S. coelicolor.* Development of satisfactory methods for assay of enzymes of glycogen synthesis. Discovery of glycogen-bound complex of enzymes of glycogen biosynthesis. Cloning and disruption of gene for glycogen branching enzyme. Cloning and sequencing of an activator gene for actinorhodin biosynthesis that may act via an antisense RNA. Cloning and sequencing of a putative transcriptional activator for actinorhodin with DNA binding properties, and recognition of a linked *act*III-like gene. Detection of an RNA polymerase form that transcribes the *actII*-ORF4 and *redD* genes. Construction of a versatile *tipA* expression vector. Isolation and characterisation of a plasmid-free derivative of the wild-type *S. coelicolor* A3(2). Generation of polyclonal antibodies against the L7/L12 and L10 ribosomal proteins. Establishment of the first database describing *S. coelicolor* gene expression as a function of the growth phase and application of cluster analysis to identify coordinately regulated families of proteins. Identification of ribosomal proteins, heat shock proteins, and actinorhodin biosynthetic proteins in the database.

WIDER CONSIDERATIONS

The aim of our programme is to interrelate a variety of physiological aspects of *Streptomyces coelicolor* (primary metabolism/storage metabolism/secondary metabolism/morphological differentiation) so that we can understand not only the switches between them but also how changes in one can affect changers in another. We are beginning to understand how *S. coelicolor* selects from among the available carbon sources; what the routes are by which the favoured carbon source, glucose, is metabolised, and how these routes change in response to the growth state; and in which circumstances carbon metabolism gets directed towards storage

compounds. At the same time, we are working backwards — from secondary and storage metabolism and differentiation — towards primary metabolism: thus, we have characterised to varying degrees some genes and proteins that regulate antibiotic synthesis or whose abundance changes in older cultures, and a future goal will be to understand how they respond appropriately to metabolic signals emanating from primary metabolism. We expect that our findings will be applicable to the genetic and physiological manipulation of industrial *Streptomyces* spp. grown for the production of antibiotics and other valuable molecules.
There has been significant mutual benefit by association with the BRIDGE teams of laboratories in (i) the University of Glasgow (coordinated by I.S. Hunter) and (ii) UMIST, Manchester (coordinated by C.P. Smith). Their studies — on (i) central metabolic pathways in *S. coelicolor* (metabolic branch points and anaplerosis; supply of malonyl-CoA for polyketide biosynthesis; the shikimate pathway; and catalase) and (ii) on the role of the *gylA* regulator in catabolite repression and actinorhodin production — are especially relevant to the BRIDGE project. Unfortunately, space limitations prevent their inclusion in this report.

COOPERATIVE ACTIVITIES

Annual co-ordinating meeting (11-13 June, 1992). At Miraflores de la Sierra, Spain, by kind invitation of Dr F. Malpartida and R. P. Mellado. All participating laboratories and the two 'associated' (non-BRIDGE-funded) teams (University of Glasgow and UMIST) were represented. Of the companies who offered support to the BRIDGE programme, representatives were present from SmithKline Beecham (UK), SmithKline Beecham (Madrid, Tres Cantos) and Lepetit (Italy).

Staff exchanges.

(i) *Working visits:* G. Blanco (Oviedo): 3 months in Paris to learn 2D gel technology and to apply it to study the regulation of ribosomal protein synthesis; E. Miguélez (Oviedo): 1 week in Norwich to discuss problems in assaying enzymes of glycogen metabolism with A. Smith and K. Chater; T. Obanye (Manchester) 2 months in Groningen for joint experiments.

(ii) *Co-ordinating visits:* L. Bystrykh, A. Alves and L. Dijkhuizen (Groningen) to Manchester (15-16.10.92), Bristol (17-18.11.92) and Norwich (19-20.11.92); C.J. Thompson (Paris) to Norwich (6-7.7.92 and 15-16.12.92); D.A. Hopwood to Paris (23-24.11.92); D.A. Hopwood to Warwick (19.3.93); P. Postma and J. Kwakman to Norwich (20-22.11.92); T.J. Simpson (Bristol) to Norwich (17.12.92).

(iii) *Exchange of materials:* PCR-amplified segments of putative *glgB* gene from Oviedo to Norwich for comparison with Norwich clone; purified actinorhodin from Bristol to Groningen and Norwich as standards; other blue/red pigments from Groningen to Bristol for structural analysis; *tipA* vector from Norwich to Paris, Madrid, Oviedo,; oligonucleotides from Norwich to Amsterdam; *Streptomyces* strains and vectors between many of the participating groups.

EUROPEAN DIMENSION

This cooperation is providing several genuine bridges. It has encouraged researchers on the basic *E. coli* model system to investigate the generality — or otherwise — of their discoveries; researchers on isolated areas of *Streptomyces* biology are learning to see their work in a 'whole organism' context; academic researchers are brought into contact with industrial scientists, and *vice versa*; and a wide range of expertise has been made available to all participants, minimising technical bottlenecks and maximizing our breadth of vision. The meetings are proving an

invaluable training ground for younger scientists, who are getting opportunities to give formal presentations to 'foreign' audiences. We hope that one outcome will be an increased fluidity of movement of post-doctoral scientists between countries. We believe that interactions on this scale, with their great benefits, are only possible because of the wholesale funding approach (i.e. because the EC is funding not only the communication between partners, but also contributes importantly to the budgets for the work). We cannot envisage comparable success from any system of nationally-based funding.

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The production and recovery of biotechnologically important proteins from the yeast *Saccharomyces cerevisiae* (BIOT CT-900165)

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OBJECTIVES

1. Studies relating to the production of heterologous proteins in yeast.
1-1. Molecular characterization of the fermentation-stage-specific gene *MOL2.*
1-2. Expression of a lactose-fermenting phenotype in brewing yeast

2. Studies relating to the recovery of heterologous proteins by secretion.
2-1. Analysis of enhanced secretion phenotypes: involvement of degradative activity.
2-2. Studies of the secretion performance of trout growth hormone II (tGHII) by *Saccharomyces cerevisiae.*
2-3. Screens for additional enhanced secretion mutants.

3. Studies relating to the recovery of heterologous proteins by cell lysis.
3-1. Further characterization of gene *SLT2* that codes for a homolog of MAP kinases and complements *lyt2* mutations in single copies.
3-2. Analysis of the complementation effects of gene *SPO12*, a suppressor of *lyt1* mutations and attempts to clone gene *LYT1.*
3-3. Characterization of a new set of autolytic strains isolated form a diploid strain of *S.cerevisiae.*
3-4. *Studies on the endo-1,3-*β-glucanase system and attempts to isolate a gene controlling production of these enzymes.
3-5. Complete the characterization of the cloned exo-1,3-β-glucanase genes *EXG1*, *EXG2*, and *SSG1.*
3-6. Use the knowledge acquired concerning the processing and secretion of vegetative exo-1,3-βb-glucanases to facilitate the production of heterologous proteins of interest.
3-7. Cloning and characterization of exo-1,3-β-glucanase-encoding genes from non-conventional yeasts.
3-8. Further development of fermenter strategies using temperature-sensitive autolysis mutants for intracellular recombinant protein recovery.

MAJOR PROBLEMS ENCOUNTERED:

1-1. The *ESP65* gene turned out to be equivalent to *HXT3*, which is being worked on elsewhere. It was therefore decided to concentrate on *MOL2*, which was found to have the additional unusual feature of being regulated by thiamine in the medium.

2-1. It was discovered that a degradative activity, apparently extracellular, associated with wild-type yeast cells, was affecting the yield of some secreted proteins, indicating an unknown factor in the enhanced secretion mutant phenotype. This, therefore, had to be investigated and resolved before any mutant phenotypes could be understood.

2-2. tGHII failed to be secreted from most strains of *S. cerevisiae*, even though it is a naturally secreted protein in trout. Although some strains showed a modest level of secretion, which was aided by growth at low temperature to aid protein folding, this was far too low to be commercially viable.

3-4. A detailed procedure, based on conventional column chromatography and FPLC, has been worked out to purify three endo-1,3-glucanses of very low abundance from *S. cerevisiae*. However, so far it has not been possible to determine the N-terminal amino acid sequence due to blocking of this N-terminal end. The extremely low abundance and instability of these enzymes complicates the solution, but attempts are being made to obtain higher amounts of them in order to remove the block and to obtain the sequence.

RESULTS

1. Studies relating to the production of heterologous proteins in yeast.

1-1. *Molecular characterization of the fermentation-stage-specific gene MOL2. (PAM)*

Two of the λEMBL3 genomic DNA clones that were previously isolated by hybridization with a *MOL2* cDNA probe were sub-cloned into pUC9 for restriction enzyme analysis. The size of inserts were 2.8kb and 3.6kb. Restriction enzyme analysis showed that the two clones were different from each other in respect to the location of restriction sites within the isolated genomic clones. The region of cDNA binding homology within these two isolated clones was determined. Using these regions of cDNA homology as probes onto a northern blot, the same pattern was observed as with *MOL2* cDNA. When a total genomic southern blot was probed with these regions of cDNA binding homology, only 3 bands were observed. Comparison of the two southern blots to each other revealed that one band from one clone was absent in the other blot, when probed with the other clone.

Further analysis of both these clones is currently in progress. A nested set of deletions for both isolated clones has been constructed using exonuclease III digestion in pUC9. Using a double stranded sequencing protocol, one clone has been sequenced and the other is expected to be completed shortly. The sequence data obtained, when compared with known sequences, shows approximately 60% homology over 200 bp (at the DNA level) to the *NMT1* (no message thiamine) gene of *Schizosaccharomyces pombe*, which is likewise regulated by thiamine. When *MOL2* expression is analysed on + or − thiamine containing media, *MOL2* expression is seen to be repressed by thiamine at a concentration of 10μM.

Gene disruption experiments of *MOL2* by one-step gene disruption are currently in progress. The transcript start site will be mapped and the promoter isolated by PCR and analysed using a β-galactosidase gene fusion.

1-2. *Expression of a lactose-fermenting phenotype in brewing yeast (WEL)*

Brewing yeast has neither a lactose uptake system nor an enzyme for breaking down lactose in the medium and so cannot utilise the sugar for fermentation and growth. A brewing ale yeast has been transformed with plasmid pVK1.1, which carries the sequence encoding the *Aspergillus niger* lactase (a secreted enzyme), flanked by the yeast *ADH1* promoter and terminator sequences. This transforms the brewing yeast to ability to grow on lactose as a sole carbon source, and, as such, acts as a dominant selectable marker. In addition, this new phenotype

enables milk-based raw materials in the fermentation process to produce novel products. Various 'worts' containing lactose (eg. yeast extract plus lactose, whey, whey permeate, wort/whey permeate mixtures) can be fermented to produce alcohol as determined in laboratory scale fermentations.

2. Studies relating to the recovery of heterologous proteins by secretion.

2-1. *Analysis of enhanced secretion phenotypes: involvement of degradative activity (CH)*

Initial studies with the mini-proinsulin secretion reporter peptide indicated that the titre of secreted insulin in the culture medium decreased upon storage. Subsequent analysis of the culture profile showed that insulin titre increased to a maximum at the end of log-phase growth (24 hours culture) and subsequently decreased to zero after 72 hours. This profile was not specific to insulin, however, as secretory wheat α-amylase showed a similar pattern. Human serum albumin and bacterial β-galactosidase were also sensitive to this pattern of degradation. These proteins are all non-glycosylated. In contrast, *Aspergillus niger* β-galactosidase, human α-amylase and yeast invertase were all resistant to degradation in the culture medium and are all glycosylated. Deglycosylation resulted in sensitivity. We have therefore been able to show that glycosylation protects secretory proteins, suggesting that this could be one purpose of this post-translational modification.

Degradation of the sensitive proteins in the medium indicated the presence of secreted proteases. However, as *S.cerevisiae* is not known to produce such activity from standard taxonomic assays, we reasoned that the activity was low, in keeping with the slow loss late in culture. Furthermore, buffering the culture medium close to neutral eliminated the degradation, indicating that the proteolytic activity was acid-activated. Certain vacuolar proteases are acid-activated and share the secretion pathway as far as the Golgi, where they are sorted and diverted to the vacuole. We therefore suspected that this may be inefficient and some proteases were being secreted (the default pathway). When vacuolar protease mutants were used as hosts, secreted proteolytic activity was eliminated, suggesting this to be the case.

2-2. *Cloning of genes identified by enhanced secretion mutant phenotype. (CH)*

Two enhanced secretion mutants with dominant phenotypes were chosen as the first subjects for gene cloning. One of these showed enhanced secretion of mini-proinsulin, but failure to secrete wheat α-amylase. The other showed an early enhanced secretion phenotype. Genomic DNAs have been prepared. λYES has been prepared as the vector; this facilitates efficient library cloning and contains a drop-out yeast shuttle plasmid. Library construction is underway.
We are also re-analysing our other mutants isolated with the insulin reporter to ensure that they are not protease mutants, before further genetic analysis and library screening. For this latter purpose, we have obtained and bulked up a YCp50 library.

2-3. *Studies of the secretion performance of trout growth hormone II (tGHII) by Saccharomyces cerevisiae. (JD)*

2-3.1. *Constitutive expression/secretion*

Intracellular expression in *S.cerevisiae* of tGHII by the *HSP12* promoter led to the production of about 100 μg/l of the recombinant protein. This protein however was highly insoluble, accumulating intracellularly as inclusion-bodies-like structures. The protein could only be solubilised by treatment by high concentration of SDS or of guanidinium chloride. As such treatments also destroyed the biological activity, new gene constructions were developed to drive the secretion of the protein in yeast.

In the pYtGH5 construct the prepro sequence of *S.cerevisiae* a factor was fused in frame to a tGHII sequence. This construct was inserted between the *GAPDH* promoter and the α factor terminator. The resulting constitutive expression cassette failed to drive the secretion of tGHII in any of the conditions and any of the yeast strains tested.

The sequence of the pYtGH5 construct was checked for the absence of mutation and for correct joining of the different fragments. The expected sequence was found.

Evidence exists that buffering of culture medium can significantly improve secretion. In addition it is known that tGHII is rapidly denatured at low pH (below 6.5). Strain BF307-10 bearing the pYtGH5 plasmid was grown at different pH in MES-buffered media. Intracellular soluble and insoluble, periplasmic and secreted protein fractions were analysed by polyacrylamide gel electrophoresis and western blotting. In all conditions tested, the recombinant yeast accumulated tGHII in the intracellular insoluble fraction only.

2-3.2. *Expression driven by a regulated promoter*
The above reported negative results could be due to a toxic effect of the recombinant protein when constitutively expressed. Therefore regulated promoters were evaluated for driving the expression and the secretion of tGHII.

The pYtGH7 construction consisted of a fusion between the signal sequence of the *Kluyveromyces lactis* toxin and the tGHII sequence under the control of a hybrid *GAPDH-UAS*^{ADH2} promoter. This promoter is tightly regulated by the carbon source. Plasmid pYtGH7 was transformed into YEGT03, an *ADR1*-protein hyperproducing strain. No secretion could be detected in any of the conditions tested. The transformed yeasts showed a low level of accumulation of intracellular insoluble tGHII.

The use of the *ADH2*-based promoter is restricted to some strains. Therefore to be able to screen different genetic backgrounds for high level tGHII secretion, a new expression cassette containing another regulated promoter was constructed. In pYtGH8 the *HSP12* promoter and the *GAPDH* terminator were used. Secretion was driven by the pre sequence of the *K. lactis* toxin. This cassette was cloned in the 2µm based yeast — *E. coli* shuttle vector pYEGT110. The resulting recombinant plasmid was introduced into the following yeast strains: DBY746, BJ2168, JRY188 and BF307-10.

Expression of tGHII in various media (conical flask experiments) was monitored by western blotting of different protein fractions. The recombinant yeasts expressed tGHII mainly as intracellular proteins (up to 100 µg/l). Strains DBY746, JRY188 and BF307-10 also produced intracellular soluble tGHII (4 µg/l). In strains DBY746 and BF307-10, the protein was secreted at low level (<1 µg/l).

2-3.3. Screening yeast strains for high level tGHII secretion.
To select the best genetic background, more yeast strains were transformed by the pYtGH8 construct. Transformed yeasts were obtained with the following strains: AB103, BJ1991, BJ3501, BJ3505, DBY745, DBY747, SHY2, SHY4 and W303-1B. Three clones from each strain were evaluated for their tGHII secretion capacity with a plate footprint immunoassay in different media (SCD-ura, SCD-ura + casamino acids, SCD-ura MES- buffered at pH 6.5, YPD and YPD MES-buffered at pH6.5). No difference in tGHII secretion could be detected when comparing the different media. Strain BJ1991 looked to be the best tGHII secretor.

2-3.4. *Optimisation of culture conditions*
Published data and non published personal results suggested that secretion and/or protein folding can be improved at low temperature. Moreover tGHII is produced and is active in trout at temperatures usually below 15°C. Secretion of tGHII by strain BJ1991 bearing pYtGH8 was compared at 30°C and 20°C in YPD medium in conical flasks. Western blotting of secreted proteins showed that at both temperatures the yeast cells rapidly secreted recombinant tGHII to a level of 100-200 μg/l. The molecular weight of the secreted tGHII was always 22 kD: the protein was apparently correctly processed and non glycosylated.

The secretion of tGHII was more quantitively assessed by radio-immuno assay. At 30°C the yeast cells secreted a maximum of 80 μ/l of tGHII. At 20°C up to 300 μg/l were detected.

2-4. ***Screens for additional enhanced secretion mutants***
2-4.1. *Mutants showing improved secretion of an inefficiently secreted protein (CH):*
These mutant screens involved the use of a heterologous plasmid construct encoding the secretory β-galactosidase (lactase) of *Aspergillus niger.* This enzyme hydrolyses X-gal in the medium to give blue haloes on plate assay. It is secreted from *S. cerevisiae* with low efficiency and colonies are frequently found that fail to secrete activity. We considered this to be an ideal phenotype for the isolation of enhanced secretion mutants. Over 20 mutants have been isolated that showed a very strong β-galactosidase secretion phenotype (i.e. more pronounced blue haloes on X-gal). Further analysis of their phenotypes and genetics is underway.

2-4.2. Secretion marker tagging of trout growth hormone (tGH) for the isolation of enhanced tGH-secretion mutants (JD & CH):
Constructs are being made to fuse tGH (which is very poorly secreted) with wheat α-amylase (which is efficiently secreted), so that the amylase activity can be used as an easily-screenable phenotype for the isolation of yeast mutants that are able to more efficiently secrete tGH.

3. Studies relating to the recovery of heterologous proteins by cell lysis.

3-1. ***Further characterization of gene LST2 and its role in cell integrity. (CN)***
Gene *LST2* codes for a serine/threonine protein kinase. Detailed analysis of the gene showed that the product is a homolog of MAP (Mitogen Activated) protein kinases. Deletion of *LST2* or transplacement of the mutant allele *slt2-F54* (lysine 54 residue, the putative ATP binding active centre, substituted by phenylalanine) resulted in strains that were autolytic not only at 37°C, but also in the presence of low concentrations (≤ 6 mM) of caffeine. Flow Cytometric measurements, by a procedure developed in our laboratory, documented very clearly the effects of the loss of *LST2* function on cell integrity with the corresponding release of proteins from the cells. Transcription of gene *LST2* was shown to initiate at a unique point located in nucleotide -56 with regard to the initial ATG codon of the ORF. Expression of the gene was analyzed with the use of *LacZ* fusions. Preliminary results showed that expression was maintained at a very low level, the promoter region showing some areas of control that seem to down-regulate expression of the gene as well as a region that regulates expression in response to yeast α-factor.

We have expressed gene *LST2* in *Escherichia coli* and polyclonal antibodies raised for further studies related to cell localization and function.

A heterologous protein expressed in yeast and whose release has been followed in fermenter experiments is bacterial CAT. In order to test the expression of

eukaryotic proteins of interest in these strains, a strategy was followed, based on PCR techniques, for cloning and expression of HLA-DPw2 genes as a single chain synthetic gene, by using a linker peptide. The synthetic gene consisted of the extramembrane domains of genes DPα and DPβ of the human HLA-DpW2 complex. The transmembrane domains are excluded and the hybrid gene was constructed in two versions, one of them carrying a synthetic peptide to favour joining of both chains. Expression and release of these proteins are currently being tested.

3-2. ***Detailed characterization of the LTY1 system and of SPO12 a suppressor of lyt1 mutations. (CN)***

lyt1 mutants were shown to be *cdc*-like mutants that lysed when grown at 37°C, osmotic stabilization with sorbitol not preventing the autolysis of the cells, contrary to what happens with *slt2* mutants. Other phenotypic alterations of *lyt1* mutants were an altered budding pattern and a deficiency in sporulation of homozygous *lyt1/lyt1* haploids. *SPO12*, essentially a gene involved in meiosis, had been cloned as a suppressor of the lytic phenotype of *lyt1* strains but not the sporulation deficiency. Various manipulations were carried out of gene *SPO12* to establish the requirements for functionality in the suppression of the lytic phenotype. Truncation of the C-terminal acid tail of the gene or a change in the charge of the protein, which are known to affect meiotic functions of the gene, did not affect the suppression of the *lyt1* phenotype. Other changes, such as of several potentially phosphorylated amino acid residues, did not affect complementation either.

New attempts to clone the structural gene *LYT1* instead of a suppressor were carried out with the use of a centromere gene bank. Preliminary results indicated that we have finally isolated *LYT1* gene. A set of clones, all of them capable of complementing the different phenotypic alterations (lysis of mitotic cells, budding changes, meiotic deficiency) that occur in lyt1 mutants have been isolated. These clones seem to bear overlapping regions and are functional when carried in a centromere vector.

3-3. ***New thermosensitive autolytic mutants from diploid yeast. (CN)***

A complete process to screen for osmotic remedial autolytic mutants from diploid yeast was conducted and followed by a preliminary genetic characterization, that involved segregation and genetic analysis of progenies. At least one strain was shown to carry a single mendelian dominant mutation leading to autolysis, which is being characterized by molecular approaches.

3-4. ***Studies on endo-l-3-***β-glucanses. (CN)

Purification of three of these enzymes has been achieved (see MAJOR PROBLEMS), but the N-terminal amino acid sequences are still to be determined. Nevertheless, several DNA clones were selected by screening for overexpression of proteins reacting with polyclonal antibodies raised against purified preparations of endo-1,3-β-glucanases. These clones are being sequenced and characterized.

3-5. ***Complete the characterization of the cloned exo-1,3-β-glucanase genes EXG1, EXG2 and SSG1. (FR)***

Work to complete the characterization of the *EXG1* and *EXG2*-encoded products has been directed to a functional analysis of the corresponding proteins through localization of the domains involved in both their correct processing and catalytic activity. With respect to the functional domains involved in catalytic activity, comparison of the predicted amino acid sequences of the *EXG1* and *EXG2*-encoded products have revealed five highly conserved regions which are located in the same relative positions in both polypeptides and may be essential for β-glucanase function. We focused on five amino acids which were totally conserved (DHHHY) and,

in previous work, we demonstrated that removal of any of the histidine residues abolishes β-glucanase activity. The role of these amino acids has now been studied on the basis of the mutants obtained by introducing single substitutions at the ^{293}DHHHY^{297} region of the *EXG1*-encoded polypeptide. Each of the 3 His residues was changed by site-directed mutagenesis into Arg, and the Tyr residue was changed to Phe. Mutations of His-296 to Arg did not result in significant inactivation; mutation of His-294 to Arg causes a 60% inactivation, and replacement of His-295 or Tyr-297 results in a dramatic loss of exo-1,3-β-glucanase activity (about 1% residual activity relative to that produced by the isogenic wild-type strain). These results suggest that the second histidine and the tyrosine are fundamental for catalysis and/or binding to the substrate, and their importance was confirmed by site-directed mutagenesis at the ^{331}DHHHY^{335} region of the *EXG2*-encoded polypeptide: the mutated proteins *EXG2*-His333 Arg and *EXG2*-Tyr335 Phe are almost totally inactive, since the maximum exo-1,3-β-glucanase activity was only 2% of the glucanase activity of the wild-type protein. Interestingly, the XHXY motif is present in the sequence of 26 reported 1,4-β-glucanases (cellulases) from distantly related organisms, such that it would be attractive to speculate that the conserved histidine and tyrosine may play an important role in a common enzymatic mechanism for carbohydrolases with different substrate specificities.

3-6. ***Use the knowledge acquired concerning the processing and secretion of vegetative exo-1,3-β-glucanases to facilitate the production of heterologous proteins of interest. (FR)***

Regarding processing and secretion of the vegetative exo -1,3 -β-glucanases, work was directed to studying the export of the *EXG2*-encoded polypeptide. Contrary to the *EXG1*-encoded glucanase, which is efficiently secreted to the periplasmic space and then released to the surrounding medium, only about 5% of the *EXG2*-encoded exo-1,3-β-glucanase is detected in the culture supernatant. Subcellular fractionation experiments showed that most of the cell-retained activity is associated with the cell-wall structure. Comparison of the predicted amino acid sequence of this exo-1,3-β-glucanase with those of other reported yeast cell-wall-associated proteins reveals a C-terminal structure characteristic of polypeptides attached to the plasma membrane through a glycophosphatidylinositol (GPI) anchor. These GPI-anchored proteins are synthesized as precursors with a serine-rich region, immediately followed by a pair of small residues (ser, Gly, Ala, Asp, Asn or Cys) positioned 10-12 residues NH_2-terminal to a C-terminal domain composed predominantly of hydrophobic amino acids; anchor addition involves a coordinated processing event in which the precursor is cleaved at the level of the small residue motif, thus removing the hydrophobic COOH-terminal domain of the nascent protein, followed by the attachment of the GPI-group to the new COOH terminus. To analyze the function of the C-terminal domain of the *EXG2*-encoded glucanase, we investigated a truncated mutant protein lacking the 69 C-terminal residues, including the serine-rich region, the putative GPI-attachment site (KNAA) and the hydrophobic carboxy-terminus. This protein was not localized in the cell-wall structure, but rather was mostly released into the medium, thus pointing to a direct involvement of the C-terminus of the primary translation product in the correct final location of the mature wild-type 1,3-β-glucanase.

Due to the facility with which the *EXG1*-encoded 1,3-β-glucanase is secreted to the culture medium, most probably mediated by protease Kex2 processing, we have been prompted to construct an expression/secretion vector based on the *EXG1*-glucanase signal peptide. Firstly, we created a recognition site for the endonuclease *Sna*B1 downstream of the strong constitutive *ADH1* promoter, as well as in the 5' non-translated leader sequence of *EXG1*; these were then joined. Secondly, we

engineered an *Eco*RI site 2 amino acids carboxy-terminal to the putative Kex2 processing site. This mutagenic event changes the N-terminal amino acid context (Asn43 → Gln, corresponding to Asn3 in the mature peptide, and Tyr44 → Phe) but does not significantly affect either enzyme activity or secretion. In order to analyse the ability of this signal peptide to direct heterologous protein to the exterior of the cell, another *Eco*RI site was engineered 2 amino acids from the mature N-terminus of a wheat α-amylase as a heterologous reporter gene. After fusion, the final construct results in a plasmid vector containing a 2μm origin of replication, the yeast *TRP1* gene as an auxotrophic marker and a ColE1 origin of replication for propagation in *E.coli*. Additionally, the a-amylase gene is directly followed by the CYC1 terminator, to prevent transcriptional read-through. The correctness of the fusion has been verified by nucleotide sequencing, and the plasmid has been introduced into *S.cerevisiae*. Analysis of a-amylase production and secretion is currently underway. Our future aim is, if amylase production proves to be significant, to replace the constitutive *ADH1* promoter with an inducible or derepressible promoter, and to try to apply the system to polypeptides of commercial and/or industrial interest.

3-7. ***Cloning and characterization of exo-1,3-β-glucanase-encoding genes from non-conventional yeasts. (FR)***

Taking advantage of the high degree of identity exhibited by the exo-1,3-β-glucanases from *S.cerevisiae*, we initiated the cloning and characterization of exo-1,3-β-glucanase-encoding genes from non-conventional yeasts. For this purpose, synthetic oligo nucleotides designed according to the conserved regions were used as primers in PCR to amplify DNA-fragments from genomic DNA from different yeast species *(Schwanniomyces occidentalis, Kluyveromyces lactis, Hansenula polymorpha* and *Yarrowia lipolytica)*. Nucleotide sequencing of the PCR-amplified products revealed a degree of homology with the already characterized *S.cerevisiae* glucanases. This indicated that they form part of the exo-1,3-β-glucanase-encoding genes in these organisms. In order to clone the complete genes, these PCR-fragments were used as probes to screen genomic libraries from the corresponding organisms. Based on results obtained by Southern hybridisation with total DNA, a partial genomic library from *S. occidentalis* was constructed by inserting *Bam*HI-*Xho*I fragments with a size ranging between 4 and 5 kb at the corresponding sites of the Bluescript KS+ vector. This library was screened with the appropriate labelled probe, and this allowed us to isolate a 4.3 kb *Bam*HI-*Xho*I fragment which was then subjected to sequencing analysis. An open reading frame 1275 bp long was found, encoding a 425 amino acid polypeptide with a calculated Mr-value of 49.130, which contains one potential N-glycosylation site. Comparison of the predicted amino acid sequence with that of the *S. cerevisiae EXG1*-encoded 1,3-β-glucanase shows a significant degree of similarity, resulting from a high percentage of identities and an accumulation of many conservative replacements: in 448 aligned amino acids, 232 residues of the *S. occidentalis* 1,3-β-glucanase (52%) are identical to residues in the *EXG1* protein, and another 46 residues represent conserved amino acid substitutions, such that there is an overall level of similarity of 62%. Regarding *K. lactis*, a putative 1,3-β-glucanase containing fragment was isolated by screening a genomic library consisting of 15-20 kb partial *Sau*3A fragments inserted into the *Bam*HI site of the λEMBL3 vector (supplied by Dr P Meacock). Southern analysis localized the putative 1,3-β-glucanase-encoding region on a 4.2 *Xho*I-*Pst*I fragment, whose nucleotide sequence is nearing completion.

3-8. ***Further development of fermenter strategies using temperature-sensitive autolysis mutants for intracellular recombinant protein recovery (DP)***

Following shake flask scale work to define the yield coefficients associated with the auxotrophic requirements of the autolytic mutants, initial studies (25L) have developed minimal and complex media and feed control strategies to enable high biomass (OD_{550} > 100) fed-batch fermentations of *lyt2* and *slt2* mutants grown at the permissive (24°C) temperature. Work is currently in progress to evaluate growth and confirm the lysis of both the mutants and transformants containing a CAT plasmid in batch culture (25L). Following successful completion of these experiments protein release in high density fed-batch fermentations will be examined.

HIGHLIGHTS/MILESTONES

(a) The discovery of thiamine as a regulator of *MOL2* gene expression.

(b) The discovery that mis-secreted vacuolar proteases have a general degradative effect on non-glycosylated polypeptides, but not on glycosylated ones. This is a very important for biotechnological production.

(c) The development of fermenter-based cell autolysis routines as a new means of harvesting foreign proteins expressed within yeast cells.

(d) The discovery of signal transduction-like and cell cycle-like regulation in cell wall integrity begins to show how cell wall synthesis control may be linked with other cell growth functions at the molecular level.

WIDER CONSIDERATIONS

Some products of this research are being evaluated by the industrial participants for potential commercial usefulness. Possibly most interesting is the autolysis system for recovering foreign proteins expressed inside the cell. As yeast cells are normally very difficult to break open, simple temperature-induced cell breakage has potential important application. On a different aspect, the genetic modification of brewing yeast to be able to ferment whey provides, in theory, new possibilities for fermented beverage production. However, such products are not yet met with public acceptability, and for the foreseeable future remain purely experimental.

COOPERATIVE ACTIVITIES

(a) **Formal meetings.**

Regularly scheduled twice-yearly meetings have continued to be highly successful forums for coordinating and furthering the project as an integrated whole.

Meeting 3: November 1992 — part 1: Luton, UK, Host: Whitbread.
part 2: Macclesfield, UK, Host: ICI

Meeting 4: May 1993 (scheduled) Liège, B, Host: Eurogentec.

(b) **Staff exchanges.**

Mr Pablo Alvarez from the group in Madrid is working for a 3-4 months period in the fermentation facilities of ZENECA (formerly ICI) under the supervision of Drs Pioli and Kara. The aim of the visit is to evaluate the biotechnological interest of the *lyt2/slt2* system in pilot plant fermenter scale. Training has been provided in large-scale industrial fermentation research practice. This has included operation and use of 30L fermentation vessels (including safety aspects), on-line gas analysis (mass spectrometer) and RQ based computer control of fed-batch fermentations.

(c) **Materials exchanges.**
WEL supplied the *A. niger* lactase plasmid to CH for secretion studies and use as a reporter for isolating enhanced secretion mutants.
CH supplied α-amylase secretion plasmid and sequence details to JD for the construction of amylase-tGHII fusions.
PM supplied a λEMBL3 genomic library to PR.

EUROPEAN DIMENSION

Scientific research in different countries often shows a bias towards certain activities, as a particular subject area is often too large, with many specialities, for all aspects to be covered within a single country. This is particularly so in yeast molecular biology. Internationally-coordinated research enables groups with different expertise to come together in combinations not possible at the national level. Furthermore, there may not be funds available to bring groups together in the same way at national level. The European Community programmes are therefore invaluable at providing new, mutually beneficial, collaborative opportunities.

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Wide domain control of primary and secondary metabolism in *Aspergilli* (BIOT CT-900169)

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OBJECTIVES

1) Fine stucture map of *A. nidulans pac*C (UK).
2) Detailed molecular analysis of binding of CREA protein to receptor sites (*prn, alc,* IPNS) (F & E).
3) Production of additional mutant *cre*A alleles *in vitro* (F).
4) Production of regulatory proteins (F & E).
5) Introduction of *A. nidulans* and *A. niger* regulatory genes into *A. niger (NL).*
6) Metabolic analysis of mixed carbon source utilisation in *A. nidulans* (wild type, mutants,transformants) and *A. niger* (wild type, mutants) (NL).

MAJOR PROBLEMS ENCOUNTERED

A)Carbon catabolite repression (CCR).
1) Expression in *E. coli* of full length regulatory proteins. (Orsay).

B)pH Regulation.
1) In view of the infeasibility of fine structure mapping of pacC (see previous report) we have made use of the single stranded conformational polymorphism (SSCP) technique as an aid to localisation of mutations for sequence analysis. We have found this technique less laborious than the carbodiimide method mentioned in the previous report. (London).

RESULTS

1. Molecular and genetic analyses

A. CARBON CATABOLITE REPRESSION

i) Definition of sites of action of the creA product for the IPNS gene (Madrid)

The transcriptional start of the IPNS mRNA has been mapped to position –106 (relative to the ATG) using primer extension analysis and cDNA sequencing. The intergenic region between the divergently transcribed ACVS gene and IPNS is 572 bp. Functional elements in the IPNS promotor have been defined in strains containing single copy integrations, at the *arg*B locus, of promotor deletions fused to *lac Z*. β-galactosidase activity has been measured in extracts from mycelia grown under penicillin production conditions leading or not to carbon catabolite repression (CCR). The IPNS promotor is mostly regulated by negative elements which act upon a high basal promotor activity provided by a short stretch of DNA around the transcriptional start. We have identified four negative *cis*-acting regulatory elements, one of which mediates CCR. This element has been named CRE (acronym for catabolite responsible element). This element, which has been reduced to 368 bpp is the target of a negatively-acting transcriptional regulator.

IPNS transcription is not regulated just by negative controls. Two positively-acting regions have been identified. One of them binds a protein from *A. nidulans* total protein extracts. Molecular characterisation of the protein responsible for this binding activity is currently in progress.

By using homogeneous CREA::GST fusion protein (containing the CREA DNA-binding domain) and mobility shift assays, we have scanned the IPNS promotor region for CREA binding sites. Ten binding sites have been identified and footprinted. The consensus sequence protected from DNAaseI digestion fits well with that described by B. Felenbok in this report (G/CYGGGG) for CREA binding sites at the *alc*R promotor, though the existence of exceptions which conform to this rule but do not bind CREA indicates that further unidentified sequences are required for efficient CREA binding.

The positions of these binding sites have been compared to the funcitonal map of the promotor mentioned above. Only one of them lies within the CRE, strongly suggesting that the other nine have no functional relevance *in vivo*. Binding of purified CREA::GST to the site present in the CRE protects 29bp from DNAaseI digestion, consistent with binding of two repressor molecules to this site. Examination of the sequence reveals two adjacent CREA binding sites. The 29bp protected region has been precisely deleted *in vitro* from the promotor, fused to *lac*Z and transformed into *A. nidulans.* A strain containing a single-copy integration at *arg*B has been identified and grown under conditions leading or not to CCR. Comparison of β-galactosidase activities with those driven by the wild type promotor strongly indicate that the only CREA site which lies in the promotor region which mediates CCR has no functional relevance *in vivo.*

We have also analysed IPNS transcription in broths containing C2 and C3 compounds as carbon sources. The IPNS message is repressed in mycelia grown in compounds which are de-repressing for other genes under *cre*A control and *vice-versa*: they are repressed in mycelia grown in certain compounds which are repressing for genes under *cre*A.

Therefore, three different lines of evidence support that CCR of the IPNS gene is not *cre*A dependant:
(i) the absence of substantial derepression in strains carrying the extreme loss-of-function mutations in *cre*A (see previous report); (ii) IPNS steady-state levels in mycelia grown in certain C2 and C3 compounds as carbon sources are not consistent with control through *cre*A; (iii) deletion of the only CREA binding site present in the CRE (the region which mediates CCR identified in this work) causes no effect on expression of the gene under carbon repressing or derepressing conditions. We conclude that a second mechanism of CCE exists for secondary-metabolic genes. The (negatively acting) regulatory gene(s) has/have not been identified most possibly because screens used to select mutations affecting CCR have been based on genes of primary metabolism. The biotechnological relevance of this second circuit of CCR is obious.

Finally, we have shown interaction between CCR and pH regulation on the control of IPNS transcription. These experiments will be completed and described in detail in the next report.

ii) Regulation of the Ethanol Regulon and the Proline Cluster (Orsay)

Our results concerning the mechanisms of carbon catabolite repression and of specific induction on two regulons namely *alc* and *prn* in *A. nidulans* have allowed a thorough dissection at the molecular level of both mechanisms. In the *alc* system,

the relationship between these two regulatory pathways has been demonstrated to occur via a competition between the specific transactivator ALCR and the repressor CREA. For the *prn* system, the results favour a model in which CREA prevents the action of a hitherto unidentified enhancer binding protein.

The Ethanol Regulon:

a) Mechanisms of specific induction:
Identification of ALCR binding sites

Specific induction of the ethanol regulon in *A. nidulans* occurs at the transcriptional level through the action of the transcriptional activator ALCR. This protein was shown to be a DNA zinc-binding protein of the binuclear cluster family (C6 class) as exemplified by GAL4. The zinc binuclear cluster motif was expressed in *E. coli* as an ALCR::GST fusion protein. Gel mobility shift assays, DNAaseI protection footprints, chemical protection and interference footprint experiments have revealed specific binding sites in the *alc*R and *alc*A promoters. These are characterised by a conserved core sequence 5'-qCCGCA-3' occurring in two different orientations: a direct repeat and an inverted repeat. The *alc*R promoter contains the two types of targets, which are adjacent, located far from the start of transcription (-460). The *alc*A promoter contains two direct repeat targets the half sites of which are separated by a variable number of nucleotides and an inverted repeat whose half sites are separated by a constant number of nucleotides (two) as in the *alc*R promoter, all clustered in the same region of the DNA. Our data suggest that the ALCR protein makes contact in the major groove of the DNA helix of the half sites as a dimer.

The ALCR binding sites are functional

For both *alc*R and *alc*A promoters, ALCR binding sites have been deleted *in vitro* and the resulting deletion transformants analysed for their ability to grow on ethanol and for their transcriptional activity.

The conclusions of these studies are:

— the ALCR binding sites are necessary to activate specific transcription of the *alc* genes; they are therefore upstream activating sequences UAS_{alc}.

— both types of targets (i.e. the inverted repeat and the direct repeat) are functional *in vivo.*

— in the *alc*A promoter the three targets are necessary to induce full transcription.

— in the *alc*R promoter the inverted repeat is sufficient.

These results are the molecular demonstration of the *alc*R auto-regulation process shown previously.

b) Mechanisms of carbon catabolite repression:
Identification of CREA binding sites

The CREA repressor was shown to contain two zinc finger-like structures of the C_2H_2 class similar to those found in Zif268, Krox20 and MIG1, the latter being involved in glucose repression in yeast.

CREA was expressed in *E. coli* as a CREA::GST fusion protein and gel mobility shift assays and footprint experiments were performed. In both *alc*R and *alc*A promoters, specific CREA binding sites have been localized which present the same GC rich core 5'-G/CPyGGGG-3'. Three are present in the *alc*R promoter

and two in the *alc*A promoter. Two interesting features characterize three of these sites: 1) they are localized in very GC rich regions of the DNA (75-80%); 2) they are in the same DNA region as the ALCR binding sites, with some of the CREA binding sites overlapping the ALCR binding sites.

Function of CREA binding sites

Deletion of the CREA binding site overlapping the two ALCR binding sites in the *alc*R promoter resulted in a derepressed deleted *alc*R transformant. Derepression of *alc*A was also observed but to a lesser extent (40%) and a small derepression of *ald*A transcription occurred.

These results indicate that the specific CREA binding sites localized *in vitro* in the *alc*A promoters are functional *in vivo*. They also give a rationale for the interaction between specific induction and carbon catabolite repression: since binding sites for the two regulatory proteins, the activator ALCR and the repressor CREA, are localized in the same DNA region, competition should occur between them, resulting in a partial derepression of the *alc*A gene.

Carbon Catabolite Repression in the Proline Cluster:

a) Identification of the CREA binding sites in the cis-acting region

This work has now been completed. We have shown that in a 24 bp region where the prn^d mutations map there are three CREA binding sites. Two of these sites have a higher affinity than the third for the CREA::GST fusion protein. The strong derepressed mutation prn^d22 impairs completely the binding of CREA to one site, whilst the weak derepressed prn^d20 impairs binding to the second high affinity site. The binding pattern of this region has been refined further by protonation interference footprinting. These results extend the consensus sequence for CREA binding to 5' G/CYGGG/AG 3'.

We have also shown that the specific regulatory gene *prn*A is weakly repressed when both ammonia and glucose are present. In contrast to the *alc* regulon, this repression is not strong enough to prevent the induction of the other genes of the cluster. Its physiological role is thus obscure. Gel retardation and footprint experiments indicate that there are two CREA binding sites upstream of *prn*A. One of these sites binds the CREA::GST fusion protein with a higher affinity than the second.

b) Evidence for an enhancer-like element in the cis-acting region

This work has been continued and we have determined that the enhancer-like region can be pinpointed to a region of 240 bp which comprises the cluster of CREA binding sites described above. There is evidence that this region not only acts bi-directionally on the divergently transcribed *prn*D and *prn*B genes.

iii) A. niger creA — Gene and Mutants (Wageningen).

a) Construction of plasmids and acceptor strains for in vivo analysis of creA function

Since the last report, progress on a number of the approaches therein listed has been achieved.
In order to examine the function of the *cre*A gene, specific recombinant plasmids and *A. nidulans* acceptor strains have been generated. These are being used to analyse and compare the effects of the *A. nidulans* and *A. niger cre*A genes on different metabolic functions in response to repressor dosage. Both the *A. niger* and *A. nidulans cre*A genes isolated from the Wageningen lambda phage library have

been successfully subcloned into vectors carrying either the *arg*B or *pyr*A genes. An *A. nidulans arg*B2, *cre*A^d30, *bi*A acceptor strain has been constructed and screening of the progeny from a cross designed to produce a *pyr*G89, *arg*B2, *cre*A^d4 strain is being done. This latter strain will be used to generate molecularly defined transformants containing potential *cre*A target genes at the *arg*B locus and which will then be transformed a second time with plasmids carrying *cre*A genes (*A. niger* or *A. nidulans*) in order to permit the study of the effects of *cre*A specificity and dosage. Using the materials noted above we will examine the influence of the *A. nidulans* and *A. niger cre*A genes on metabolic pathways in *A. nidulans* and in addition study the effects of these genes on targets introduced into *A. nidulans*, such as the *A. niger pel*A gene (see below).

*arg*B/*cre*A_{nid} and *arg*B/*cre*A_{nig} recombinant plasmids have been used to transfrom the *A. nidulans arg*B2, *cre*A^d30, *bi*A strain. Transformants have been tested for their resistance to various levels of allyl alcohol — 1mM, 5mM, 25mM; all retain the biA marker and show resistance to allyl alcohol. With regard to the latter, it was noted that in increasing amounts of allyl alcohol the transformants tended to show a progressively poorer level of conidiation and a greater tendency towards extensive hyphal growth. DNA from the transformants is being isolated to investigate the genomic location and relative copy numbers of the *cre*A genes.

The *A. nidulans pyr*G89, *arg*B2, *cre*A^d4 strain will be used to obtain transformants carrying the *A. niger* pelA gene (encodes PLII), via selection for arginine protrophy, and characterise several of these with regard to number and genomic location of *pel*A genes. Subsequently, a second transformation with the *cre*A/*pyr*A plasmids (selection by uridine protrophy) will be performed and the role of both the A.nidulans and *A. niger cre*A genes on the expression of *pel*A will then be analysed in these doubly transformed strains.

Previous data from our lab (Kusters pers comm) has indicated that the *pel*A of *A. niger* is subject to glucose repression but that when transformed into a *cre*A^+*A. nidulans* strain it escapes this effect. In addition, nucleic acid/protein binding studies (see previous report) have shown the presence of in vitro $CREA_{nid}$ binding sites. In order to obtain some initial data on dosage effects of the *A. niger cre*A on the *pel*A gene a cotransformation experiment has been conducted. *A. nidulans* strain WG164 (*arg*B2, *y*A2, *w*A3) has been transformed with the following plasmid constructs:

1) (*arg*B/*pel*A) + (*cre*A_{nig})
2) (*arg*B/*pel*A)
3) (*pel*A) + (*cre*A_{nig}) + (*arg*B)

A selection of the arginine prototrophs obtained from each transformation experiment has been grown in *pel*A inducing and repressing conditions and samples of medium run on western blots against a PLII antibody. No cross-reactive bands have been seen in the media — glucose or pectin — in which the untransformed recipient strain (WG164) has been grown; several transformants generated from 1) above appear to demonstrate glucose repression of PLA production i.e. cross-reactive bands occur in the pectin medium but nothing is observed in glucose medium whereas others show bands in both types of medium; from 2), some transformants show the presence of a cross-reactive band in pectin but not in glucose thus appearing to demonstrate glucose repression! Others of type 2) (the majority analysed) show the presence of cross-reactive bands in both pectin and glucose media; of those analysed from 3), half show no cross-reactivity in either medium whilst the others show bands only in the pectin medium. DNA has been isolated

from these transformants and is being analysed for *cre*A gene structure and copy number in order to compare this to *pel*A expression.

b) CREA Antibodies

Two anti-$CREA_{nid}$ antibodies of mouse origin have been generated using the *A. nidulans* GST::CREA expression construct referred to in the preceeding sections. Western blots to thrombin digests of the GST::CREA fusion protein indicate that one antibody is directed against both the CREA and GST moieties of the fusion protein and that the other is predominantly directed against CREA epitopes. These antibodies will facilitate the purification of CREA protein by affinity chromatography and permit the study of its expression and any post-translational modifications that may occur.

c) A. niger creA mutants

An *A. niger cre*A mutant has been isolated by UV_A pseudoreversion of *are*A (see last report). Recovery of the *are*A phenotype by both *cre*A_{nig}/*pyr*A and *cre*A_{nid}/*pyr*A plasmids has enabled us to show that this mutation occurs in the *cre*A gene. This strain will allow us to conduct complementary studies to those being undertaken in *A. nidulans* and investigate which metabolic pathways come under *cre*A control in *A. niger* as this is largely unknown. The isolation of additional *A. niger cre*A mutants is currently in progress in order to facilitate an analysis of allele specificity.

d) Biochemical Analysis of A. nidulans creA Mutants

Biochemical analyses have shown that fructose-6-phosphate reductase activity is about 2-3 times elevated in *cre*A^d4, *cre*A^d25 and *cre*A^d30 mutants and that hexokinase activity is increased by 1.5 — 2 fold in *cre*A^d4 and *cre*A^d30, the two most extreme phenotypes. In *cre*A^d30, the activities of aldolase, enolase and glyceraldehyde-3-phosphate dehydrogenase are found to be consistently lower and may be due to impaired growth of this mutant. With regard to intracellular metabolite levels, a 25%-80% increase in glucose-6-phosphate is observed in all three *cre*A mutants analysed. The implications of these observations for transduction of the carbon catabolite repression signal are not yet understood but we expect that these observations will be important to unravel the mechanism at the biochemical level.

B. pH REGULATION

i) Fine Structure mapping of the A. nidulans pacC gene (London)

Resolution of a compression in the *pac*C (wild type) sequence towards the 3' end of the coding region altered the reading frame in this region so that the predicted amino acid sequence is 659 (rather than 573 as reported last year) residues long. This brings the 18bp repeats into frame (see previous report) and they now seem likely to encode an activation domain.

Partially aided by SSCP, mutational sequence changes for several mutations have been determined. Mutations from the major phenotypic class of mutant *pac*C alleles include frameshifts, chain termination mutations and a 203bp deletion all in the 3' portion of the coding region. Two phenotypically wild type revertants of one chain termination mutation and one of another have been sequenced. Two restore the wild type sequence while the third replaces the chain termination codon with a (phenotypically undetectable) missense codon. Two mutations in the

other *pac*C mutant class are frameshifts removing much more of the predicted protein.

The phosphate-repressible acid phosphatase encoding *pac*A and alkaline protease encoding *prt*A genes have been cloned and partially sequenced. pH regulation has been similarly demonstrated for the *pac*C transcript, consistent with autogenous regulation.

A fusion protein containing the *pac*C zinc finger region and glutathione S-transferase has been expressed in *E. coli*. In collaboration with the Madrid partner laboratory, a PacC binding site has been identified by footprinting in the IPNS promotor. A very similar sequence ocurs in the *pac*C promotor.

ii) Analysis of the *A. niger* pacC gene (Wageningen)

The *A. niger pac*C related sequence referred to in last year's report has been used to construct a recombinant plasmid carrying the *arg*B gene from pILJ16. The resultant construct has been used to transform two *A. nidulans pac*C mutants (*pac*C's 11 & 14), both of which have *arg*B2 backgrounds. Positive selection was made on minimal medium at pH 6.0 — only transformants expressing *arg*B will grow and in addition only those in which the *pac*C mutations have been complemented will show restored conidiation. Of the *arg*B$^+$ transformants obtained about two-thirds showed restoration of conidiation, demonstrating complementation of the *A. nidulans pac*C11 and *pac*C14 mutations. This identifies the gene in question as *pac*C of *A. niger*. The 5.5kb *A niger pac* C BamI fragment has itself been fine structure mapped with a number of restriction enzymes. At present we have about 4kb of sequence data, of which just over 2.5kb is now known to be *pac*C structural gene information — sequence data and comparison to the *A. nidulans pac*C sequence (Joan Tilburn, London) has enabled us to determine the approximate location of the *pac*C gene on the *A. niger* BamHI fragment.
Recently, a potential *A. nidulans pac*C target gene — an acid protease — has been isolated and is currently being sequenced.

2. Metabolic analysis and physiological pH regulation studies

Metabolic Analysis of Aspergillus species (Wageningen)

For primary and secondary metabolism studies using ^{13}C-NMR spectroscopy an extensive ^{13}C-chemical shift reference map of the most common fungal cell metabolites has been constructed in order to identify intermediates of cell metabolism in extracts of different *Aspergillus* strains grown on various carbon sources. Additionally some very specific intermediates, most of them known from bacterial galacturonate degradation routes, have been included in this reference list.

Several different phosphorylative and non-phosphorylative galacturonate degradation routes have been described in bacteria but little information is available on the metabolic fate of galacturonate in fungi. This is of interest since the degradation pathway of galacturonate and therefore also of pectin leads to the formation of intermediates common with glycerol metabolism.

The *A. nidulans* mutants WG262 and WG222 are unable to utilize D-galacturonate as sole carbon source in contrast to the wild type WG096 strain (Uitzetter *et al.* J. Gen. Microbiology (1986) 132 1167-1172). In natural abundance ^{13}C-NMR extract spectra of the WG262 and WG222 mutants respective accumulation of two different, unidentified cell metabolites could be observed when transferred from

sucrose to D-galacturonate containing media. These cell intermediates were absent in sucrose grown cells and in the wild type incubated under both conditons.

Comparison with reference measurements and literature showed that the accumulation products in both mutants are not identical to intermediates found in bacterial galacturonate catabolism (tagaturonate,altronate, 2-keto-3-deoxygluconate). Identification of the fungal intermediates is in progress using both ^{13}C-NMR and ^{1}H-NMR spectroscopy and HPLC analyses.

HIGHLIGHTS / MILESTONES

1) The pattern of pH regulation of *pac*C transcript levels and the emerging pattern of *pac*C mutant sequence changes underline the importance of establishing the phenotype of a pacC null mutation (London).

2) The functionality of the *in vivo* CREA and ALCR binding sites has been confirmed (Orsay).

3) It has been shown that IPNS transcription is under CCR and that this mode of regulation is not mediated through *cre*A, the repressor which is responsible for CCR in primary metabolism. It is concluded that a second, unidentified mechanism, must exist, possibly specific for genes of secondary metabolism. Functional analyses of the IPNS promotor, a model secondary metabolic promotor, have been performed using reverse genetic techniques. Identification of *cis*-acting regulatory elements represents a milestone in understanding how transcription of a penicillin structural gene is regulated (Madrid).

4) Complementation of both *pac*C and *cre*A mutations of *A. nidulans* by the equivalent genes isolated from *A. niger* has been achieved, thereby confirming the function of the *A. niger* sequences. A set of recombinant plasmids has been constructed which will facilitate the introduction of these regulatory genes into *A. niger* strains carrying the appropriate auxotrophic markers. In addition, physiological effects early in glycolysis leading to increased levels of G6P and hexokinase and F6P reductase activities have been observed in the *A. nidulans creA* mutants analysed (Wageningen).

WIDER CONSIDERATIONS

1) The inferred existence of a second circuit of CCR opens an obvious field of future research to identify the regulatory genes implicated. The identification of *cis*-acting regions in the IPNS promotor, together with the (expected) co-regulation of the divergently transcribed ACVS gene, opens the possibility of the use of reverse genetics to eliminate undesired controls in penicillin production by *Plectomycetes*.

2) Significant modifications to the model for pH regulation (Arst *et al.*) are anticipated although the basic tenet that *pac*C encodes a transcriptional regulator acting as an activator at some promotors and a repressor at others is clearly consistent with all of the recent results.

3) The isolation of *A. niger cre*A$^-$ mutants will facilitate the identification of genes/metabolic pathways, both primary and secondary, that are subject to *cre*A regulation in this organism and permit a comparison to the well studied systems of *A. nidulans*. This in turn may lead to opportunities to modify the expression of target genes by manipulating promotor elements and/or exchanging genes between these species.

INDUSTRIAL PARTICIPATION

Representatives of Antibióticos (León, Spain) assist in the periodic meetings of the project. M. A. Peñalva visits frequently Antibióticos's factory to discuss scientific advances with technical staff of this company. Ciba Geigy AG (Basel — Switzerland) has assisted in organizing the 2nd annual project meeting.

COOPERATIVE ACTIVITIES

J. Tilburn spent two weeks in Madrid and cooperated with M. A. Peñalva and E. Espeso in establishing the relationship between CCR and pH regulation. Telephone contact between London and Madrid to advance in this topic is continuous and fluent. M. A. Peñalva spent one month in Paris as Visiting Professor, using this opportunity for continuous discussion with C. Scazzocchio and B. Felenbok on CCR and *cre*A. M. A. Peñalva and C. Scazzocchio have obtained EMBO's support to organise a Workshop on the Molecular Biology of Filamentous Fungi, to be held in Sept. 1993. Exchange of strains is continuous between all groups and not subject to restrictions.

H. Arst visited the Orsay laboratory and examined a doctoral thesis in June —92. J. Tilburn spent two weeks in March '92 in the Madrid laboratory, working with M. Penalva and E. Espeso to produce the PacC protein. The Madrid and London laboratories have collaborated on determining PacC binding sites. The Wageningen and London laboratories are collaborating on the *A. niger pac*C sequence analysis. M. Gielkens, a Wageningen undergraduate under supervision of the Wageningen laboratory, spent 6 months (from 1/10/92) in the London laboratory doing his degree project. R. C. Novais from the Orsay laboratory spent 6 months (from 1/7/92) in the London laboratory.

I. von Recklinghausen presented a poster at 'The First European Workshop on Biotechnology Applications of Microinjection, Microscopic Imaging and Fluorescence' (BAMMIF), London, 21.-24.04.1992 and visited the London laboratory for a day to receive some *A. nidulans pal*-strains for the Wageningen laboratory. I. von Recklinghausen also visited the FEBS Advanced Course 'Applications of NMR Techniques to Probe Metabolism in Yeasts and Other Organisms' held in Oeiras Portugal (06-18.09.1992). There a talk on 'The application of ^{31}P-NMR spectroscopy and spectrofluoremetry with pH-sensitive dyes to the measurement of intracellular pH in the hyphal fungus Aspergillus nidulans' was given.

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Stability of genetic information in *Bacillus* (BIOT CT-910268)

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OBJECTIVES

- Studying interactions of a rolling circle replication protein and the cognate origin.
- Characterisation of plasmid segregational stability functions.
- Construction of strains carrying PBSX lysis gene in the chromosome.
- Characterisation of basic replicons of large plasmids.
- Cloning of the *Bacillus* subtilis topoisomerase I gene.
- Analysis of the fidelity of Φ29 replication.
- Studying parameters affecting stability of genes inserted in *B. subtilis* chromosome.

MAJOR PROBLEMS ENCOUNTERED

Instability of *B. subtilis* genes cloned in *E. coli* has slowed down considerably several lines of research, such as isolation of the entire topoisomerase gene or analysis of the phage PBSX lysis gene. The instability could be due to toxicity of the gene products and strategies to either tightly control gene expression or to clone inactivated genes are being presently tested. Replication regions from large plasmids were very difficult to isolate and analyse, also due to their instability.

RESULTS

Interaction of rolling circle replication protein and the cognate plasmid origin (INRA)

Active site of the replication protein (Rep) of the plasmid pC194 was investigated by site- specific mutagenesis. A tyrosine essential for full protein activity was previously identified at a position predicted by sequence alignments with the phage Φχ174 replication protein. However, the mutant protein retained ~5% of activity. The residual activity was not abolished by mutating three neighbouring amino acids carrying a hydroxyl group (two serines and a tyrosine). This raised a possibility that the phosphodiester bond in the replication origin is not attacked by a hydroxy-amino acid but by a water molecule (as is the case for various exonucleases) and that the role of the critical tyrosine is to properly orient the participants in this reaction (water, phospho-diester bond). Since carboxy-amino acids are known to be involved in such reactions their role in Rep protein activity was examined. Three such amino acids are conserved in all known Rep proteins of the pC194 family, one aspartic acid and two glutamic acids. Change of the aspartic acid to alanine did not affect Rep activity whereas change of either glutamic acids to alanine abolished the activity completely. The mutant proteins bind the origin as

efficiently as the wild-type protein, which suggests that their nicking activity was affected. The involvement of glutamic acid in the activity of an RCR Rep protein, possibly by orienting a water molecule, is novel and unexpected.

A mutation was introduced in the replication origin of plasmid pC194, at the nick site. Wild type replication protein had very little activity on the mutated origin whereas mutant proteins, in which the active site tyrosine was replaced by phenylalanine, had low but equal activity on the wild type and mutant origins. This indicates that mutant proteins have relaxed sequence specificity.

Segregation stability functions (Trinity College, Technical University, INRA)

The first step leading to plasmid segregational instability is the appearance of a plasmid-free cell in the population of plasmid-carrying cells (the next step, enrichment of plasmid-free cell in the population is not addressed here). Conventional analysis considers that any cell in the population has the same probability of becoming plasmid-free. To challenge this assumption a test was developed, allowing to visualise selectively only the cells which have lost the plasmid. Fluctuation test analysis indicated that a small sub-population of cells is much more likely to lose plasmids than the remainder of the cell population. This leads to an important conclusion, that the mathematical models currently used to formalise plasmid maintenance are inadequate.

This observation was extended to chromosomal mutations, which also do not occur with a similar probability in all cells. The factors which affect this probability are under investigation.

Segregational stability of RCR plasmids depends, in part, on their single-stranded replication origins. These origins are required to convert plasmid single-stranded DNA into a double- stranded form. To analyse this process an *in vitro* replication system was developed, using single-stranded origin of plasmid pBAA1. The present system allows DNA synthesis in an origin-dependent manner. It will be used to investigate the efficiency with which the origins convert single stranded to double stranded DNA.

A comparative analysis of single-stranded plasmids of the pC194 type was performed. Four groups of plasmids from Gram-positive bacteria were identified: a *Bacillus* group, two *Staphylococcus* groups and a *Lactobacillus* group. In addition, single plasmids isolated from *Nostoc, Shigella* and *Treponema* species are highly divergent from each other and from the plasmids identified in Gram positive species. The double-stranded origins of replication (with the exception of pNostoc) and the putative active site of the replication proteins display significant similarities between all plasmids. In contrast, the single-stranded replication origins appear to be host-specific. In the case of the plasmid isolated from *Shigella* the single- stranded origins are similar to those previously found on single-stranded coliphages. This suggests that the single-stranded origins might be a feature which determines the host range of a given plasmid.

Plasmid stability is affected by formation of oligomeric forms. This process is counter-acted by site-specific resolution systems, which resolve oligomers into monomers. We have analysed the resolution system of the theta-replicating plasmid pAMβ1, comprising the resolvase gene and the *res* site. The site is carried on a ~120 bp DNA segment, and contains three repeats of 14 bp, two of which are in inverted orientation and are separated by 2 bp, whereas the third is in direct orientation relative to the first repeat and is separated by 33 bp from the second. Resolution presumably occurs between the first two repeats and is abolished by a

mutation in this region. Resolution system of a pAMβ1-related plasmid pIP501 was also analysed and found to cross-react with the resolution system of pAMβ1.

The pAMβ1 resolvase has been overproduced in *Escherichia coli* and *Bacillus subtilis* and shown to bind to the *res* site in cell-free extracts. Site-specific mutagenesis of the residues thought to be critical for the enzyme activity is being carried out.

Lysis gene of the phage PBSX (Novo-Nordisk, Trinity College)

5.0 kb of PBSX DNA including the lysis gene were sequenced and 4 ORFs were detected. The lysis gene has not been identified yet, due to instability of the singly cloned ORFs in *E. coli* and *B subtilis*. The instability might be due to synthesis of toxic gene products which could not be controlled tightly enough. To better control expression of these ORFs, a system of gene control based on PBSX functions is being built up. For this purpose a *B subtilis* strain in which most of the phage late operon (~22 kb) is deleted was constructed. The strain carries only the late phage promoter and the last 2.35 kb of the late operon. Temperature induction of the phage genes results, however, in inhibition of the cell growth, possibly because of the phage replication activity, and strains resistant to inhibition are currently being constructed. They will be used to examine the four phage ORFs for the presence of the lysis gene.

Replicons of large plasmids (INRA, Trinity College)

Replication region of large, theta replicating plasmid pAMβ1 was mapped to a segment encoding only the replication protein and origin. This segment displays unidirectional, thermosensitive theta replication, which is dependent on the replication protein and has a broad host range and is thus indistinguishable from that of the parental plasmid. Surprisingly, replication of pAMβ1 is initiated by DNA polymerase I, thus resembling initiation of replication of plasmids related to ColE1. However, ColE1-like plasmids do not encode a replication protein and plasmids which encode replication proteins do not require DNA polymerase I. We conclude that pAMβ1 is a representative of a hitherto unknown plasmid class, which at present includes at least a dozen members from numerous genera of gram- positive bacteria.

Sequence analysis of a region downstream of the basic pIP501 replicon revealed an integrated rolling circle plasmid which is not present in pAMβ1. Its replication is suppressed in an unknown way and can be restored by excision from pIP501.

Replication regions of several large plasmids were cloned, but displayed high level of instability, which precluded their further characterisation.

***B. subtilis* topoisomerases (Groningen University, INRA)**

Two segments of the *topI* gene of *B subtilis*, of 300 and 1000 bp were obtained by PCR, using primers selected by aligning topI sequences from different microorganisms. Sequencing of the segments revealed convincing homology with the *E. coli topA* gene. The attempts to clone the entire *B subtilis* gene in *E. coli*, using high or low copy number plasmids or λ phage were not successful. Complementation of the thermosensitive *E. coli topA* mutation also failed. It is possible that the *B subtilis* gene product is toxic for *E. coli* and we therefore plan to clone the gene in two parts.

Inactivation of the *topI* gene by double or single crossing over with the cloned segment interrupted by a Cm^R gene was not successful. This suggests that the *B sub-*

tilis topI gene might be essential and cannot be inactivated by the strategy used. Since in *E. coli* absence of TopI can be compensated for by increasing the amount of another topoisomerase, TopIV, we are searching to clone the *B subtilis* Top IV subunits, using the PCR approach. Overproduction of *B subtilis* TopIV will be attempted, followed by inactivation of the *topI* gene.

The *E. coli topIII* gene is reportedly involved in certain DNA rearrangements. An internal segment of the *B subtilis topIII* gene was cloned by PCR, the gene was mapped and inactivated by double crossing over. The mutant is presently being characterised.

Φ29 replication (CSCI)

Φ29 DNA polymerase mutants have been isolated in three regions at the carboxyl part of the protein conserved among α-like DNA polymerases, characterized by the motifs Dx_2SLYP, Kx_3NSxYG and KxY (regions 1, 2a and 4, respectively). The mutant proteins Asp^{249}->Glu, Ser^{252}->Arg or Gly, Leu^{253}->Val, and Pro^{255}->Ser in region 1, Asn^{387}->Tyr, Ser^{388}->Gly, and Gly^{391}->Asp in region 2a, and Ly^{498}->Thr or Arg, and Tyr^{500}->Ser in region 4, were purified and characterized. The results obtained indicate that the conserved amino acid motif Dx_2SLYP is involved in metal and dNTP binding, motif Kx_3NSxYG is involved in template-primer binding and dNTP selection, and motif KxY is involved in primer utilisation.

Using synthetic oligonucleotides we have carried out a mutational analysis of the Φ29 DNA right end to evaluate the effect of nucleotide changes at the replication origin and to determine the precise initiation site. The results obtained indicate that:

(1) there are no strict sequence requirements for protein-primed initiation on single-stranded DNA;
(2) initiation of replication occurs opposite the second nucleotide at the 3' end of the template;
(3) a terminal repetition of a least two nucleotides is required to efficiently elongate the initiation complex; and 4) all the nucleotides of the template, including the 3' terminal one, are replicated. A sliding-back model has been proposed in which a special transition step from initiation to elongation can account for these results.

The fidelity of Φ29 DNA polymerase in protein-primed initiation and DNA polymerisation has been studied. The results obtained indicate that DNA polymerisation catalyzed by Φ29 DNA polymerase is a highly accurate process. Conversely, the fidelity of protein-primed initiation is quite low in terms of both insertion fidelity and mismatch elongation discrimination. Even more, the 3'->5' exonuclease activity of Φ29 DNA polymerase is not able to act on the TP-dAMP complex, precluding the possibility that a wrong dNLP covalently linked to TP can be excised and corrected. The sliding-back mechanism proposed can account for the maintenance of the sequence at the ends of Φ29 DNA.

Stability of genes inserted in the chromosome (INRA)

Activation of a RCR origin in the *B subtilis* chromosome stimulates deletion and amplification of repeated sequences in its vicinity. To study the effect of activation of a theta origin we inserted pAMβ1 replicon in the chromosome. The origin was not functional at the high temperature (51°) and was activated at low temperature (37°), as shown by electrophoretic and electron microscope analysis. Both recom-

bination and amplification were only marginally affected by origin activation. This indicates that the type of replication a genome undergoes influences highly its stability.

HIGHLIGHTS/MILESTONES

(1) Acidic amino acids involved in the activity of the Rep protein of a RCR plasmids were identified.

(2) Occurrence of frequent plasmid loss from a small subpopulation of cells was revealed, which questions the validity of the currently used mathematical models to describe plasmid segregational instability.

(3) A novel class of plasmids was identified, having features of two distinct previously known plasmid classes.

(4) Segments of several topoisomerase genes were cloned and a topoisomerase III mutant was obtained.

(5) Amino acid motifs involved in DNA polymerase activity were identified.

(6) A sliding-back model of initiation of DNA synthesis in phage Φ29 was elaborated.

WIDER CONSIDERATIONS

(1) Loss of plasmids from the host cells is affecting usefulness of microorganisms in biotechnology. The observation that a small subpopulation of cells is very prone to lose a plasmid raises two types of important questions:
   (i) what makes these cells different from all the others;
   (ii) how to prevent appearance of such cells. Concepts and technics to deal with these questions should be developed.

(2) Replication is the process which the entire genome of an organism must undergo each generation, and, beyond learning about effects of replication on DNA stability it is important to understand the process itself in great detail. Two hitherto unknown modes of initiation of replication have been revealed, in phage Φ29 and plasmid pAMβ1. They might be paradigms for initiation of replication of numerous genomes in different organisms.

COOPERATIVE ACTIVITIES

A plenary meeting was held in Jouy in Josas on June 12-13, 1992. Two meetings between Novo Nordisk and Trinity college took place, on March 25-27, 1992 and December 17-18, 1992 and one scientist from Novo Nordisk is working at Trinity college. Collaborative efforts are being carried out by INRA and the University of Groningen. Contacts are maintained between different laboratories by telephone, mail and the exchange of materials. Close contacts between the T project on lactic bacteria and the present program are entertained, *via* the University of Groningen and the INRA laboratories.

EUROPEAN DIMENSION

The greatest benefit of the program is to bring together scientist with complementary expertise, which would otherwise not consider interacting.

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Physiology and molecular genetics of amino acid production and secretion by corynebacteria: Flux of intermediates and feedback control mechanisms (BIOT CT-910264)

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OBJECTIVES

— Molecular mechanism of replication of plasmids from corynebacteria;
— Investigation of conjugal competence in corynebacteria;
— Genome mapping and genome plasticity in *C. glutamicum*;
— Sigma factors in coryneform bacteria;
— Characterization of central pathways and TCA cycle key enzymes in *C. glutamicum*;
— Characterization of phage-derived integrative vectors;
— Molecular genetics of amino acid biosynthesis: The arginyl tRNA synthetase gene (argS) from *Brevibacterium lactofermentum*;
— Metabolic engineering to improve tryptophan productivity.

RESULTS

1. Molecular mechanism of repiclation of plasmids from corynebacteria

To determine the region(s) implicated in the replication of pBL1 *(ori)*, several fragments of pBL1 were cloned into the vector pULMJ20. Seven constructions (pULMJ201, 202, 204, 205, 206, 207, 209) were studied. The minimal region able to replicate in *B. lactofermentum* was a fragment *Hin*dIII-*Sph*I of 1813 bp. Computer analysis of this sequence shows three ORFs in this region implicated in the replication of pBL1.

In order to know if pBL1 used the rolling circle (RCR) model to replicate, we tried two strategies:

1) pBL1 was introduced in another host to observe the accumulation of CNAss;
2) several pBL1 constructions were tested to find some of them without minus origin (M.O.).

The absence of a functional M.O. results in a large accumulation of internediate DNAss.

To establish that these single strands were circular intermediates of RCR, it was necessary to demonstrate that they consist of only one pBL1 strand. We synthesized two ADNss probes from each pBL1 strand to demonstrate that the band of DNAss would hybridize with only a specific probe. Lysates from *C. glutamicum* (pULMJ600) and *B. lactofermentum* (pULCF23 and pULMJ600) were assayed using as probes the two specific oligonucleotides. Positive hybridization was obtained with only one of the two probes. These results proved that pBL1 replicates via RCR.

2. Investigation of conjugal competence in corynebacteria

We could identifiy a set of exogenous effectors which influence the transfer efficiency in interspecific matings. Since we knew, that restriction-deficient strains of *C. glutamicum* showed optima transfer results, we reasoned that conjugational competence might be induced by impairing the restriction system of the recipient cells. We therefore tried to clone the gene(s) which are involved in the restruction of foreign DNA in *C. glutamicum*. Out of a genomic library we could isolate a plasmid which is able to complement the restruction deficient strain *C. glutamicum* RM3. A detailed investigation of the complementing DNA region revealed the presence of one open reading frame *orf*1 that is essential for the complementation. *ofr*1 is indeed coding for an enzyme involved in degradation of foreign DNA.

3. Genome pamming and genome plasticity in *C. glutamicum*

The megabase-mapping of the *C. glutamicum* ATCC 13032 chromosome was conducted with several 8-base cutters. Of these, the enzymes *PMe*I, *Pac*I, and *Swa*I had the fewest cuts, whereas *Srf*I, *Asc*I, *Sse*83871, *Not*1, and *Sfi*I all had more than 25 cuts per chromosome. The 'bottom-up' approach was started with the construction of cosmid and phage libraries of *C. glutamicum*. Both banks contain thousands of clones and were successfully checked for complexity with several gene probes.

4. Sigma factors in coryneform bacteria

The major aim of this research is to characterize *B. lactofermentum* sigma factors: their structures, function and evolutionary relationships with other prokaryotic sigma factors. Sequence alignment of the principal sigma factors of *Escherichia coli* and *Bacillus subtilis* revealed that certain regions are highly conserved (the *rpo* box). At least three different sigma factor genes were detected in *B. lactofermentum* genome using the *rpo*D and the cloned *S. griseus hrd*B fragments as probes. Similar results ware obtained when the source of DNA was *Corynebacterium glutamicum* or the nocardioform bacteria *Rhodococcus fascians* (formerly *Corynebacterium fascians*).

5. Characterization of central pathways and TCA cycle key enzymes in *C. glutamicum*

The TCA cycle enzyme 2-oxoglutarate dehydrogenase eomptes with glutamate dehydrogenase for the common substrate 2-oxoglurarate and thus is involved in directing the carbon flux at this branche point of the TCA cycle. The specific activity of 2-oxoglutarate dehydrogenase has been determined in cell extracts from *C. glutamicum* after growth on different media. Acetate kinase (AK) and phosphotransacetylase (PTA) are responsible for activation of acetate and thus are the initial enzymes for acetate catabolism of *C. glutamicum*. Both enzymes have been shown to be present in *C. glutamicum* and seem to be regulated by the carbon source.

The *C. glutamicum ace*A and *ace*B genes coding for the inducible enzymes isocitrate lyase (ICL) and malate synthaxe (MS), respectively, were isolated, sequenced and expressed in *C. glutamicum*.

The physiological role of glutamate dehydrogenase (GDH) of *C. glutamicum* was studied with respect to glutamate biosynthesis and glutamate secretion. For this purpose the corresponding *gdh* gene was used

(i) for gene directed mutagenesis of *C. glutamicum* resulting in a GDH-negative strain and
(ii) for construction of *gdh*-iverexpressing *C. glutamicum* strain.
Analysis of both recombinant strains revealed that GDH is dispensable for grotwh and glutamate production of *C. glutamicum.*

6. Characterization of phage-derived integrative vectors

Temperate phage ∅AAU 2, infecting *Arthrobacter aureus* was previously isolated from samples of soils. Analysis of the lysogenic state revealed the ∅AAU 2 could integrate into the chromosomes of its host thanks to the presence of a unique attachment site (*att P*). Integrative functions were searched for in the rgions surrounding *att p*. Mini-mu PR13 insertions carried on p5510 allowed the localization of two distinct functions: a repressor-like function on the left side and an integrate-like function on the right side. Deletion of the former did not affect integration.

Although essential integrative functions seemd to be encoded by the p5510-phage insert, the study of a ∅AAU 2 spontaneous virulent mutant led us to the isolation of another integrative function, dfistant from*att P* on the phage restriction map. Expression of this new function we called RIN (Regulation of Integration) seems to be crucial for establishment of lysogeny.

7. Molecular genetics of amino acid biosynthesis: The arginyl-tRNA synthetase gene (argS) from *Brevibacterium fermentum* is located in the upstream region of the lysA gene

The *lys*A gene encoding diaminopimelate decarboxylase is the last gene of the lysine biosynthetic pathway in coryneform bacteria and also in a wide range of microorganisms. We have found an open reading frame in the upstream region of *B. lactofermentum* from which the *lys*A gene is expressed. The potential translation product of this gene gave an identity of 30% and a similarity of 50% with the *E. coli* arginyl-tRNA synthetase. To demonstrate biochemically that the product of this unknown gene was an arginyl-tRNA synthetase (*arg*S gene), we subcloned it in a bifunctional vector for *E. coli*-Corylebacteria (pUL610M). Crude extracts of *B. lactofermentum* cells transformed with the latter construction showed a 4-fold higher arginyl-tRNA synthase activity than untransformed *B. lactofermentum* cells.

8. Metablolic engineering to improve tryptophan productivity

Transcription of the tryptophan operon is blocked by the interaction of the aporepressor/corepressor complex with the *trp* operator sequence. We have applied a gel retardation assay base don the ability of the repressor to retard the electrophoretic mobility iof an 175 bp DNA fragment containing upstream regulatory sequences (O-P) of the *B lactofermentum trp* operon, using protein concentrations in the micromolar range. The intesity of the retarded band is roughly dependent on the tryptophan concentration present.

The aporepressor has been partially purified by using gel filtration, ionic exchange (DEAE-Sephacel), mono Q), and affinity chromatography (heparine-agarose, P11 phosphocellulose). Using semipurified fractions we have estimated a M.W. of 27.5 kd for the aporepressor.

As an initial approach to improve the Trp productivity in *Corynebacterium glutamicum* industrial strains we looked at the feasability of chromosomally amplifying key genes involved in Trp biosynthesis. Two clones were amplified, the DAHP gene and a deregulated Trp operon. Fhes amplification events resulted in

increased copy number (approx. 10) for both target genes as evidenced by Southern analysis. Increased copy number was accompanied by increased specific enzyme activity for both DAHP synthase and anthranilate synthase. Strains which gave the best enzyme activities for both clones were assayed for Trp productivity.

To address the problems of genetic stability, Rec A^- strains of *C. glutamicum* and *B. lactofermentum* were constructed. This approach can now be applied to the construction of recombination deficient industrial strains to allow stable gene amplification for overproduction of desirable metabolites.

HIGHLIGHTS / MILESTONES

The mechanism of plasmid replication (rolling circle model) in corynebacteria has been established.

— Factors that influence conjugal competence in corynebacteria have been defined.

— A DNA restriction activity has been found in cell extracts of *C. glutamicum.*

— Pulse field gel electrophoresis has been used for genome mapping and genome plasticity studies in *C. glutamicum.*

— Genes for RNA polymerase Sigma factors have been cloned and characterized.

— The *int* sequences of phages of some corynebacteria have been isolated and integrative vectors constructed.

— The repressor of the tryptophan operon has been characterized by DNA-binding studies.

— The genes for isocitrate lyase and malate synthase of *C. glutamicum* have been cloned and characterized.

— Glutamate dehydrogenase of *C. glutamicum* has been found to be dispensable for growth and for glutamate production.

— Metabolic engineering studies have been carried out to improve tryptophan productivity.

WIDER CONSIDERATIONS

Significant advances have been made by the five groups involved in the project on the basic techniques for genetic manipulation of amino acid producing corynebacteria and related Gram-positive microorganisms. In particular the molecular mechanism of plasmid replication in corynebacteria has been elucidated, effectors that influence conjugal competence in corynebacteria have been defined and pulse-field gel electrophoresis has been used to construct encyclopedias of corynebacterial DNA.

Phage-derived integrative vectors have been constructed what is of great interest from the industrial point of view.

A DNA-binding assay has been used to purify the repressor of the tryptophan operon of *B. lactofermentum*.

A significant advance has been made in the isolation of genes encoding the RNA polymerase Sigma factors which will be very useful for optimization of gene expression. Enzymes for acetate metabolism (which are of great interest since many

corynebacteria use acetate very efficiently) have been purified and the corresponding genes were cloned.

The advances in basic techniques have been applied to improve production of tryptophan by the isolation of the 5-methyltryptophan resistance determinant. These results have opened new perspectives for metabolic engineering to improve tryptophan productivity.

COOPERATIVE ACTIVITIES

A close collaboration network has been established between the five groups involved. Two scientists from the group of Martín (Dr. Luis M. Mateos and Ms. C. Fernández) have been working in Bielefeld. A new scientist (Mr. A. Correia) is working together with Drs. Pühler and Kalinowski in chromosome mapping by PFGE. Integrative vectors were provided by Blanco to Pühler and Sahm. Dunican made «MIU»rec«D» minus strains of *C. glutamicum* and *B. lactofermentum* by gene disruption which were made available to all the groups.

LIST OF JOINT PUBLICATIONS WITH TRANS-NATIONAL AUTHORSHIP

Publications between the pühler's group and the Martin's group with the scientific work of L.M. Mateos and C. Fernández are being prepared for publication.
A review article on the 'Present status of genetic manipulation in corynebacteria' is being written together by Drs. Dunican, Pühler, Sahm, Banclo and Martín under the coordination of Drs. Dunica and Martín.

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Valorisation of non-conventional yeasts of industrial interest: Exploration and molecular engineering of their genetic constituents (BIOT CT-910267)

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OBJECTIVES

Participant 1: Cloning of *K. lactis* genes involved in the *rag* mutations isolated during the previous period; mapping and characterization of the newly cloned genes; regulatory studies of these genes; heterologous gene expression on killer plasmids.

Participant 2: Expression of the *K. lactis* ADH genes under fermentative and respiratory metabolism; construction of ADH mutant strains by gene disruption techniques; determination of the physiological role of the ADH isozymes; analysis of regions flanking ARS sequences and analysis of factors which might interact with them and participate in the initiation complex.

Participant 3: Development of genetics of *Hansenula polymorpha* by construction of heterothalic strains and isolation of auxotrophic and sporulation mutants; analysis of a constitutive glycolytic promoter (GAPDH) from *H. polymorpha*; optimization of expression levels for the model *K. lactis* system (quantitation of RNA and protein levels, determination of optimal transcription levels for maximal product yield, dependence on cell physiology.

Participant 4: Analysis of the modification that blocks the amino terminus of proteins and study of its influence on secretion; influence of glycosylation on secretion; analysis of the precursors, signal peptide; development of supersecretor mutants; formation of hybrids with membrane proteins; uracil and lactose permeases; characterization of cloned genes of *K. lactis* and *Y. lipolytica*, involving DNA sequencing, transcript mapping and gene disruption experiments; cloning of the genes involved in secretion by functional complementation of the mutants.

Participant 5: Characterization of cloned *K. lactis* genes, involving DNA sequencing, transcript mapping and gene disruption experiments; assessment of gene expression under industrial fermentation conditions.

Participant 6: Cloning by complementation of *K. lactis RAG* loci previously identified; definition of metabolic deficiencies in *rag* mutants; genetic and physiological analysis of the catabolite repression mutants to find out the pleiotropic ones; attempt of cloning genes affecting catabolite repression.

Participant 7: Cloning *Y. lipolytica* genes by complementation using genomic library on replicative plasmid and molecular characterization; generating conditional sec ts mutants and their characterization; expression of heterologous genes on the killer plasmids.

Participant 8: Comparison of the performance of the different *K. lactis* strains engineered in year 1 in terms of growth characteristics and product yield; selection of the systems giving the highest product yield and a satisfactory strain stability for further optimization in fed- batch and chemostat experiments; construction of derivatives of the best performing system by varying the transcription signals of the expression cassettes.

Participant 9: Analyze secretion processes and secretion capacity of selected *Kluyveromyces* strain; characterization of cloned regulator sequences and construction of expression vectors; optimization of host/vector system of selected commercial *Kluyveromyces* strain; evaluation of engineered yeasts (*Kluyveromyces* and *Hansenula)* for possible application.

MAJOR PROBLEMS ENCOUNTERED

— The delay of the second funding caused serious problems for some of the participants.

— Strong expression of genes on linear plasmids has not been obtained so far. Development of genetics of *Hansenula polymorpha* meets some difficulties.

RESULTS

Participant 1:

(i) Cloning and characterization of glycolysis genes. Using the previously obtained *rag* series mutants of *K. lactis*, several genes involved in glycolysis have been cloned and their sequences determined. *RAG5* codes for the hexokinase of *K. lactis* and appears to regulate the transcription of *RAG1* gene coding a glucose transporter. This unique hexokinase of *K. lactis* offers a tool to study the regulatory functions of the multiple kinases of *S. cerevisiae*. A few other regulatory genes have been isolated including that for pyruvate decarboxylase and a transcriptional regulator of respiratory enzymes. The first low-affinity glucose transporter gene of *S. cerevisiae* has also been cloned by complementation of *K. lactis rag1* mutation, showing the possibility of cloning redundant genes by heterologous complementation of a non-redundant system.

(ii) Mapping studies. A number of cloned genes have been physically mapped to individual chromosomes. Auxotrophic mutations were genetically characterized and allelism and linkage studies are in progress. An auxotrophic marker sequence was established. A yeast-yeast shuttle bank was constructed and proved to be useful for trans-specific gene cloning.

(iii) A mutant promoter with increased activity was discovered in the killer plasmid, and its sequence determined. The capacity of the killer plasmids as a vector for secreted production of a human protein was examined. Expression was limited by the weakness of the plasmid promoter activity. New linear plasmids have been discovered from several non-conventional species of yeast, and their nature was studied in relation to the killer plasmids.

Participant 2:

(i) There are four alcohol dehydrogenase genes in *K. lactis*. While KlADH1 and KlADH2 are essentially constitutive, KlADH3 is induced by respiratory substrates except ethanol and repressed by glucose. In contrast, KlADH4 is specifically induced by ethanol and is not repressed by glucose. The basis of these regulatory mechanisms is being examined. Single and multiple mutations of the four *ADH* genes have been generated by which the roles of individual genes in *K. lactis* physiology are studied.

(ii) The structure-function relation of *K. lactis* ARS elements is examined on KRF4 isolated in this laboratory and on KARS12 and KARS20 from participant 03. KRF4 and KRS20 can sustain replication of plasmids in both *K. lactis* and *S. cerevisiae*, whilst Kl12 replicates only in *K. lactis*. All KARSs have been subcloned and the ARS function more precisely localized within 100-300 bp regions which have been sequenced. Several nearmatches to the *S.cerevisiae* consensus have been found in all cases, as well as sequences which could bind transcription factors. Binding with *S.cerevisiae* ABF1 has been demonstrated for KARS20. The sequences important for ARS function have been investigated more in detail by site directed mutagenesis. The relation of these sequences with active replication origins is also in progress by means of two-dimensional gel electrophoresis of DNA.

Participant 3:

(i) The *H. polymorpha* methanoloxidase (*MOX*) promoter could be shown to be similarly regulated in *S. cerevisiae*. The *ScADR1* gene is involved in derepression of this gene suggesting a strong conservation in glucose repression and in the regulation of peroxisomal functions between both yeasts. *Cis*-acting regulatory elements conferring *ADR1* regulation have been identified and attempts to clone the H.polymorpha homologue of *ADR1* by complementation in *S. cerevisiae* are in progress.

(ii) The promoter of the glyceraldehyde phosphate-dehydrogenase gene (*GAPDH*) from *H. polymorpha* has been isolated and characterized. It directs high levels of constitutive gene expression and its appliation for heterologous gene expression is currently under investigation.

(iii) The model *K. lactis LAC4* (beta-galactosidase) promoter was further analyzed. It could be converted into a stronger or weaker promoter by manipulating the rate of expression of its transcriptional activater LAC9 which represents the *K. lactis* homologue of GAL4. Fermentation studies are in progress in collaboration with participant 8. Manipulation of the concentrations of additional regulatory components revealed a delicate balance between the galactokinase, LAC9 and the negative regulator GAL80. The regulatory function of *GAL1* (encoding galactokinase) in the induction of lactose/galactose metabolism was further analysed. Mutations that affect either regulatory or kinase activity of the galactokinase could be isolated. The *KlGAL80* was cloned and by disruption of this gene, the *LAC4* promoter was converted into a strong constitutive promoter. A region within the *LAC4* promoter (BCR) confers LAC9 independent transcriptional activation during growth on non-fermentable carbon sources both of *LAC4* and of heterologous genes. An inducible activity binding to the BCR sequence has been identified. Its specificity suggested an involvement in the regulation of another cloned *K. lactis* gene. This possibility is currently being investigated in collaboration with participants 2 and 8. The *RAG1* gene encoding a low-affinity glucose transporter could be isolated in a screen for regulators of glucose repression

of *LAC4*. Its influence on the metabolism during growth on glucose is is examined in collaboration with participant 1.

Participant 4:

(i) A repressible acid phosphatase has been purified from *Kluyveromyces lactis* 2359/152. The ezyme is a heterogeneous glycoprotein with an optimum pH of 4.3 and an apparent molecular weight lying between 90 and 150 kDa. Treatment with the enzyme Endo-H allows one to infer that the protein moiety has a molecular weight of 60 kDa. The analysis of aminoacids of several peptides has been carried out and oligonucleotide cloning probes were designed. By PCR, the gene has been cloned.

(ii) Two DNA pieces responsible for regulating resistance to copper in *Yarrowia lipolytica* have been isolated and both have been sequenced. One of them had an open reading frame, 411 codons long, and has been found to code for a transcriptional activator similar to those described for *S. cerevisiae* and *Candida glabrata*. The other clone contained two open reading frames, in opposite orientation, which appears to code for two proteins of 54 and 55 aminoacids. This result provided a model that is different from those previously described for fungal metallothioneins. The bidirectional promoter region is being analyzed.

Participant 5:

In the first year of the programme, we isolated two categories of *K. lactis* genes which show growth phase dependent expression. A homologue of *HSP12* gene is induced towards the end of exponential growth and in response to heat shock; *K. lactis* homologues of the *MOL1* and *MOL2* are transiently induced on entry into stationary phase during growth in molasses, but completely repressed in YPD. In the second year, these genes were characterized in detail by sequencing, by gene disruption and by expression studies in a variety of media. The component that repress the expression of *MOL1/MOL2* in YPD was identified as thiamine (vitamine B1). Effects of added thiamine on the expression of these genes suggest a likely function for *MOL1* and *MOL2* gene products in thiamine biosynthesis. This is supported by DNA sequence data for the *K. lactis MOL2* coding region which we found to be 60-65% identical to the thiamine repressible gene nmt1 of *Schizosaccharomyces pombe*. This gene is known to be involved in the synthesis of the pyrimidine moiety of thiamine. This function is therefore implied for the *K. lactis MOL2* gene, although this has yet to be confirmed by gene disruption. Disruption of *K. lactis* chromosomal *MOL1* gene in a haploid strain created a thiamine auxotroph which could grow when the thiazole precursor was added. Therefor *MOL1* probably encodes an enzyme involved in thiazole synthesis. Sequence data have also been obtained for most of the coding region of the *K. lactis HSP12* homologue and for several hundred bp upstream of a putative translation start site. Experiments to complete the sequencing of these genes and to map their transcripts are now in progress. Comparison of promoter sequences with other yeast heat shock and thiamine sensitive genes in conjunction with promoter deletions will be used to localize elements responsible for the observed expression characteristics of these genes in *K. lactis*. In particular we wish to investigate the expression of reporter genes under the control of thiamine responsive elements to assess whether this can form the basis of a regulatable system for heterologous gene expression in *K. lactis*.

Participant 6:

The role of the *RAG1* and *RAG2* genes in carbon catabolite repression has been analyzed. Strains carrying the null *rag1* allele but not the null *rag2* allele, display

significantly reduced sensitivity to carbon catabolite repression. On the basis of the parameters of catabolite repression and of the selection strategies previously defined, several catabolite repression mutants have been isolated. The genetic characterization of the isolated mutants defined six complementation groups. Four of them display Rag$^-$ phenotype. Two of the genes involved in carbon catabolite regulation have been cloned and one of them sequenced. The structural gene of D-lactate dehydrogenase has been cloned by complementation of a mutant of *S. cerevisiae*, and sequenced. The regulation of the expression of this gene has been studied.

Participant 7:

(i) By analysis of twenty seven isolates of *Y. lipolytica*, at least ten different karyotypes were distinguished with a number of chromosomal DNA bands ranging between 4 and 6. Some bands corresponded to more than one chromosomes, and some strains appeared aneuploid. Chromosomal repartition of a given set of genes was highly variable, explaining initial difficulties in setting up a genetic system in this yeast. Laboratory strains show reproducibly five chromosomes ranging between 1.5 Mb and 5 Mb. Chromosome separation was improved on a standard laboratory strain and a collection of 30 cloned genes is used to establish a preliminary linkage map. Ribosomal DNA patterns appeared particularly complex with at least 4 loci scattered along the five chromosomes.

(ii) The *SEC14* gene has been cloned. Unlike in *S. cerevisiae, SEC14* appears non-essential for secretion in *Y. lipolytica*, although it codes for the major phosphatidylinositol transfer protein and its product is localized to the Golgi complex. *SEC14* null mutation disrupts the dimorphic transition between the yeast and mycelial forms in all genetic background tested. Homologs of *SEC61, SEC62* and *SEC63* (the products of which form part of the ER translocon) were looked for by PCR or using functional complementation of *S. cerevisiae ts* mutants. A *SEC65* homolog was fortuitously identified in participant 4's laboratory. These genes will be tested for linkage to genes identified as synthetic lethals in the presence of a *SCR2^{ts}* mutation.

(iii) New vectors were constructed containing a piece of ribosomal DNA, the *XPR2* gene encoding a secreted protease, and deleted versions of the *URA3* gene. Deleted versions retaining less than 12 bp of the 5'flanking sequence did not generate transformants when integrated at *XPR2* locus, but produced a few when targeted to the rDNA. These were shown to contain multiple copies of the vector (30 copies or more), thus providing the first multicopy vector system in this yeast. Integrations occurred at different rDNA loci in individual transformants. Transformants were unstable when copy number exceeded 20, and the instability varied in different clones.

Participant 8:

The last report dealt with the construction of a series of isogenic, recombinant *K. lactis* strains that harbored a mammalian reporter gene under control of various homologous and heterolgous yeast promoter sequences, either integrated at a defined chromosomal locus (*RAG2*) or carried on a multi-copy vector derived from the plasmid pKD1. The analysis of transcript levels and product yields revealed that all promoters so far examined (*ScPGK, ScGPD, KlLAC4, KlADH3* and *KlADH4*) gave by far superior yields (at least 10 to 20-fold) when the reporter gene was carried on a pKD1 derived expression vector. This clearly demonstrates that expression levels in *K. lactis* can be strongly influenced by modulating the copy number of the target gene. Furthermore, expression levels of the reporter gene were a function of the transcriptional signals used in the expression units described

above. *KlLAC4* and *ScGPD* represent the strongest and the *KlADH3* the weakest promoters. Although not quite as efficient as *KlLAC4*, the *KlADH4* promoter showed very promising expression levels and was studied further because it represents an interesting alternative to the lactose-galactose regulon in terms of transcriptional regulation. In contrast to the regulated *ADH* system in *S. cerevisiae*, *KlADH4* is not subject to glucose repression and is induced by the presence of ethanol in the culture medium. The *KlADH4* promoter has been isolated by participant 2 and is currently object of a joint study. In order to evaluate the usefulness of the *KlADH4* promoter for heterologous gene expression, shake flask as well as high-cell density fermentation studies were carried out using recombinant *K. lactis* strains expressing the reporter gene under control of the *KlADH4* transcriptional signals. The expression vector was based on the multi-copy plasmid pKD1 and harbored the *KlPGK* gene for plasmid stabilization. The host strain was a *pgk* disruption mutant of *K. lactis* strain Y721. Secretory expression of the heterologous protein was achieved by using a mammalian secretion signal. Plasmid stability was determined by sequential shake flask cultivation of recombinant cells using M9 medium supplemented with yeast extract and lactose plus ethanol as carbon source (10g/litre each). The percentage of plasmid-containing cells as well as the production of the foreign protein remained constant over at least 200 generations. Cell growth and protein production were determined with various media. In shake flasks, one obtained up to 300 mg of foreign protein per liter. High-density fermentations in 2 litre fermentors were carried out with a defined synthetic medium, with ethanol as the carbon source. Fed-batch conditions were achieved either by coupling the carbon dioxide evolution rate (CER) to the nutrient feed-rate, or, after initial batchwise growth and consumption of the C-substrate, by adding the feed medium according to a pre-programmed time-based feeding profile deduced from previous experiments. Growth as well as production kinetics were found to be well correlated to the amount of C-substrate added. At a biomass of 69 g/liter (dry cell weight), the yield (Yx/s) was determined to be 45%, and the protein production reached 840 mg/liter. Similar fed-batch experiments carried out with a complex medium with yeast extract and ammonium acetate as nitrogen source and ethanol as carbon source yielded over 1 gram of secreted heterologous protein per liter.

Participant 9:
In previous studies, it has been shown that *K. marxianus* is a yeast with superior fermentation capacities. As a model system for the production of heterlogous proteins, the expression of the inulinase gene was studied. Inulinase production is subject to glucose repression. The enzyme is partially secreted into the culture medium, partially retained in the yeast cell wall. Under optimal conditions at very high cell densities, over 2 grams per liter of the enzyme is produced. In order to use the promoter and secretion signal sequences of the inulinase gene for the expression of heterologous genes, it was decided to clone this gene. The enzyme was purified and partial aminoacid sequences were determined. From the deduced nucleotide sequences, oligonucleotides were synthesized and used as primers in a PCR reaction using total *K. marxianus* DNA. DNA fragments including the complete inulinase coding sequence and flanking sequences were isolated and sequenced. The nucleotide sequence showed, as expected, a homology with *S. cerevisiae* invertase gene. The transcription initiation site was determined. The inulinase gene contains a prepro sequence for secretion of the enzyme. The promoter and signal sequences were cloned in front of the plant alpha-galactosidase gene in a pKD1-like vector. When the construct was introduced into *K. lactis*, the expression and secretion of alpha galactosidase were comaprable to an analogous *S. cerevisiae* system (using PGK promoter and invertase signal sequence). This result shows that

the inulinase expression and secretion signals are functional for heterologous proteins at least in *K. lactis*. The positive result was obtained using rich media (YEPD). Using continuous cultures with minimal media and sucrose as carbon source (normal expression medium for inulinase in *K. marxianus*), the expression of alpha-galactosidase was very low. The future plans are to test the inulinase promoter and signal sequence in combination with the plant alpha galactosidase gene in *K. marxianus* and to investigate the negative results in minimal media using the inulinase promoter.

HIGHLIGHTS/MILESTONES

- A few regulated genes have been found promising for heterologous gene expression in *Kluyveromyces*.
- Regulatory responses to glucose in *K. lactis* were quite different from what has been known from studies on *S. cerevisiae*.
- Many new regulated gene expression systems are being developped in all the target species of non-conventional yeasts (galactokinase, *GAL80*, first low-affinity glucose transporter of yeast, ethanol-regulated *ADH4*, growth phase-associated genes and thiamine-regulated genes in *K. lactis*, morphogenesis-associated gene and copper resistance gene in *Y. lipolytica*, etc.).

WIDER CONSIDERATIONS

The International Conference on Non-Conventional Yeasts held at Basel in August 1992 is most relevant to the present project. The ELWW workshop 'Biology of *Kluyveromyces* V' was held as a satellite meeting of the 16th International Conference on Genetics and Molecular Biology of Yeast, Vienna, August 1992. Next workshop 'Biology of Kluyveromyces VI' will be held in Siena (organized by participant 6 and I.R.I.S.) in June 1993. *K. lactis* research has established one of the most promising non- conventional yeast systems (genetics, reverse genetics, secretion, tool for gene expression analysis). *K. lactis* gene vector systems for cloning, expression and secretion, as well as host strains have been distributed worldwide in answer to many requests.

COOPERATIVE ACTIVITIES

(1) Coordination. The participants held a coordination meeting at the occasion of the Workshop 'Biology of Kluyveromyces V', Vienna. Another meeting was held at the coordinator's place on March 13 1993.

(2) Working visits.
Visits between participant laboratories were multiple and frequent. Two cases were long stays involving one predoctoral and one post-doctoral fellows.
Participant 1 to participants 2, 5 and 7
Participant 2 to 1 and 8
Participant 3 to 1
Participant 4 to 1 and 7
Participant 5 to 1
Participant 6 to 1 and 2
Participant 7 to 1
Participant 8 to 1, 2 and 3

(3) Exchange of materials (strains, plasmids, chemicals).
Participant 1 with participants 2, 3, 4, 6 and 8.
Participant 2 with participants 1, 3, 6 and 8.

Participant 3 with participants 1, 2 and 8.
Participant 4 with participants 1 and 7.
Participant 5 with participant 7.
Participant 6 with participant 1 and 2.
Participant 7 with participants 4 and 5.
Participant 8 with participants 1, 2, 3.

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Molecular and genetic analysis of genes controlling flower development (BIOT CT-900171)

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OBJECTIVES

The aim is to isolate and study key genes controlling floral morphogenesis in an experimentally suitable system and eventually to compare them with their counterparts in other species. *Antirrhinum* provides an excellent model system since two key genes have already been isolated; it has well-characterised transposons and an extensive range of mutations affecting floral development have been documented. A range of molecular and genetic tools will be established in *Antirrhinum* to facilitate gene cloning and analysis. These include: isolation of a set of active transposons by trapping them in previously characterised genes; construction of a combined restriction fragment length polymorphism and genetic map; genetic characterisation of floral mutants, construction of double mutants and development of a transformation system. These resources will then be exploited to isolate genes by transposon-tagging or differential cDNA cloning. In addition, the possibility of using genes from *Antirrhinum* to study their counterparts in other species such as Pisum sativum will be investigated. The results of this research should make a major contribution to the isolation of key plant genes and to our understanding of flower development.

RESULTS

1. Transposon-trapping

One feature of the *Antirrhinum* system, of great importance for gene isolation, is the availability of cloned transposons (short segments of DNA that can move around the genome). If a mutation in a gene is caused by insertion of a well characterised transposon, the affected gene can usually be isolated. It is therefore essential to isolate and characterise all active transposons that are present in *Antirrhinum* lines routinely used for transposon mutagenesis. The approach adopted in this programme is to trap the active transposons in previously isolated genes. For example, the *incolorata (inc)* gene is required for red pigmentation of *Antirrhinum* flowers; *inc* mutants have pale or white flowers. So far, 16 different *inc* mutants have been recovered by the Norwich group by screening mutagenic lines for pale or white-flowered plants. Because the *inc* gene has been cloned, it was possible to analyse the *inc* mutations at the molecular level. They showed that most of the mutants were caused by insertions of members of the CACTA family of transposons at different positions in the *inc* gene. Eight of the transposons have been shown to correspond to previously cloned transposons (Tam1 has been trapped once, Tam2 once, Tam4 twice, Tam5 three times, Tam6 once). Of the remaining eight transposons, two have been cloned and are now being sequenced. These will be available as probes in transposon-tagging experiments. In addition to *inc*,

many other loci are also being used as traps in this way: five transposons have been trapped at the *plena* locus, two of which are Tam3 and one is Tam7 (Norwich), three at *squamosa* (Koeln), three at *globosa*, one Tam1, one Tam7, one Tam9 (Koeln) two at *deficiens* one Tam7, one Tam8 (Koeln), two at *sepaloidea*, both Tam3 (Norwich) and two at *floricaula*, one Tam3, one CACTA type (Norwich). We have therefore been able to establish a 'library' of transposons available for general use. In addition, because many of the trapped transposons show DNA sequence homology at their ends, a general PCR strategy for selecting for insertions in genes is being investigated (Norwich).

2. Targetted tagging

Many of the mutants in *Antirrhinum* have arisen from general transposon-mutagenesis programmes so that several different types of transposon could be responsible for a mutation. An alternative approach is to try and target a specific transposon to the gene of interest. This may be possible because several plant transposons have been shown to preferentially transpose to chromosomal positions closely linked to the site of excision. To test the feasibility of this approach, an extensive crossing programme is being carried out by the Lisbon group to try and select for events in which the transposon Tam3 has moved from the pigmentation gene *pallida (pal)* to *divaricata (div)*, a locus closely linked to *pal*, which controls flower shape. About 3000 progeny from a cross between *div* and 100 different Pal^+ revertants have so far been screened for new *div* mutants. Many of the revertants should carry copies of Tam3 in the vicinity of *div* and by growing them at 15°C, Tam3 transpositions should be favoured. Because the Pal^+ revertants were heterozygous for the *pal* allele carrying Tam3 and the *div* parent was homozygous for a stable recessive *pal* allele, it was possible to confirm that Tam3 transposition had indeed been very high since about 85% of the progeny were Pal^+. Most of the progeny had flowers with a shape intermediate between *div* and wild-type, as expected because *div* is semidominant. However, 4 plants had flowers with a near-normal shape. Molecular analysis revealed that each of these 4 plants carried an extra copy of a locus closely linked to Div^+ (< 1cM). In one of these plants it was possible to show that the previously mapped chromosomal region *pal-div-flo* had been duplicated. In the progeny derived by selfing this plant, 2 novel genotypes carrying either 3 copies of Div^+ or 2 copies of *div* and 1 of Div^+, were identified. Interestingly, the plants with 3 doses of Div^+ had a novel phenotype which may reflect overexpression of *Div*. They are now trying to analyse the novel genotypes cytologically. However, taking full advantage of the material generated in the programme requires a *div* clone and therefore obtaining a Tam3 insertion at *div* remains as their main objective.

3. Construction of an RFLP map of *Antirrhinum*

The previous section illustrates how the ability to localise genes on a genetic map can be of enormous value for gene isolation and analysis. Although a good genetic map of *Antirrhinum* exists, it is of limited value for the rapid assignment of map positions to newly cloned DNA sequences. To circumvent this problem, the Koeln group has constructed a combined genetic and Restriction Fragment Length Polymorphism (RFLP) map for *Antirrhinum*. Crosses have been carried out between inbred lines of *Antirrhinum majus* and *Antirrhinum molle*, two species that are likely to reveal RFLPs. 96 F2 progeny from these crosses have been probed with more than 100 molecular markers (these include randomly selected cDNA clones and clones of known loci, which were provided in part by the Norwich group). This analysis has already allowed the map position of 100 cloned DNA

sequences to be determined. Because some of these cloned sequences correspond to previously mapped genetic loci, it has also been possible to start aligning the RFLP and classical genetic maps. So far, five out of the eight *Antirrhinum* chromosomes, mapped in the past by classical methods, have been assigned to RFLP linkage groups. The validity of the RFLP map was confirmed by comparison of linkage data obtained by analysis of two independent F2 populations.

The map produced has already proved invaluable to all *Antirrhinum* scientists. In the future, the Koeln group wish to further refine the map using a novel method (AFLP), developed by KeyGene in Holland. They have also carried out an extensive EMS mutagenesis in *Antirrhinum* to try and generate further genetic material that will be of general use.

4. Construction of double mutants

The analysis of how genes interact to control flower development can be greatly aided by studying the phenotypes and properties of plants that carry mutations in several genes (e.g. double or triple mutants). The Norwich, Koeln and Lisbon groups are involved in this aspect of the programme. For example, the Norwich group has constructed and analysed: *cyc;ovu, def;ple, sep;ple, glo;ple, flo;glo, flo;s-qua*. The phenotypes obtained have had important implications for models of how flower development is controlled. The Lisbon group has made *cyc;div* double mutants and are incorporating a third flower shape gene, *dichotoma* in their analysis. Many other mutant combinations have also been made in the collaborating laboratories. In addition, screens for mutations that suppress or enhance the phenotype of genes such as *flo* (Norwich) or *def* (Koeln) have been initiated.

5. Transformation of *Antirrhinum*

The ability to modify and introduce genes into plants provides a very useful tool for the analysis of gene function. Although genes isolated from *Antirrhinum* can be introduced into related species, such as tobacco, it is not yet possible to re-introduce them into *Antirrhinum*. This is because there is no routine method for transformation and regeneration of *Antirrhinum*. One aim of the programme is to try and develop such a method.

Initial work of the Birmingham group centred on the use of *Agrobacterium tumefaciens* and various experiments were carried out to optimise T-DNA transfer. Since transfer rates are known to be influenced by a range of factors, including the concentration of certain phenolic compounds, the Birmingham group performed transformations using combinations of different phenolics, wild-type *A. tumefaciens* strains and varieties of *Antirrhinum*. In general, optimal transformation (as measured by the rate of production of opine-producing tumours) was found with strains C58 or A281. Transformation was favoured by low pH and the inclusion of acetosyringone in the co-cultivation medium, although maximal regeneration of the least susceptible variety was obtained with a combination of high pH and the addition of syringaldehyde. Further transformations were made with a range of disarmed vectors containing the reporter gene *gus* and a selectable marker gene (encoding kanamycin resistance). Following large-scale experiments, no transformed plants were regenerated. The conclusion was that, using the protocols developed, the frequencies of both regeneration and *A. tumefaciens*-mediated transformation were quite low.

More recently, the Birmingham group have turned their attention to the use of *Agrobacterium rhizogenes* for transformation. This bacterium induces the produc-

tion of hairy roots rather than tumours but, for some plant species, it has been possible to regenerate plants from these transformed roots. A protocol has been developed that allows the production of hairy roots from a wide range of *Antirrhinum* varieties. So far, it has only been possible to induce these roots to form on excised hypocotyl segments. Using *A. rhizogenes* that contains a binary vector, it has been possible to produce *gus*-positive hairy root clones which can also be selected on plates or in liquid culture on the basis of their kanamycin resistance. A low rate of shoot regeneration from some hairy root clones has been observed for a few varieties of *Antirrhinum*. Some of these shoots have been transferred to soil and one individual, with *gus*-positive leaves is currently growing in a glasshouse. This individual displays the distorted morphology characteristic of plants derived from hairy roots and it is not known yet whether it is capable of setting seed.

Two types of experiment are currently being used in an attempt to optimise this transformation system. A number of bacterial cell lines containing modified Ri (root-inducing) plasmids have been obtained; in these either TL, TR or one of the *rol* genes has been deleted or inactivated. Use of these modified strains may lead to altered hormone balances in the hairy roots produced and this may favour shoot production. In a second approach, cytokinins are being added to the medium on which the roots are growing in an effort to enhance the frequency of shoot formation.

From the initial work with *A rhizogenes*, it seems clear that genetically engineered *Antirrhinum* plants can be produced; the problems now being addressed are those of 1) increasing the frequency of shoot formation from hairy roots, 2) expanding the range of varieties in which this occurs, and 3) overcoming the difficulties resulting from the typically distorted phenotype of plants produced from hairy roots.

6. Technology transfer between plant species

Understanding the genetic control of flower development in *Antirrhinum* should provide a model for studies in other species. To investigate the possibility of extending expertise to other plants, the Valencia group is using *Antirrhinum* DNA probes for the analysis of genes controlling flower development in pea. The genetics of pea has been very well researched and many mutants are available. One of these gives flowers in which carpels (female organs) grow in place of stamens and petals are sepaloid; a phenotype rather like that of the *deficiens* mutant of *Antirrhinum*. The Valencia group is using the *deficiens* gene to isolate its counterpart in pea to test whether the phenotypic similarities between the mutants in these species reflect the action of a common gene. They have constructed a cDNA library from pea flowers and probed it with a *deficiens* clone. 24 positives were isolated and these are currently being characterised. They have also isolated several cDNA clones which show differential expression in ovaries when the giberillin, GA3, is applied. This is of particular interest because effects of GA3 on ovary development have been demonstrated in pea.

In parallel with this work, they have also been characterising the histology of pea floral mutants and screening for monoclonal antibodies which will specifically recognise cell types in different floral organs. From an initial screen of monoclonal antibodies against extracts from pea flowers, they obtained one antibody that recognised the large subunit of Rubisco. They were able to use this to identify sepals due to the low amount of Rubisco in the other floral organs, allowing the transformation of petals to sepals in the *deficiens*-like mutant to be readily

visualised in flower sections. A second monoclonal recognised an antigen present in petals at high levels relative to other organs.

One problem encountered in the screens for organ-specific antibodies was the presence of large amounts of proteins common to all flower parts. To alleviate this problem, they have used several strategies to eliminate common proteins from the extracts before they are injected into mice. Results with subtracted carpel extracts have been encouraging and led to the isolation of several ovule-specific antibodies. A similar approach is aslo being extended to stamens. However, in this case there is the further complication of abundant proteins that may be immuno-dominant. Anthers contain an abundant 27kD protein, located in the pollen sacs, that is present at very early stages of development. To eliminate this protein from anther extracts by immunoprecipitation, they have raised a monoclonal antibody that will recognise it. This antibody has also been useful as a marker for identifying anther cell types in homeotically transformed organs in pea. The antibodies obtained from these screens may be valuable for studying *Antirrhinum* as well as pea, illustrating that exchange of materials and expertise between groups working on different species may be very valuable.

HIGHLIGHTS AND MILESTONES

1. Isolation and characterisation of several new plant transposons.
2. Construction of a combined RFLP and genetic map for *Antirrhinum*.
3. Characterisation of novel gene interactions controlling flower development.
4. Characterisation of monoclonal antibodies recognising floral organs
5. Successful transformation and regeneration of *Antirrhinum*

WIDER CONSIDERATIONS

The form, physiology and function of the flower is of central interest to plant developmental and reproductive biologists. Furthermore, the flower is of major agronomic importance both for the efficient breeding of crops and because many plant products are derived from flower seeds and fruits. European scientists have made major advances in the molecular and genetic analysis of flower development through studies on *Antirrhinum* (the garden snapdragon). By studying mutations that cause *Antirrhinum* flowers to develop abnormally, they have been able to start unravelling how genes control the development of normal flowers. The technology is also being extended to other species, such as pea, so that processes general to all plant species are being uncovered.

This programme has brought together expertise in different European laboratories to develop the most effective tools required for exploiting and extending the *Antirrhinum* system. They have been successful in isolating several novel transposons (jumping genes) from this species and have developed a molecular map of its chromosomes. These tools can now be used to help scientists isolate and study key genes controlling flower development.

COOPERATIVE ACTIVITIES

The success of this programme depends on the effective combination of complementary skills and resources in the participating laboratories. The disciplines covered include: molecular biology, classical genetics, tissue culture, developmental and comparative biology. Each laboratory alone would be unable to develop all the

tools necessary but in combination it is hoped that will firmly establish a powerful and generally useful model system. The particular exchanges that have occured are:

1. Meetings
So far we have had two major meetings, one held in Koeln from 17/4/91-21/4/91 and one in Valencia from 13/5/92-16/5/92. These involved presentations from all of the participants. A report on the first meeting was published in Flowering Newsletter (see below). The next meeting will be held in Norwich from 26/5/93-30/5/93.

2. Materials
Many clones of genes have been freely exchanged between participants, including clones of *flo, ple, def, glo, squa, del, pal, olive, inc.* In addition, cloned transposons exchanged include Tam1, Tam2, Tam3, Tam4, Tam5, Tam6, Tam7, Tam8 and Tam9. Many genetic stocks have also been freely exchanged, including those carrying mutations at *flo, ple, glo, squa, inc, div, pal, def.*

3. Staff exchanges
J. Almeida spent July 1991 working with the Norwich Group.
S. Hantke from the Norwich group worked for 3 weeks with the Koeln group in December,1991.
M. Mooney and P. McSteen from the Norwich Group visited the Birmingham group for 2 days in September, 1991.
E. Andrade from the Lisbon group has joined the Koeln group to work on a PhD.
P. McSteen and J. Roberts from the Norwich Group visited the Birmingham group in October1992
M. Rocheta from the Lisbon Group came to work with the Norwich Group for 1 month in March 1993.
M. Rodriguez from the Valencia Group spent two months with the Kolen Group in 1992.

JOINT PUBLICATIONS

Carpenter, R., Coen, E.S., Huijser, P., Schwarz-Sommer, Z., Sommer, H. (1991) The genetic control of flower development in *Antirrhinum Flowering Newsletter* **12**, 38-41.
Bradley, D., Carpenter, R., Sommer, H., Hartley, N. & Coen, E. (1993). Complementary Floral Homeotic Phenotypes Result From Opposite Orientations of A Transposon at the *plena* Locus of *Antirrhinum*. Cell, **72**, 85-95.

In addition, in January 1992, we submitted a report on the programme to be published as a brochure by the Commission.

The molecular basis of cell-cell interactions in self-incompatibility (BIOT-90-0172)

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OBJECTIVES

The objectives set for the second year of this project as defined by the original workplan deviate from the results achieved in a number of respects. It was proposed to transform potato plants with an SLG coding sequence (now generally termed S-RNAse in the *Solanaceae*), and to monitor production of the mRNA and protein products. As reported in our previous (1992) progress report, however, this work must be preceded by a definition of the promoter sequences necessary for high-level, authentic expression of the S-RNAse, and experiments in this direction have been carried out in 1992. A further objective was the development of an *in vitro* bioassay for S-RNAse action. In this connection we have made substantial progress, both in the definition of reliable *in vitro* culture conditions for potato pollen, and in S-RNAse purification, but have not yet carried out bioassay experiments due to priority of other experiments. As planned, a cDNA bank from pollen mRNA has been prepared and screened for S-RNAse-homologous clones and for S-allele-specific clones (see results). A YAC library for potato is now available and is being screened with a number of single copy probes. Highly conserved S-RNAse sequences have been employed to clone an S-RNAse sequence from a related species, *Lycopersicon esculentum*. This clone is of interest in two respects

(i) to make use of the tomato RFLP map and YAC banks;
(ii) to understand how the tomato S-locus has been inactivated. This work precedes attempts to introduce SI into tomato, and the corresponding experiments are now planned for the final year of the contract.

MAJOR PROBLEMS ENCOUNTERED

The outstanding problem remains clear evidence for the nature of the pollen S-gene product. Although other research groups report an S-RNAse sequence is expressed gametophytically in microspores at the time of tapetal degeneration, our analogous PCR experiments have been negative. Additionally, no pollen transcripts have been identified on the S-RNAse genomic clones for alleles *S1* and *S2* from potato. These clones outside of the S-RNAse coding region are composed of highly repetitive sequences, making 'walking' in the lambda library unrealistic.

RESULTS

Characterization of S-locus defined potato plants suitable for transformation and molecular biology (Wageningen).

Tuber-bearing S-homozygous lines (for S1, S2, S3 and S4) have been identified and propagated. The S3 and S4 homozygotes include lines carrying the 'compatibilizing factor' tS1. Seed lots have been obtained, which consist of pure, unmixed S-heterozygotes (such as only S1S2, or only S1S3, etc.). A readily transformable dihaploid line, A16, has been identified. As this line is SC and possesses an unknown S-allele, other readily transformable lines of known S-genotype have been screened out. Thus far, these lines are also SC, but attempts are being made to select SI progeny from these lines. The lines have been made available to the other BRIDGE partners.

Potato transformation with S-RNAse sequences(Wageningen)

The S2-RNAse genomic clone sequence in pBin19 has been transferred into the diploid potato clone A16. The plants are now ready for analysis and crossing onto SI partners.

Genetics of interspecific incompatibility *S. tuberosum/S. verrucosum* (Wageningen)

Interspecific crosses between S. verrucosum and S. tuberosum have been carried out and their fertility in crosses with acceptor and non-acceptor tuberosum lines investigated.

Ultrastructural analysis of fertilization in SI potato lines (Siena)

The distribution of SK2 and S2 (S-RNase S2) was investigated in pistils of potato clones of known S-genotype using antibody prepared from FPLC-purified SK2 and from an *E. coli*-expressed GST-S2 fusion. SK2 is located in the intercellular matrix of potato styles where it accumulates to very high levels. The pattern of accumulation was identical in four different potato clones. In contrast, SK2 was concentrated in the cytoplasm of stigma secretory cells underlying the papillae. SK2 is a medium component playing an unknown role in facilitating pollen tube growth. In view of its sequence identity and enzymatic activity as a chitinase, it may be involved in defence against fungal and/or insect pathogens. Monoclonal antibodies are being prepared against SK2 to enable broader comparisons of its distribution to be made.

The distribution of S2 showed a qualitatively similar pattern to that of SK2 except that no cytoplasmic labelling was seen.

Transgenic plants expressing S2 in pollen have been developed (Köln). These are being analysed by immunocytochemistry (Siena) to establish precisely the cellular localization of the protein.

Molecular characterization of the gametophytic two-locus self-incompatibility system in rye (Hannover)

As reported in the 1992 progress report, the involvement of phosphorylation in the rye SI reaction is being investigated. The phosphorylation kinetics in germinated as well as ungerminated pollen grains differs significantly after *in vitro* treatment of the pollen with either 'cross' or 'self' stigma eluates, with pronounced phosphorylation following 'self' eluate treatment of pollen grains. The difference was most distinct 90 seconds after the start of the phosphorylation reaction, which

corresponds to the timeframe of an *in vivo* SI reaction. Self-compatible mutants displayed significantly less phosphorylation in untreated pollen grains compared to SI genotypes. Three polypeptides which selectively failed to be phosphorylated in pollen of self-fertile mutants are regarded as promising candidates for SI-related pollen components.

Application of different tryosine- or PKC-specific protein kinase inhibitors and the Ca^{2+} antagonists verapamil and La^{3+} to isolated stigmas resulted in an inhibition or delay of the SI response upon *in vitro* self-pollination. On the other hand, application of the Ca^{2+} influx-promoting ionophore A 23187 resulted in a higher proportion of pollen tube inhibition in genetically compatible combinations.

The results obtained so far suggest that in rye the recognition event may occur before germination within the first 90 seconds after the initial contact between the pollen grain and the stigma surface and that loss of self-incompatibility in sf mutants may in part be the result of protein kinase deficiency in these mutants.

A model for the SI mechanism in grasses was established which comprises the independent, additive activation of pollen-born membrane-bound S- and Z-specific receptor kinases by binding of their stigma-born soluble ligands with the involvement of Ca^{2+} as second messenger.

Expression of S-RNAse sequences in transgenic plants (Köln)

A 5.6 kb promoter fragment of S2-RNAse was transformed as a β-glucuronidase (GUS) reporter into the homologous host potato (cv. Desirée) and *Nicotiana tabacum*. In potato, only transgenic plants expressing GUS activity in pollen, but not in the style, were identified, suggesting the possibility of cosuppression as one explanation, although this effect is usually only seen in a small fraction of transformants carrying a particular construct.

In *Nicotiana tabacum*, GUS activity was seen in both pollen and pistil, however, the pattern of expression in the pistil was unexpected, in that a much stronger staining of the stigma was seen than of the transmitting tract. It is known that the S-RNAse mRNA and protein are essentially restricted to the transmitting tract in the potato host, implying that the 5.6 kb promoter is being regulated aberrantly in tobacco. One reason for the difference might be the lack of a transacting factor in the transmitting tract which is associated with SI, as *N. tabacum* is SC. Conversely, the 5.6 kb promoter is very active in pollen despite our failure to detect S-RNAse transcripts in potato pollen. This phenomenon is probably associated with the GUS reporter gene since similar observations have been made with other promoters. The experiments are being repeated using other reporter constructs, and different combinations of 5', 3' and genomic coding sequences to check for the possible presence of cis-acting elements in these regions.

Establishment of pollen cDNA banks (Nijmegen and Köln)

In order to identify the S-locus component encoded by the male gametophyte, representative cDNA banks from pollen at different developmental stages are necessary. This effort has been divided in the programme; in Nijmegen, a cDNA bank has been prepared to *in vivo* germinated potato pollen. Pollen was germinated on potato pistils, and the pistils used for cDNA library construction. The bank is being screened with various probes including a fertilized-unfertilized pistil subtractive probe. A number of style-derived cDNA clones which are pollination-induced have been characterized.

The Köln group have prepared a library (estimated 6×10^4 independent inserts) from *in vitro* germinated pollen from one S-genotype (b4). This has been screened with a subtractive probe prepared using biotinylated RNA from a second S-genotype (W). Again, a number of cDNA clones are being analysed further although so far none show a qualitative hybridization difference between a b4 and a W cDNA probe as we had hoped.

Application of SI studies to tomato (Zaadunie and Köln)

The main objective of our collaboration, to change the S-phenotype of tomato by transformation, has been approached in three ways.

(1) To investigate the factors affecting authentic and high-level expression of the S-RNAse in transgenic plants
(2) to optimize transformation protocols for tomato (see also 1991 report) and
(3) to clone the tomato (*L. esculentum*) S-locus. The latter exercise is helping us to understand better why *L. esculentum* is self-compatible. A probe for the locus has been obtained by PCR .Its location on chromosome 1 of tomato was verified by RFLP mapping (carried out by Dr. Martin Ganal, Gatersleben, Germany). This probe will now be employed to isolate much larger regions of the S-locus in this crop species.

HIGHLIGHTS/MILESTONES

Encouraging progress was achieved in understanding the significance of phosphorylation changes in the rye SI reaction. This may provide a more promising route to the identification of S-locus components in this species than direct isolation of pistil proteins as carried out for *Solanaceae* and *Brassica* species.

WIDER CONSIDERATIONS

It is clear from the work of this BRIDGE group and publications in the field in 1992 that the pollen-pistil interaction is complex and involves many more components than hitherto assumed. In order to exploit SI we will need to understand how the pistil and pollen react to each other also in a compatible situation at the cellular and biochemical levels. The characterization of transcripts appearing during fertilization is one approach to elucidating this process. The results should have consequences for our understanding of other fertilization barriers such as interspecific incompatibility.

COOPERATIVE ACTIVITIES

The project is based on the use of potato clones of defined S-genotype which have been provided to Siena, Köln and Nijmegen by Wageningen. Yi-Qin Li from Siena visited Wageningen and Nijmegen for collection and analysis of material in April 1992. Dr. Thompson and M. Ficker from Köln visited Wageningen to supply clones and discuss the analysis of transgenic plants in November 1992. Antibodies and a potato genomic bank were supplied by Köln to the Nijmegen group during 1992, and antibodies were also provided to the Siena group for immunocytochemistry. A. Bartalesi from Siena visited Köln in December 1992 in order to isolate stylar proteins using FPLC. Dr. Thompson met with Prof. H. Dickinson and colleagues in Oxford in March 1993 and discussed exchange of materials and methods. In April 1992 a mid-contract joint meeting was held in Köln between this project and that on sporophytic SI (BIOTCT90-0174) coordinated by Prof. Dickinson. In addition to reviewing research in each project, joint services were set up for clone and antibody distribution. During the meeting, guest speakers Te-hui Kao (Penn.State

Univ., USA) and Jeff Schell (MPI Köln) gave external perspectives on our research. An EC-sponsored training course on Plant Reproductive Biology was run by the Siena group in January 1993, also involving participants from the Köln, Nijmegen and Wageningen SI BRIDGE groups.

EUROPEAN DIMENSION

The involvement of groups from several countries in this project has enabled a 'critical mass' of research in gametophytic SI to be brought together which includes techniques and expertise not available in any single lab. Thus the limited national funding for SI research in each country is potentially better utilized. A specific example would be the immunocytochemistry being carried out in Siena using materials from Köln and clones from Wageningen.

LIST OF JOINT PUBLICATIONS/PATENTS WITH TRANS-NATIONAL AUTHORSHIP

Li, Y.Q., Tiezzi, A., Eilander, R., Thompson, R.D., Cresti, M. Cytochemical localization of the abundant pistil protein (Sk2) in potato (*Solanum tuberosum*). *Sex. Plant Reprod.* (submitted).
Li, Y.Q., Bruun, L., Pierson, E.S. (1992). Periodic deposition of arabinogalactan epitopes in the cell wall of pollen of *Nicotiana alata* L. Planta **188**: 532-538.

OTHER PUBLICATIONS/PATENTS

Herpen, M.M.A. van, de Groot, P.F.M., Schrauwen, J.A.M., van den Heuvel, K.J.P:T., Weterings, K.A.P., Wullems, G.J. (1992). *In vitro* culture of tobacco pollen: gene expression and protein synthesis. *Sex Plant Reprod.* **5**: 304-309.
Pierson, E.S., Cresti, M. (1992). Cytoskeleton and cytoplasmic organization of pollen and pollen tubes. *Int. Rev. Cytol.* **140**: 73-125.
Rutten, T.L.M., Derksen, J. (1992). Microtubules in pollen tube subprotoplasts: organization during protoplasts formation and protoplasts outgrowth. *Protoplasma* **167**: 231-237.
Thompson, R.D., Kirch, H.-H. (1992). The S-locus of flowering plants: when self-rejection is self-interest. *Trends in Genetics* **8**: 381-387.
Weterings, K.A.P., Reijnen, W.H., van Aarssen, R., Kortstee, A., Spijkers, J., van Herpen, M.M.A., Schrauwen, J.A.M., Wullems, G.J. (1992). Characterization of a pollen-specific cDNA clone from *Nicotiana tabacum* expressed during microgametogenesis and germination. *Plant Mol. Biol.* **18**: 1101-1111.
Weterings, K.A.P., Reijnen, W.H., Wijn, G., van de Heuvel, K., Appeldoorn, N., de Kort, G., van Herpen, M.M.A., Schrauwen, J.A.M., Wullems, G.J. (1992). Molecular characterization of the pollen-specific genomic clone NTPg303 and *in situ* localization of expression. *The Plant Cell*, in press.

Gamete differentiation and fertilization (BIOT CT-900180)

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OBJECTIVES

Exploitation of cDNA libraries, derived from mRNA extracted from Petunia ovules and isolated embryo sacs. Identification of specific sporophytic ovular and specific gametophytic (embryo sac) sequences. In vitro translation of ovular (sporophytic and gametophytic) and leave (sporophytic) RNA. SDS-PAGE analysis of in vitro translation products. Cytological characterization of Petunia embryo sac development for future in situ hybridization experiments (Collaboration PARIS-WAGENINGEN).

Testing of Fucus gamete plasma membrane labelling antibodies for labelling activity with Petunia gamete plasma membranes (Collaboration BIRMINGHAM-WAGENINGEN).

Cytological analysis of isolated and in situ embryo sacs, egg cells and sperm cells of Petunia. Isolation of Petunia sperm cells. (Collaboration SIENA-WAGENINGEN).

MAJOR PROBLEMS

Due to limited financial support, the available manpower remains a limiting factor. This seriously hampered progress. Petunia has bicellular pollen, that can be easily germinated in vitro. However, in spite of many efforts we could not (yet) induce sperm cell formation in these in vitro pollen tubes. For that reason, immunobiological and in vitro fusion experiments could not be started, since these require large numbers of isolated sperm cells.

RESULTS

SDS-PAGE analysis of in vitro translation products of total RNA from ovule-derived protoplasts and other cell types showed products ranging in size from 10 to 100 dalton. Several protein bands were only detected in products derived from ovular RNA. As a first step in the exploitation of the cDNA libraries, homology cloning was used to isolate members of potential embryo sac and ovule specific protein kinase sequences. Seven partial cDNA clones were obtained encoding short sequences containing invariant amino acids characteristic of the catalytic domain of serine/threonine kinase.

Testing of Fucus gamete plasma membrane labelling antibodies did not reveal any cross reactivity against Petunia gamete plasma membranes.

Chlorotetracycline and fluphenazine were used as fluorescent probes to visualize the distribution patterns of membrane-calcium and the calciumreceptorprotein calmodulin in the various cell types of in situ and isolated embryo sacs of Petunia and Nicotiana.
Mass isolation of Petunia sperm cells from in vitro grown pollen tubes failed. Most of the in vitro grown pollen tubes fail to form sperm cells.

HIGHLIGHTS

The actual construction of cDNA libraries from gametophytic mRNA extracted from isolated embryo sacs. The *in vitro* translation of gametophytic mRNA. The identification of several gametophytically expressed sequences and gametophyte specific translation products.

COOPERATIVE ACTIVITIES

A plenary meeting was held at Paris on 1992-03-06 to discuss the project activities, and to organise and initiate the activities in 1992. The meeting was attended by Doris F.P. (Dublin), Theunis C.J. (Wageningen), Van Went J.L. (Wageningen), Hoekstra F. (Wageningen), Cresti M. (Siena), Oleson P. (Copenhagen), Kranz E. (Hamburg), Callow J.A. (Birmingham), Kreis M. (Paris), Ferrant V. (Paris), Dunwell J. (Bracknell).

- Research visit M. Kreis (Paris) at Wageningen on 14 and 15 april.
- Research visit M. Cresti and G. Cai (Siena) at Wageningen from 1 to 9 june.
- Training period H. vd Maas (Wageningen) to Hamburg from 1 to 15 march.
- Training period V. Ferrant (Paris) to Wageningen in april.
- A plenary meeting was held at Wageningen on 1 and 2 june to organize the future activities of the project group in view of the preparation of the BIOTECH programme, and to prepare a research proposal. The meeting was attended by Kreis, Cresti, Kranz (Hamburg), Van Went, Hoekstra and Callow.
- Presentation of the project group and results at the Eucarpia meeting at Angers.

JOINT PUBLICATIONS

Ferrant V., Van Went J.L., De Vries S., Kreis M. (1992) Construction of PCR cDNA libraries from Petunia ovules and isolated embryo sacs. In: Dattee Y (ed) Book of poster abstracts XIIIth Eucarpia congress on Reproductive biology and plant breeding. Imp Malgogne, Angers pp 41-42.
Ferrant V. et al. (1993) Embryo sac and ovule cDNA libraries of *Petunia hybrida L.* (in preparation).
Tirlapur U.K., Van Went J.L., Cresti (1993) Visualization of Membrane-calcium and calmodulin in embryo sacs in situ and isolated from *Petunia hybrida* and *Nicotiana tabacum*. Ann Bot. **71:** 161-167.
Van Aelst A.C., Pierson E.S., Van Went J.L., Cresti (1993) Ultrastructural changes of *Arabidopsis thaliana* pollen during final maturation and rehydration. Zygote. **1:** 173-179.

Molecular genetics and physiology of self-incompatibility in *Brassica* crops (BIOT CT-900174) (SMA)

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OBJECTIVES
(set out under target themes, and identifying participants)

1. **Identification and characterisation of the male component of the *S*-locus (Managing contractor: Lyon)**
   - Polymerase chain reaction (PCR) cloning of putative male sequences (Lyon)
   - Construction of libraries for 'walking' (Durham)
   - Characterisation and cloning of putative male determinants (Oxford and Durham)
   - Immunoscreening of expression libraries; cloning of sequences of interest (Nijmegen)

2. **Biochemistry and physiology of the SI response (Managing contractor: Oxford)**
   - Pollen coating molecules; structure and function (Oxford)
   - Physiological and structural responses in the stigma (Oxford)
   - Polypeptide changes in pollen following self and cross pollinations (Oxford)
   - Comparisons with SI physiology in sugar beet with other species (Maribo Seeds)

3. **Organisation and expression of the *S*-locus, relationships between *S*-alleles (Managing contractor: Norwich)**
   - Molecular studies of mutated SI lines (Lyon).
   - Molecular genetics of *S*-receptor kinases and associated phosphatases (Norwich, Birmingham)
   - Construction of packaged libraries for chromosome 'walking' (Durham)
   - Development of *S*-allele specific probes (HRI; Wellesbourne)

4. **Transfer of *S*-alleles to new lines (Managing contractor: Norwich)**
   - Refinement of transformation strategies (Norwich, Birmingham)

MAJOR PROBLEMS ENCOUNTERED:

1. **Male component**
   - For genomic libraries; reliability of P1 packaging system (Durham)
   - Fractionation of low Mr pollen coat proteins; now achieved (Oxford)
   - Cloning and segregation of pollen coat genes owing to large family size (Oxford)
   - Availability of *S*-specific monoclonals; further help received from Lyon (Oxford)

2. **SI response**
   - Microscopic methods for determining structural aspects of the stigmatic response to pollination (Oxford)

3. **Organisation of *S*-locus**
   - Problems with P1 packaging system; see above (Durham)
   - Inefficient transformation strategies; see below (Birmingham and Norwich)
   - Continued ignorance of male determinant (all participants)
   - Low levels of *S*-receptor kinase in anthers. Methods now developed (Lyon)

4. **Transfer of *S*-alleles**
   - **Transformation strategies in** *Brassica*. These remain unreliable and inefficient. *B.napus* works well but *B.oleracea* remains intractable. Collaboration with other UK groups in the programme has helped, and promising new methods are being investigated.

General
The quality and pace of research still remains affected by delays in receiving funds from the EC at the beginning of the programme. Many institutes would not allow positions to be advertised before funds had arrived, and thus many participants could not commence work until early 1992. An extension to the initiative has been requested.

RESULTS

1. **Male component**

Sporophytic pollen coating peptides have been further characterised at **Oxford** (Doughty *et al.* 1993). These interact with the SLSG (*S*-locus specific glycoprotein) expressed in the stigma and may act either as cofactors or the male determinant itself. High resolution 2D gel work, combined with FPLC and reverse phase HPLC has established the presence of a large family of these peptides, and sequence data is currently being acquired for the single member of the family in each *S*-genotype which interacts with the SLSG. A panel of monoclonal antibodies has been raised to this class of pollen coat proteins and, once the active peptide has been identified for each genotype, immunolocalisation and inhibition experiments will commence. Interactions with the *S*-receptor kinase (*SRK*) are also being investigated. In collaborative work with **Durham**, the 3' end of a cDNA clone coding for the active peptide from S29 has been cloned and is now the subject of further joint experiments to investigate gene expression and its relationship to SI.
Work on pollen coat proteins is also under way in **Nijmegen**. Following a visit by Dr Ruiter to **Oxford** to learn techniques, he has used an immunological approach to demonstrate the accumulation of three proteins in the exine. These results were obtained by screening a cDNA expression library, prepared using mRNA from anther tissue of *B. oleracea*, with an antiserum raised against exine proteins. The sequence of one cDNA shares 90% homology with the profilin mRNA from birch

pollen, while analyses of the other proteins failed to reveal homologies of any significance.
In **Lyon**, Mark Cock (post-doc and now permanent INRA researcher) has confirmed the presence of transcripts homologous to the *SLG/SRK* genes in anther tissues by the polymerase chain reaction (PCR). He is currently sequencing the anther PCR fragments to determine the level of homology between the male and female S transcripts. Due to the very low level of expression of these messengers, M. Cock will be attempting their tissue localization by using *in situ* hybridization techniques.
Work by the **Durham** group to package *Brassica* DNA with a view to 'walking' along the *S*-locus from the SLSG genes to the male determinant is described under (3) below.

2. The SI response

General aspects of pollination physiology continue to be studied at **Oxford**. EM cytochemistry, combined with physiological experiments involving inhibitors of coating and stigmatic enzymes, reveal that components of both coat and the stigma surface are required for water release. Coating esterases act on water soluble factors of the pellicle to first permit hydration of the coating (so-called coating conversion) and then the grain. A series of interspecific pollinations within the Cruciferae, and other groups with dry stigmas suggests the *Brassica* stigma is very highly evolved, with only very close relatives, having the enzymology to extract water from it. *Brassica* pollen, on the other hand, will extract water from most other dry stigmas — underlining the strong evolutionary pressure for the development of pollination systems that exclude pathogenic spores.

Further EM studies have shown that compatible pollination is followed by activation of the stigmatic cytoplasm and an expansion of a specialised outer layer of the papillar wall subjacent to the grain. These observations are consistent with pollen enzymes passing into the stigmatic wall. Interestingly, treatment of stigmatic papillae with isolated coat induced a conspicuous reaction with the wall outer layer being massively expanded, and seen to contain electron-opaque membranous vesicles — apparently already in the wall or released by the female protoplast. Although this stigmatic response is characteristic only of compatible matings, it is considered to be significant in inter- rather than intraspecific interactions — since it seems that following selfing, development does not proceed as far as the release of these signals (Elleman *et al.* in preparation). Other experiments demonstrate the discriminatory power of the stigma; for example, the addition of trace levels of pollen protoplast (not intine) proteins to the isolated coat result in the synthesis of callose, rather than wall expansion and vesiculation (Elleman et al. in preparation). Interestingly, this behaviour mimics a characteristic stigmatic response to self-pollen carrying weak *S*-alleles. Techniques have also been developed to study pollen polypeptides synthesised (and phosphorylated) within minutes of contact with the stigma. This has involved considerable refinement of 2D IEF/PAGE systems.

Recent investigations at **Durham** have shown conclusively that okadaic acid effectively knocks out *Brassica* self-incompatibility without affecting normal pollination. Okadaic acid is a potent inhibitor of serine/threonine protein phosphatases type PP1 and PP2A. Okadaic acid treated self-incompatible lines are rendered compatible and exhibit normal self-pollen germination and growth; compatible lines are unaffected by the treatment (Scutt *et al.* 1993).

Parallels between the SI system of *Brassica* and the mechanism operating in sugar beet continue to be studied at Maribo Seeds. At the protein level, western blots (containing protein from sugar beet styles or leaves) have been made. These blots have been sent to T. Gaude (**Lyon**) who has probed the blots with an antibody against one of the *S*-proteins in *Brassica*. The antibody reacted with a protein from sugar beet styles approx. 43 kd in size. Using the same antibody a strong immunological reaction has been shown in the transmitting tissue of cross sections of sugar beet styles.

3. Organisation and expression of *S*-locus

Characterisation of the S-receptor kinase (SRK) and S-phosphatase gene families

At **Norwich** a DNA fragment of 7,981 bp encoding a putative *SRK* gene from the S_{63} line has been sequenced. The gene structure is homologous to the previously characterised SRK_6 and SRK_2 alleles and predicts an 859 residue *SRK* protein which carries intact motifs specifying active site and ATP-binding domains. This receptor kinase probe detects an *S*-linked RFLP but may not represent the authentic SRK_{63} gene. The clone was used to isolate homologous sequences from an S_{29} genomic library, the results indicating that the *SRK* is one member of a large (approx. 20 copies/genome) multigene family. Six of these were partially sequenced; five encoded potentially functional proteins and one was a pseudogene. One sequence was found to be 99% identical to the S_{63} kinase, suggesting a direct allelism. Most of the clones carried *SLG*-like receptor domains, suggesting that the previously described *S*-sequence complexity is largely made up of receptor kinase genes. The *SLG* (and *SLR1*) genes would thus be evolutionary derivatives, through duplication and over-expression of receptor domains. Using primers synthesised to conserved regions of plant/animal type I serine/threonine phosphatases, we have amplified an authentic transcript from S_{29} stigma mRNA by 3'RACE-PCR. Using this probe, we now intend to screen for *S*-linked RFLPs and gauge gene polymorphism between *S*-homozygotes to see if a phosphatase gene might be another co-adapted member of the complex *S*-locus.

Control of the S-locus expression in a self-compatible line

Work in **Lyon** has demonstrated that the *P57Sc* self-compatible line exhibits stigma *SLG* products the expression of which is similar to the spatial and temporal pattern described for *SLG* of normally self-incompatible *Brassica* plants. A cDNA clone presumably encoding the *Sc*-SLG has been isolated and the complete amino-acid sequence deduced from the nucleotide sequence reveals this glycoprotein to be highly homologous to the pollen recessive *S2*-allele glycoprotein (Gaude *et al.* 1993). Besides, a cDNA encoding the *SLG*-Si product of the self-incompatible *P57Si* line has been also isolated and sequenced. The Si allele belongs to the S allele dominant series. The search for homology between pollen recessive S-alleles (ie. *S2*, *S5* and *S15*) and the *P57Sc*-allele has been carried out by using the molecular probes established from the *Sc*-SLG products (monoclonal antibodies and *Sc*-SLG cDNA). This analysis clearly indicated strong homology between the *Sc* and *S15* allele. This work was undertaken with the collaboration of Dr D. Ockendon (**HRI, Wellesbourne**) who supplied the 'pollen recessive' seeds.

S-locus promotors

At **Birmingham** work has continued to focus on the analysis of a *Brassica oleracea* S-gene promotor. Having shown that 1.5kb of the S63 SLR1 promotor was able to direct tissue specific and temporally regulated expression of the GUS reporter gene in transgenic tobacco, a deletion analysis of the promotor has been carried

out so that the elements responsible for this pattern of expression may be identified.

It has been found that 500bp of the promotor, contiguous with the translation start site, are sufficient to direct expression of a reporter gene in the pistil and pollen of transgenic tobacco. Nucleotide sequence analysis has revealed the presence of several motifs within the 500bp SLR1 promotor that may be responsible for the observed pattern of expression. These include elements that have homology to pollen specific motifs from other genes. Further SLR1 promotor constructs have been made to test the activity of these motifs, following amplification of promotor fragments using PCR. The pattern of reporter gene expression directed by these promotor fragments is currently being analysed. Results so far indicate that a 110bp promotor is able to direct a low level of expression in tobacco pollen, but not in pistil tissue. Results from the PCR generated SLR1 promotor deletions have shown that the elements required for gene expression in the pistil are situated between -220bp and -500bp to the translation start site. Furthermore, an element that is able to silence SLR1 directed expression in the pistil has been located to within a 150bp fragment situated further upstream of the functional 500bp promotor. The results are from experiments using the heterologous host, tobacco. *Brassica* plants have now been transformed with some of the SLR1 constructs and will be analysed shortly.

Currently, upstream sequences are being isolated from other members of the *Brassica* S-gene family using inverse PCR. We are particularly interested in SLG and SRK promotors from plants containing alleles that are either very high or low in the dominance series. The molecular basis of the dominance relationship of the S-alleles is being investigated to determine whether dominance is controlled at the level of transcription. Our approach will involve looking at the level of S-gene transcripts present in the stigmas of S-heterozygotes, and an analysis of the level of transcription directed by the promotors of the corresponding genes.

Mapping the S-locus

At Durham the construction has commenced of a genomic library containing relatively large fragments in order to span the S-locus. Our original YAC (Yeast Artificial Chromosome) approach was unsuccessful for technical reasons and lack of time. A second strategy has involved the use of the P1 phage cloning system which theoretically should be capable of cloning reasonably large sized genomic fragments in the range of 65 kB — 95 kB. The latest P1 vector, pAD10-SacBII possesses many new characteristics

(i) Facilitates the cloning of large fragments and library screening.
(ii) Has 2-3 rare cutting sites flanking the cloning site.
(iii) Has T7 and SP6 promotors for production of chromosome walking probes.
(iv) Involves an easy protocol for the isolation of cloned DNA.

To facilitate the construction of the library a novel approach has been developed to clone as large fragments as possible and which avoids the need for a series of partial restriction digests of the starting genomic DNA. The strategy consisted of several steps:

(1) isolation of 150 kB — 200 kB DNA;
(2) size fractionation by sucrose gradient centrifugation;
(3) T4 DNA polymerase filling/trimming of the ends and their dephosphorylation by CIAP;
(4) ligation of primer adaptors containing restriction sites of interest and
(5) purification of the final product and its ligation into the vector.

Following this procedure an S29 P1 genomic library has been constructed combining recombinants from 3-4 independent packaging reactions. While this approach has been partly successful the quality of the packaging extracts provided by the manufacturer NEN Dupont was somewhat variable. Therefore, the size of the libraries varied substantially and the average insert size has also proved to be less than expected. The final pool of about 15000 clones (representing only a partial library) has been screened with the probes specific for *SLG*29 and the kinase domain of *SRK* (supplied by Martin Trick, **IPSR, Norwich**) and six independent clones have been isolated. Further analysis of these clones has revealed that they contain kinase sequence but none of them appear to hybridize to the SLG29 probe. After subcloning the genes have been hybridized to genomic blots containing restriction digests of DNA from *Brassica oleracea* F2 segregants from crosses between S29 and S5 lines. These blots were prepared earlier to show the allelic relationships between SLG29 and SLG5 (See Scutt, C.P. and Croy, R.R.D. (1992) Mol. Gen. Genet. 232, 240-246).

One particular HindIII digest has proved to be quite successful revealing clear specificity of the probes and confirming their linkage to the S-locus. Further experiments need to be done to isolate more SI-specific genomic clones followed by probing with anther and stigma specific cDNA probes.

S-locus specific probes

At **HRI: Wellesbourne** good progress has been made in developing a system for identifying S-alleles based on DNA amplification by PCR. Primers have been designed which give preferential amplification of SLG sequences, which are thought to be the main determinants of S-specificity, but it is difficult to totally avoid amplification of other members of the S-sequence family. An alternative approach has been to use a pair of 'universal' primers which potentially amplify all members of the S-gene family, and which give good amplification products with all of the 40 S-allele homozygotes tested. Using four carefully chosen restriction enzymes, almost all the 40 alleles can be uniquely identified by their banding patterns. In some cases the patterns may represent both SLG and SRK sequences, but as both are tightly S-linked they should be usable for S-allele identification. Another possibility is to use probes representing part of the SLG sequence, and these have been made for four S-alleles. Although these probes do not seem to be as specific as originally hoped, they will readily distinguish the three most recessive S-alleles from the rest, and offer considerable potential for further development. Study of banding patterns in an F2 population showed that the S-allele constitution as determined by DNA analysis perfectly matched that obtained by pollen tube growth tests. In S-heterozygotes it is important to be able to identify both of the S-alleles present, and this will be considerably more difficult that identifying the single S-allele in S-homozygotes. In one S-heterozygote using the 'universal' primers, only one of the alleles could be detected, but by choosing a different pair of primers both could be detected.

The S-locus of sugar beet

At **Maribo Seeds** the use of degenerate primers from a maize kinase sequence has enabled the amplification by PCR of genomic DNA sequences from two different sugar beet families. In both cases two PCR products were obtained. The size of the products were 600 bp and 1200 bp, respectively. These PCR products have been cloned and 20 clones have been sequenced. The 1200 bp product did not show any identity to any other known DNA sequences, whereas the PCR product

600 bp in size (SBkin600) shared identity with the self-incompatibility genes (SLGs) and to the self-incompatibility linked kinase (SLK) from *Brassica*. There was no allelic variation among the different sequenced SBkin600 clones, but Southern hybridization of the SBkin600 sequence to genomic DNA from a number of different sugar beet lines showed polymorphism. A genomic Lambda Fix library from sugar beet leaves has been constructed. This library has been probed at medium stringency with the purpose of isolating DNA sequences which share identity to SBkin600 — of which some could be SI-alleles of the sugar beet. A number of hybridizing Lambda clones have been identified. Further isolation and characterization of the clones is ongoing. The DNA work is done in collaboration with R. Thompson (**MPI**).

4. Transfer of S-alleles

At **Norwich** the *Agrobacterium*-mediated transformation system with *Brassica napus* has been further refined but efficiencies are still low (around 5%). Experiments have centred on a gene ablation strategy and targeting the *SLR1* gene, with the aim of elucidating its function in compatible and incompatible plants. Both sense and antisense constructs consisting of the $SLR1_{63}$ promotor driving appropriately oriented *SLR1* cDNAs have been delivered into *B. napus* Westar. Addition of up to 3 sense copies did not perturb endogenous gene expression or the compatible phenotype. Transgenic antisense *napus* and diploid *oleracea* plants are now flowering and are about to be assayed. Very recently, we have discovered that the *alboglabra* S_5 line, studied by the **Durham** group, is extraordinarily responsive to regeneration, with more than 50% of the infected explants producing green shoots. If we verify a commensurately high transformation rate then an excellent experimental system for manipulating *S*-genes in a diploid background would then become available.

WIDER CONSIDERATIONS

The system by which *Brassica*, an important European crop, prevents inbreeding continues to be studied in a programme involving 11 institutes, universities and industries from 4 member states. Collaboration through exchange of materials, information and staff has added considerable value to the project, and has directly resulted in progress being made in the search for the male 'factor', the structure of the 'S' locus which in male and female tissues controls the process, and the development of probes to identify plant lines with different specificity. Joint meetings have proved especially valuable for exchange of ideas and evaluating progress. Only integrated groups of this size and width of expertise, can hope to challenge the current supremacy of large American laboratories.

COOPERATIVE ACTIVITIES

1. Meetings

A very successful two-day joint meeting of all participants of both this programme and the gametophytic SI group (coordinated by Dr R. Thompson) was held in Cologne, hosted by the Max Planck Institute. A further workshop on cell signalling was planned for September 1992, but had to be cancelled. We had applied to the Commission for the necessary funds to cover the cost of 'outside' speakers but the Commission moved too slowly for the project to be practical.

2. Newsletter

SINEWS continues to be produced at regular intervals. Recent editions have contained an expertise and facilities register, in addition to the usual news, results, publications, data bases of the various 'S'-sequences, and lists of DNA clones and specific antibodies available to members of the network.

3. Visits between laboratories

(a) Dr A. McCubbin spent a second week in Lyon to identify by immunochemistry the SLG and SLR1 glycoproteins of the S25 and S63 lines used in **Oxford.**
(b) John Nielsen from **Maribo Seeds** spent ten days in the **Oxford** laboratories working on *in situ* techniques. Whilst not himself a participant in the programme this visit was as a direct result of collaboration under BRIDGE.
(c) Dr Richard Thompson visited Professor Dickinson in **Oxford** for two days in March 1993 to discuss future research strategy.
(d) Dr A. McCubbin visited the University of Hupio (Finland) to work with Drs Karenlampi and Hokko.

4. Exchange of materials

(a) INRA supplied P57Si seeds to Dr D. Ockendon, **HRI Wellesbourne**, for S-allele determination.
(b) The cDNA sequences or clones of the P57Sc and P57Si-SLG have been provided by INRA to Durham, **HRI Wellesbourne** and **Danisco.**
(c) **Oxford** supplied sequence information (7kD peptide) to **Durham** to enable a study of this protein at the gene level.

5. Network specialist services

As part of our collaboration under BRIDGE a number of services and materials are offered by individual labs to other participating groups either free or 'at cost'. These specialist services are in addition to many others listed in the Expertise and Facilities Register (see above).

1. ***HRI, Wellesbourne***
(a)Distribution of material homozygous for known S-alleles.
(b)Checking S-allele constitution of material being used for molecular work.
(c)Running S-sequence family data base.

2. ***Cambridge Laboratory***
(a)*Brassica* genomic and stigma cDNA libraries which are available to members of the programme.
(b)The most comprehensive sequence data base/analysis computing facilities in Europe.

3. ***INRA/Lyon***
(a)Antibodies (anti-SLG and anti-SLR1, polyclonal or monoclonal) are available for use in the lab at Lyon.

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Molecular control of genetic instability in regeneration of crop plants (BIOT CT-900154)

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OBJECTIVES

The EC BRIDGE group have been utilising a number of different techniques to detect somaclonal variation in cultures and also to study its causal factors. These techniques differ in sensitivity and are based on morphological, cytological, biochemical and molecular methods. In addition, the group have continued to direct efforts towards determining the causes of the variation. Some members of the group have also looked at some possible applications of somaclonal variation for crop improvement. This EC contract is a concerted action, enabling the group to keep in close contact and meet annually. The research activities decribed below have been supported by funds from outside the contract which the labs have attracted separately. Consequently, there are few joint publications with trans-national authorship.

MAJOR PROBLEMS ENCOUNTERED

Although there are many problems to be solved in controlling somaclonal variation it is currently difficult to raise much interest in this area of biotechnology. This is largely because the variation has not proven to be as useful for crop improvement as some researchers (outside of this group) originally promised. Nevertheless, somaclonal variation remains a serious problem in biotechnology and should therefore continue to receive due consideration and funding support from the EC. Without this support it will be difficult to continue progressing in this area.

RESULTS

Dr Karp and co-workers at Long Ashton Research Station, UK have been studying the behaviour of repeated DNA sequences in cereal cell cultures and regenerants. In collaboration with Prof. Kaltsikes and Dr. Bebeli of the Athens Agricultural University, they have analysed progenies of 50 regenerated plants of a selected line of rye in which most of the telomeric heterochromatic blocks had been eliminated by selection. *In situ* hybridisation with a probe for a telomere sequence family detected changes in the heterochromatin of three regenerants. One regenerant segregated two independent changes, both of which appeared to be amplifications. Dr Karp has also carried out cytological studies of cell suspensions and dividing protoplast lines of diploid and tetraploid species of wheat to examine the influen-

ces of genotype and ploidy on chromosome instability. In a collaborative effort with Dr M.R. Davey at Nottingham University, UK, long-term cell suspensions of five *Triticum* species of differing ploidy levels (*T. aestivum* (AABBDD hexaploid), *T. monococcum* (AA diploid), *T. tauschii* (DD diploid), *T. durum* and *T. dicoccum* (both AABB tetraploids) were studied. The diploids were found to be the most stable and the hexaploid the least stable, but genotype was very important.

The group of Prof Lörz and Dr Brown in the Institute für Allgemeine Botanik, Germany, have used RAPDs to screen R0 regenerants of two cultivars of *Triticum tauschii* and two cultivars of *T. aestivum*. In all the cultivars tested, differences in the patterns of RAPD fragments occurred both between the regenerants and the controls, and also between the different regenerants, although the plants showed little variation in morphology . Many of the sequences associated with this variability are being cloned and examined as potential markers for hypervariable loci. Dr Brown has also successfully applied PCR and RAPD approaches to single isolated protoplasts. This advance means that the fate of both endogenous DNA and transformed sequences can be followed directly after protoplast transformation through to the production of whole plants.

Prof Buiatti's group at the University of Florence, Italy, have been concerned with the molecular characterisation of variation in tissue cultures. Recent studies have examined the relationship between somaclonal variation and physiological modifications occurring during cell development and induction by stress in tomato (*Lycopersicon esculentum*). In one series of experiments comparative studies were carried out on tomato leaf tissue and somaclones grown *in vitro* on different auxin and cytokinin equilibria and exhibiting varying degrees of hormone autotrophy (habituation). Callus growth and habituation capacity were determined by monitoring growth rate for 90 days on a basal medium without hormones, or supplemented with (1) 0.4 mg l^{-1} 2,4-D and 1 mg l^{-1} kinetin, or (2) 1 mg l^{-1} 2,4-D and 1 mg l^{-1} kinetin, or (3) 0.8 mg l^{-1} 2,4-D and 0.2 mg l^{-1} kinetin. DNA amplification was analysed using rDNA and an anonymous tomato repeated sequence (pG11). Methylation levels were assessed with a series of isoschizomers and genetic variability with the use of three short (10 mer) oligonucleotide primers. rDNA multiplicity was found to be lower in all somaclones, but higher in the habituated line, compared with leaf tissue. Significant differences in multiplicity of the pG11 sequence were observed between clones maintained on the same medium and between the same clone on different medium. Methylation levels were higher in the leaf and in the habituated clone than in the auxotrophic somaclones and 2,4-D was found to have a general hypomethylating effect. RAPD analyses revealed a high level of genetic variation between somaclones and in the same somaclone between sub-clones growing on different media, but no correlation was found between specific variants and growth and differentiation levels.

The stability of foreign genes in transgenic *Nicotiana tabacum* plants following regeneration has been investigated by the group of Prof. Sala at the University of Pavia, Italy. Restriction enzyme and Southern blot analysis of 66 regenerated plants revealed no detectable alterations in transgene structure or insertion site. RAPDs have been used to evaluate the extent of DNA changes in embryogenic and non-embryogenic cell suspension cultures of rice and in transformed rice plants and their progenies. The results show great diversitites in RAPD fingerprints from DNA of leaves compared with DNA from cultured cells. However, the RAPD fingerprints produced by DNA prepared from the leaves of transgenic plants were generally similar to those of control rice leaves, although some peculiarities were revealed.

Dr. Pijnacker, at the University of Groningen in the Netherlands, and Prof. Wersuhn, at Humboldt University in Germany, have been studying the causes of chromosome instability in potato, tobacco, wheat and *Vicia faba* cell cultures using a variety of cytological techniques. Numerical chromosome mutations result from various events such as endoreduplication, restitution, spindle fusions, acytokinesis (euploidy), and non-disjunction (aneuploidy). Structural variation through chromosome and chromatid breakage, results in translocations, inversions, duplications and deletions, some of which may in turn lead indirectly to aneuploidy. Certain structural aberrations are not recovered because they only cycle a few times (eg, dicentric chromosomes). More chromosome variation occurs in cultures compared with regenerated plants but levels of numerical and structural aberrations can be very high in regenerants. Among the possible changes occurring at high frequencies, are aneuploidy, polyploidy and translocations.

Dr. Pijnacker has been using C-banding, N-banding and BrdC-Giemsa to study karyotypic changes in cultures and to identify the genome combination of somatic hybrids. C-bands of potato were observed to change in size in cultured cells, particularly the nucleolus organizer (NOR) region. In collaboration with Dr Waara, University of Uppsala, Sweden, an analysis of tetraploid and hexaploid somatic hybrids of potato revealed numerical and structural chromosome mutations, the latter mainly in the NOR-region. Chromosome loss enabled the mapping of the IDH-I gene on the longest chromosome, and aneuploidy and genome number variation could be correlated with phenotypical characters. Dr Pijnacker has also developed a method of using sister chromatid exchanges as a sensitive means of assessing levels of somaclonal variation in leaf explant cultures of potato, tobacco and *Vicia faba*, and in the first two mitotic divisions of wheat embryogenic cells in cultures. These studies have shown that sister chromatid exchanges provide a useful means of testing the effects of media components (for instance growth regulators and sugars) and also for studying genotype effects.

Dr Henry and his colleagues at the University of Paris-Sud, France have been studying the genetic control of somatic embryogenesis and somaclonal instability in wheat. A Chinese Spring (CS) wheat line which produces long-term somatic embryogenesis *in vitro* was used in reciprocal crosses with a nonembryogenic variety (Aquila). Somatic embryogenesis was not expressed in the F_1, indicating that it is a recessive trait, and in the F_2 segregation ratios suggested the control of a few complementary genes. Disomic and aneuploid stocks (36 ditelosomics, 7 nullitetrasomics) of CS were then compared for their ability to undergo somatic embryogenesis after two months of *in vitro* immature embryo culture. Genes affecting somatic embryogenesis and regeneration were located to the whole homeologous chromosome groups and, in particular, the absence of chromosome arms 1AL, 3AL and 3BL and 3DL practically suppressed somatic embryogenesis and regeneration capacity. Various tissue culture-induced variations were observed in the plants regenerated from the CS aneuploid stocks. Short-term regenerants produced more plants with abnormal chromosome complements (about 14%) than euploid CS (3.5%). Most of these arose from unbalanced chromosome numbers in the starting immature embryos. The frequency of plants with altered chromosome numbers increased with culture time to nearly 80% in both the control and the aneuploid lines. A few chromosome arms were shown to reduce (1BL, 2DL, 5AS) or to enhance (1AS, 1BS, 2AS, 4DS, 5DS) instability after long-term culture.

At the Athens Agricultural University in Greece, Prof. Kaltsikes and Dr Bebeli have carried out an assessment of selfed progenies of regenerated plants of rye and triticale in extensive field trials. Heritable variations were observed in several

characters, including flowering time, yield, height and number of tillers. Some plants which were superior to the parental lines were identified. Good chances for successful selection were indicated by the fact that all the regenerant families showed greater amounts of total and genetic variance for most agronomic traits. Somaclonal variation also changed the correlations between agonomic traits, thus making it easier to break unfavourable correlations.

At the Institute of Grassland and Environmental Research in Aberystwyth, UK hundreds of somaclones have been regenerated from a single cell suspension and cell suspension-derived protoplast culture of grasses. A normally stable and polymorphic isozyme, phosphoglucoisomerase (PGI/2) was used to label individual homeologous chromosomes of the hexaploid *Festuca arundinacea* ($2n=6\times=42$) and a pentaploid hybrid between *Lolium multiflorum* and *F. arundinacea* ($2n=5\times=35$). There was a direct relationship between time in cell suspension and frequency of aberrations at the PGI/2 locus. Mutations included deletion of one or more PGI/2 alleles (not always related to chromosome loss), a change from active to null alleles and in some *F. arundinacea* regenerants, modification of the electric charge/mass ratio to form two novel PGI/2 alleles which were absent in the original genotype. The pentaploid *Festulolium* hybrid has been used in a breeding programme to introduce drought resistance genes from *F. arundinacea* into *L. multiflorum*. Chromosomal breakage and rearrangements arising as a consequence of the culture phase were shown to increase levels of interspecific chromosome pairing in regenerated *Festulolium* hybrid somaclones above that found in the plant before culture. Although the chromosomal aberrations reduced fertility in the hybrid, the cell culture phase should enhance interspecific gene transfer.

At the Universidad de Complutense in Madrid, Dr. Vázquez and colleagues have analysed morphological changes in barley (*Hordeum vulgare*), rye (*Secale cereale*) and *S. vavilovi*. Only 1% of the barley regenerants and 50% of the rye regenerants showed some heritable variation. The most frequent changes in *Secale* were chlorophyll deficiencies — 9% of the regenerated plants were albinos, and 11% of the green regenerated plants segregated chlorophyll abnormalities in their progenies. Other variations included the occurrence of supernumerary flowers in each spikelet and polyembryony. An extensive study of the inheritance of these variations revealed a suprisingly high frequency (60%) of dominant mutations. Plants which carried more than one mutation occurred at equally high frequency. It was also noted that frequencies of morphological variants differed between cultivars and appeared to be genotype dependent. Almost 30% of the regenerated plants had a modified chromosome number, including a haploid regenerant, and 30% of apparently normal diploid regenerants segregated some progeny with chromosome aberrations.

HIGHLIGHTS / MILESTONES

PCR and RAPD techniques have been applied to single isolated protoplasts enabling the detection of changes in endogenous genes and transgenes immediately after transformation through to the regeneration of plants. The analysis of sister chromatid exchanges has been used to assess frequencies of somaclonal variation in cultured cells under different *in vitro* conditions. Diploid and tetraploid *Triticum* species were found to be more stable than hexaploid breadwheat in cell suspensions.

WIDER CONSIDERATIONS

Somaclonal variation has lost much popularity in recent years, largely because its contribution to plant breeding had been disappointing. Nevertheless, it remains a phenomenon of considerable importance in biotechnology. The production of plants that are not true-to-type reduces the commercial value of tissue culture-derived products and often means that somatic hybrid and transgenic plants cannot be used directly. Despite our increased understanding somaclonal variation still remains a largely unavoidable consequence of plant tissue culture and there is therefore still an urgent need for more fundamental studies of the underlying mechanisms involved.

COOPERATIVE ACTIVITIES

A meeting was held in Tuscany, Italy, hosted by F. Sala from 28-31 May 1992. Progress in the research and prospects for future funding were discussed.

PUBLICATION

Karp A (1993): Are your plants normal? Genetic instability in regenerated and transgenic plants. Agro Food Ind Hi-Tech.

The molecular biology of the cell-to-cell movement of plant viruses in relation to plasmodesmatal function (BIOT CT-900156)

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PARTICIPANTS:
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(04) S. BEN-TAHAR, BIOCEM, Aubière, F
(05) I. GARCIA-LUQUE, CIB-CSIC, Madrid, E

OBJECTIVES

(A) Are there different mechanisms in the cell-to-cell spread of the viruses being studied in the project?
(i) Mutagenesis of the CMV movement protein.
(ii) In vivo localization of the movement proteins of various viruses.

(B) What are the basic interactions between the virus, movement proteins and plasmodesmata?
(i) Isolation and characterization of putative receptors, raising of antisera, subcellular localization
(ii) Study of the TMV L and Ltb1 system. Continuation of the mutagenesis of MP genes.
(iii) Transformation of plants with MPs and mutated MPs. Biochemical analysis of expressed MPs. Binding studies for host receptors.

(C) What is the physical structure of plasmodesmata and how do receptor proteins fit in?
(i) Microinjection studies on transgenic plants and with *in vitro*-expressed MPs. Study of tissue specificity and symplastic domains.
(ii) High resolution electron microscopy of plasmodesmata in infected and transgenic plants.

(D) Methods for blocking viral movement through plasmodesmata.
(i) Transformation of tobacco with CMV MP constructs.
(ii) Seeking suitable host for transformation with BNYVV MP constructs.
(iii) Testing transformants for the effects of transformation on cell-to-cell movement of viruses.

MAJOR PROBLEMS

The major problems were technical and are identified in the results section.

RESULTS

(Principal investigator name and laboratory number in parentheses; investigator marked with * is associated with, but not funded by, the project).

Subcellular location of movement proteins.

The movement protein (MP) of cucumber mosaic virus (CMV), termed the 3a protein, was expressed in *E. coli* and an antiserum raised against it. This antiserum detected a protein of the correct size (30 kD) in western blots of proteins from infected plants. When extracts from infected plants were fractionated, the 3a

protein was detected in both the crude membrane (Triton X-100 soluble) and cell wall fractions. In contrast to the MPs of tobacco mosaic virus (TMV) and alfalfa mosaic virus (AlMV), there was more CMV 3a protein in the crude membrane fraction than in the cell wall fraction. No 3a protein was found in isolated nuclei, a result which differs from the report of MacKenzie and Tremaine (1988, *J. Gen. Virol.* **69**:2387) and which is more in accord to current thinking about the functions of MPs. In a time course study on inoculated tobacco leaves the 3a was detectable in the crude membrane fraction earlier (12 h) than in the cell wall fraction (Garcia-Luque, 05).

In an analysis of tobacco plants transformed with deletion mutants of the AlMV MP (P3) about half of P3Δ[36-81] (deletion of amino acids 36-81) was found in the cell wall fraction, the rest being in a membrane fraction. By contrast, P3Δ[113-156] and P3Δ[213-243] were found almost exclusively in the cell wall fraction. This is to be compared with the previous finding that P3Δ[1-12] was almost entirely in cell walls and P3Δ[1-77] was undetectable in cell walls. It appears that the region of amino acids 36-81 is involved in cell wall localization (Colburn, 03).

Genomic location of movement protein genes in different viruses.

Grapevine fan leaf virus (GFLV) RNA 1 is infectious to protoplasts which shows that it encodes the replicase functions but requires RNA 2 for systemic infection of *Chenopodium quinoa*. This indicates that the cell-to-cell movement functions are within the 122 kD polyprotein encoded by RNA 2. This 122 kD protein is processed *in vitro* into three proteins, the N-terminal 28 kD protein of no known function, a 38 kD protein showing amino acid similarity to TMV P30 MP and the C-terminal 56 kD viral coat protein. By microsequencing, the cleavage sites at the N-terminus (Cys_{257} /Ala_{258}) and C-terminus (Arg_{605}/Gly_{606}) of the 38 kD protein have been delimited. Using this information the complete 38 kD gene has been cloned and expressed as a fusion protein and an antiserum is currently being raised against it (Pinck*, 03).

Properties of movement proteins.

An in-frame 300 bp deletion was made in gene 1 which encodes the MP of an infectious construct of cauliflower mosaic virus (CaMV). Agroinoculation of turnip leaf discs with this mutant showed that it was defective in cell-to-cell movement but retained its ability to replicate (Maule*, 01). This demonstrated that CaMV gene 1 encodes the MP. As both the mutant and wild-type moved systemically in double infections of turnip plants the MP can act *in trans*.

Immunofluorescent staining of Chinese cabbage protoplasts infected with CaMV using anti-MP serum revealed long thread-like structures extending from the protoplast surface. Electron microscopical examination of immunogold-labelled protoplasts showed extracellular tubular structures containing the gene 1 product (P1). Western blotting indicated that the P1 in the tubules was the full-length protein and not the smaller product which accompanies the full-length one in infected plants (Maule*, 01).

Eight mutant AlMV MPs (P3) carrying in-frame contiguous deletions have been expressed in *E. coli* and some of them have been purified on CM-trisacryl columns. After renaturation 6 out of the 8 proteins bound RNA in the same conditions as wt P3 (in the presence of 0.1-0.15% Tween 20) but mutants P3Δ[36-81] and P3Δ[244-300] did not bind RNA at all. However, it is considered that the only true binding region is amino acids 36-81, the same region implicated in cell wall localization. The C-terminally deleted protein aggregates very easily and sticks to

plastic as does to some extent P3Δ[215-243]. It is thought that the C-terminus is involved in stabilizing the conformation of P3, but is not directly involved in RNA binding.

Attempts are being made to develop a system which would enable transcripts of AlMV RNA 3 (which encodes the MP and coat protein) to be analyzed. This system involves co-inoculating purified RNAs 1 and 2 (which encode the replicase functions) with RNA 3 transcripts. It would enable an analysis to be made of the effects of mutations of both the MP and coat protein on the systemic movement of the virus. The main problem that has been encountered is in obtaining preparations of RNAs 1 and 2 not contaminated by RNA 3 (Colburn, 03).

In last year's report the difficulties in identifying host proteins with which TMV P30 interacted were noted. One possible reason was that the denaturation of the host proteins affected their capacity to interact with P30. However, attempts to detect possible interactions with non-denatured proteins have also proved unsuccessful. The observation, reported below, that actin is associated with plasmodesmata led to a series of experiments exploring the possibility that P30 binds to purified actin and to cytoskeleton 'cages' which comprise actin fibres and microtubules; no binding was detected. In view of this lack of progress it has been decided to put more effort into a new aspect, that of tissue specificity of the action of TMV P30 (Hull, 01)

Ultrastructure of plasmodesmata.

In last year's report we described the new method for preparing clean cell wall fragments containing intact plasmodesmata. These have been examined in detail by electron microscopy of replicas. The plasmodesmata appear to have structure including a 'tail' extending from the neck region which is removed by treatment with Triton X-100. Treatment with proteases does not seem to affect the plasmodesmatal structure whereas 8M urea appears to remove all the substructure. Extracts from these cell wall preparations made sequentially with Triton X-100, boiling in SDS and then extracted with 8M urea, have been electrophoresed into protein gels. The profile of proteins extracted with SDS differs from that of the Triton X-100 extract but not from that of the urea extract. Wall protein preparations have been injected into rats. The resultant monoclonal antibodies will be used to probe western blots and for immuno-electron microscopy to determine if any are plasmodesmata-specific (Roberts, 02).

Dr. R. Overall* (University of Sydney, Australia) studied the distribution of actin in relation to plasmodesmatal structure. Her initial results suggested a co-location of actin fibres and the plasmodesmatal neck region (02).

Experiments were performed on the injection of dyes of different molecular sizes into cell of transgenic *N. tabacum* cv Xanthi and Xanthi nc and of *N. benthamiana* expressing AlMV P3 and observing the spread of the dye to adjacent cells. These dye-exclusion studies showed that P3 slightly increases the molecular size exclusion limit of epidermal plasmodesmata from about 1000 Da for normal plants to 4000 Da for plants expressing P3 or P3Δ[1-12]. Occasionally the plasmodesmata of epidermal cells of normal plants allowed the passage of 3000 Da molecules. This result is surprising as the increase in molecular size exclusion is significantly less than that effected by the TMV P30 (Roberts, 02 + Colburn, 03).

Virus movement proteins in transformed plants.

The CMV 3a gene was cloned in the sense and antisense orientation downstream of the duplicated CaMV 35S promoter with the TMV Ω translation enhancing

sequence and upstream of the nopaline synthase (nos) terminator (05). The chimeric genes were sent to participant 04 who cloned them between the neomycin phosphotransferase (NPTII) and β-glucuronidase (GUS) genes in a binary vector. Both the sense and antisense constructs were introduced into the disarmed *Agrobacterium tumefaciens* strain LBA 4404 which was used to transform tobacco leaf explants. The young regenerated plantlets were then screened for GUS activity and the GUS+ plants rooted twice on kanamycin. An antiserum against the CMV 3a protein detected a protein of 30 kD in western blots of proteins from the plants transformed with the sense construct. This protein was not detected in the control plants expressing NPTII and GUS genes and comigrated with the MP from CMV-infected tobacco plants. Northern blots of total RNA from the antisense transformants probed with a specific cDNA revealed a transcript of about 1.3 kb which was the expected size. Seed from transformed lines expressing each construct at high, medium and low levels have recently been sent to participant 05 for further analyses (Garcia-Luque, 05 + Ben-Tahar, 04).

The CMV 3a gene was mutated to create adjacent 10 amino acid deletions between amino acid positions 124 and 224 (05). These mutants are being cloned into the transformation vector and transformed into tobacco plants (04).

In last year' report the reasons for not transforming sugar beet with the putative MP (P42) of beet necrotic yellow vein virus (BNYVV) were discussed. The possibility that BNYVV infects another transformable plant *Arabidopsis thaliana* was investigated but the virus did not infect any of several ecotypes tested (Richards*, 03). In experiments to examine the possible complementation of the P42 the gene (from participant 03) was cloned into a binary vector and transformed into tobacco leaf explants. The only virus-specific protein detected in these transformed tobacco plants had a molecular weight of 35 kD. BNYVV-infected *Chenopodium quinoa* contained a virus-specific protein of the expected size of 42 kD; *E. coli* expressed the BNYVV gene as a 42 kD and a 35kD protein. It thus appears that in both tobacco and *E. coli* the BNYVV gene product is processed (Ben-Tahar, 04).

All the deleted AlMV P3 genes described above have now been introduced into *N. tabacum* cv. Xanthi (genotype nn) behind the CaMV 35S promoter. All except for 4 of these constructs have also been transformed into *N. tabacum* cv. Xanthi nc (genotype NN). The reason for transforming both cultivars is that, although the strain of AlMV used infects both systemically, it induces the formation of fluorescent compounds and PR (pathogenesis-related) proteins in cv. Xanthi nc but not in Xanthi. As it has been shown that AlMV RNA3 was the primary determinant of symptoms in Xanthi nc, it was hoped that the transformation experiments might give further information on the different reactions of the two cultivars.

The expression of the mutated proteins was monitored by western blotting. In the Xanthi series 5 of the mutants (P3Δ[21-35], P3Δ[36-81], P3Δ[82-112], P3Δ[215-243] and P3Δ[244-300]) were expressed. In the Xanthi nc series thus far P3Δ[36-81], P3Δ[113-156] and P3Δ[215-243] have been shown to be expressed.

An interesting difference was noted between the Xanthi and Xanthi nc transgenic plants expressing P3Δ[36-81]. All the expressing Xanthi plants looked normal. In contrast, all the expresser Xanthi nc were stunted, their leaves were very small and had chlorotic spots; this phenotype is reminiscent of the 'invertase' phenotype (von Schaewen *et al.*, 1990, *EMBO J.* 9:3033). It is thought that these transformed Xanthi nc plants might have a problem with sugar transport and storage (possibly

phloem loading) which might originate from an interaction between the N-gene product and P3Δ[36-81]. If this is so it would mean that the N-gene is involved in some way with sugar transport and that it might be possible to detect the N-gene product by differential binding to the P3Δ[36-81] in Xanthi and Xanthi nc extracts (Colburn, 03).

HIGHLIGHTS/MILESTONES

(1) The localization of the cell wall and nucleic acid binding domains of AlMV P3.
(2) The subcellular localization of CMV 3a protein, showing that it is not nuclear.
(3) Tubular structures associated with CaMV P1.
(4) Plasmodesmatal exclusion size in AlMV P3 transgenic plants.
(5) Phenotype of Xanthi nc transfromed with construct from AlMV P3 gene.

WIDER CONSIDERATIONS

This year has shown steady progress in a topic which is attracting much international interest. This BRIDGE group is one of the major players in this field and has the advantage over other research groups in the breadth of its coverage as well as depth. This breadth derives from the cooperation between five laboratories. Serious consideration has to be given to the continuation of this project after BRIDGE. It is of great importance to European research that we, and other similar projects, can continue the momentum that derives from its cooperative nature.

COOPERATIVE ACTIVITIES

— A. Poirson (from 03) visited K. Robert's lab from 15th July to 12th Aug. and from 29th Nov. to 20th Dec. to do the dye-coupling experiments with A. Turner.
— C. Vaquero (from 04) visited K. Robert's lab from 1st to 18th Dec. to do immunogold labelling experiments with A. Turner.
— December 13th — 16th 1992. Group meeting at JII Norwich in conjunction with the BRIDGE meeting on Molecular Plant Pathology. The group meeting was attended by all the participants and there was a detailed analysis of the progress and problems associated with the project. Future experiments and collaborations were planned.
— Meeting of most of the group in association with the 2nd International Workshop on Basic and Applied Research in Plasmodesmatal Biology, Oosterbeck, The Netherlands, September 1992.
— Four issues of the group newsletter, View through the Plasmodesma, bringing news and information to members of the group.

EUROPEAN DIMENSION:

This project is in the forefront of the subject primarily because the involvement of five laboratories enables a much broader perspective to be taken of the subject. One of its strengths is the ability to make comparisons between different virus systems which will lead to a better understanding of mechanisms of virus movement. This, in turn, could lead to strategies for conferring broad spectrum resistance to viruses in plants.

PUBLICATIONS

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Viry, M., Serghini, M. A., Hans, F., Ritzenthaler, C., Pinck, M. and Pinck, L. (1993). Biologically active transcripts from cloned cDNA of genomic grapevine fanleaf nepovirus RNAs. *Journal of General Virology* **74**:169-174.

Genes required for pathogenicity of bacteria to plants and application of knowledge in biological control of diseases of crops (BIOT CT-900168)

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OBJECTIVES

hrp genes of *Pseudomonas* and *Xanthomonas* (required for pathogenicity and resistance induction): sequencing, characterisation, crystallisation and biochemical function of products, control of expression, role in pathogenicity.

avr genes of *Pseudomonas* and *Xanthomonas* (determine inability to colonise certain hosts):
sequencing, characterisation of products, role in non-host interactions, structure-function relationships, regulation of expression.

rpf genes of *Xanthomonas* (regulate pathogenicity functions): characterisation of RpfC protein, sequencing of *rpfN* gene, interaction of RpfN protein with promoters of pathogenicity genes, biochemical functions of other *rpf* genes.

hrp mutants of *Pseudomonas solanacearum*: use as biological control agents against bacterial wilt.

RESULTS:

hrp genes

LBMRPM:
A protein has been identified in cultures of *P. solanacearum* which acts as an elicitor of the hypersensitive response on tobacco. This protein is not produced by most *hrp* mutants mapping within transcription units 1-4, but is still produced by strains harbouring a mutation in units 5 or 6. An active compound, having similar properties, is found within the cells of most mutants mapping in units 1-4, indicating that the corresponding genes control the secretion of the elicitor through the bacterial cell envelope. The synthesis of this elicitor is dependent on a functional *hrpB* gene which codes for a positive regulator also required for the transcription of other *hrp* genes located within transcription units 1-4.

The elicitor compound has been purified close to homogeneity and the corresponding structural gene has been identified. This gene is located in a region adjacent to the left hand end of the *hrp* gene cluster which had previously been shown to be *hrpB*-regulated. Sequencing of the structural gene for the elicitor is underway and mutants for this gene are being made.

Sequencing of the *hrp* gene cluster of *P. solanacearum* has been extended to 19266 base pairs covering transcription units 1 to 4, allowing identification of a minimum of 19 ORFs. New homologies have been found with proteins involved in the secretion of Yop proteins from *Yersinia*, but also with proteins from *Shigella flexneri* required for the extracellular presentation of Ipa proteins, thus reinforcing the likelihood that *hrp* genes encode the components of secretion machinery specific for pathogenicity factors.

IGF:
The DNA sequence of the entire 25 kb *hrp* region of *X. campestris* pv. *vesicatoria (Xcv)* was completed. 20 *hrp* genes which are organized in six loci are predicted to encode proteins most of which seem to be localized in the bacterial envelope. Strong sequence conservation between putative Hrp proteins from *Xcv* and *P. solanacearum* (LBMRPM) was found. In addition, there are striking similarities to proteins from the mammalian pathogens *Shigella* and *Yersinia* which are involved in secretion of pathogenicity factors. We propose, therefore, that pathogenicity determinants might be conserved between plant and mammalian pathogenic bacteria. In contrast to *hrp* genes from *P. s.* pv. *phaseolicola*, factor Sigma 54 does not seem to be required for *hrp* gene expression. Subcloning of the *hrp* promoters did not reveal any common or conserved sequences that might be involved in regulation of transcription activation. Several Hrp proteins could be expressed in *E coli*. One of the Hrp proteins could be visualized in protein extracts of *Xcv* using a polyclonal antibody generated against the Hrp protein that was expressed in *E. coli*.

IMBB:
(A) Structural studies of the HrpR and HrpS proteins of *P. syringae* pv. *phaseolicola*.
The aim is to purify (better than 90%) milligram quantities of both proteins for the growth of X-ray quality crystals. For both proteins the pT7-7 expression system was used; HrpR and HrpS are insoluble, forming inclusion bodies, a common problem encountered when proteins are expressed in *E. coli*. A purification protocol was developed and yielded sufficient quantities of very pure protein for crystallographic purposes. Both proteins exist in dimeric form, but they also form assemblies of higher order. The crystallization of HrpS and HrpR has not been very successful so far, due to the instability of the proteins, which after a short period of storage are (self?) cleaved to fragments of 10-22 kDa. In addition, extensive aggregation occurs as the protein samples reach concentrations which are necessary for crystallization. An attempt was made to co-purify HrpS and HrpR, as experimental data suggest heterodimer formation between HrpS and HrpR; heterodimerisation could then lead to a stabilization of both proteins in solution. Although there is some indication for a HrpS-HrpR interaction, extensive aggregation also occurred. A possible explanation of the observed aggregation effects would be the inability of the refolded proteins to form native disulfide bridges; we are presently working with reduced and oxidized glutathione in order to overcome this problem. In addition we are experimenting with a new expression system, based on the use of thioredoxin as a gene fusion partner. Thioredoxin fusions when overexpressed in *E. coli* often remain soluble.

(B) Identification of 'Harpin' genes
The HrpN protein of *Erwinia amylovora* is the prototype of a class of Hrp proteins (harpins) that cause necrosis in plants. Thus far, no such proteins have been identified as products of *hrp* genes in *P. syringae* pathovars. We have evidence that a protein encoded by a gene in the *hrp* cluster of *P. syringae* pv. *phaseolicola* encodes

a 'harpin'. Strong amino acid sequence similarity exists between the published $HrpN_{EA}$ protein and the predicted polypeptide of an open reading frame in a *hrp locus of P. syringae* pv. *phaseolicola* that has been genetically characterized. The similarity may also extend to the tertiary structure level, and, if substantiated, this could give insight into the structure of plant receptors believed to be involved in the hypersensitive response. Two approaches are being followed.

(1) The *P. syringae* pv. *phaseolicola* HrpN protein is being purified for structural studies, using the thioredoxin fusion expression system.
(2) Using degenerate primers for the polymerase chain reaction presumed *hrpN* gene fragments are being isolated from other pathogenic bacteria.

Avirulence (avr) genes
IGF:
We previously demonstrated that the function and specificity of the avirulence protein AvrBs3 from *Xcv* which governs the interaction with certain resistant pepper cultivars is determined by the repeats present in the internal region. The AvrBs3 protein and a derivative with specificity for resistant tomato cultivars were overexpressed using the triple lacUV promoter. This led to a much faster HR induction by the bacteria but unfortunately not to the detectable production or secretion of specific elicitors of the plant hypersensitive response. Due to instability of the Avr protein the purification has to await further characterization of the properties of the protein.

UWE:
Expression constructs of the *avr* gene determining race 2 specificity in *Pseudomonas syringae* pv. *pisi (Psp)* have been made using the vectors pMAL-c2 and pMAL-p2 in collaboration with J.W. Mansfield at Wye College.
DNA sequencing of a second *avr* gene determining race 3 specificity in *Psp* has shown the presence of two potential ORFs, head to head, one of which is probably the race 3 avr gene. The latter potential ORF comprises 575bp; searches have failed to identify any similar known DNA or protein sequences in the databases. We are completing this sequencing and will then subclone the ORF to confirm that its activity is correlated with the race 3-specificity. Using a larger cloned fragment we have shown by sequential inoculation of an F_2 population from a cross of pea cvs. Kelvedon Wonder X Belinda that there is co-segregation of resistance to *Psp* race 3 and to a *Psp* race 4-like strain harbouring the gene, confirming the gene-for-gene nature of the interaction.

Non-host resistance: In collaboration with J.W. Mansfield (Wye) and J.D. Taylor (HRI) we have shown that a novel avirulence specificity can be conferred in *Psp* towards pea by cloned DNA from the bean pathogen *P. syringae* pv. *phaseolicola (Psh)*. Transposon mutagenesis together with subcloning data, implicated two regions, separated by several kb, in determining the avirulence phenotype. We are currently attempting to confirm what may be a two-gene system responsible for a single avirulence specificity.

Plasmid transfer and race specificity: A major step forward with this area of work has resulted from a simple technical advance permitting clear visual demonstration of whole plasmids present in *Psp*. This has shown that the race 6 strains harbour two plasmids of approx. 120 and 70kb. The derivation of race 4-like strains involves deletion of DNA from both replicons to give two smaller plasmids of approx. 59 and 64kb, respectively. In studies with plasmid RP4 transconjugants of *Psp* race 6, it was apparent that fertility inhibition *(fi)* was being exerted against RP4; this *Fi+* phenotype was absent from *Psp* race 4-like transconjugants. We therefore obtained some incP2 plasmids (since members of this class of plasmids were known

to be responsible for *fi* against RP4) and introduced these into *Psp* race 4-like strains to see whether they might restore the suppression of the A4-like phenotype. None of the transconjugants restored the suppression, but incP2 transconjugants appeared to have undergone recombination between the incoming plasmid and the resident plasmids, such that both resident plasmids increased in size; this also resulted (in some individual transconjugants) in the restoration of fluorescent pigment production and novel cultivar specificities. We are currently attempting to confirm the changes associated with the acquisition of incP2 plasmids; to identify and clone the putative suppressor of the A4-like gene; and to determine the mechanism by which race changes occur upon acquisition of incP1 plasmids.

Regulatory genes
SL:
Synthesis of pathogenicity factors (extracellular enzymes and polysaccharide) in *X. campestris* pv. *campestris (Xcc)* is regulated positively by the genes *rpfA-H* and negatively by *rpfN*. Sequencing shows that *rpfC* and *rpfG* are members of the two component, sensor-regulator prokaryotic gene family. The *rpfC* coding sequence was cloned into the expression vector pET-15b and the protein produced as a fusion product. The pure protein was obtained by immobilised metal affinity chromatography and proteolytic cleavage to remove the N-terminal fusion peptide. Antibody is being produced to allow study of RpfC *in vivo*. Purified RpfC cannot be phosphorylated *in vitro*. However incubation of *Xcc* extracts with ATP gives phosphorylation of a protein of the same size as RpfC. During purification RpfC associates with a protein of *E. coli*. This has been purified and the N-terminal peptide sequence determined. We shall attempt to determine whether this is in a regulator protein in *E. coli* related to Rpf proteins. If so, the association might represent an example of regulatory 'cross-talk'.

The gene *rpfF* has been found to be involved in synthesis of a diffusible substance. Enzyme production by *rpfF* mutants is restored by the diffusible factor. Experiments are in progress to determine the nature of the diffusible factor and its role in regulation.

The negative regulatory locus *rpfN* contains a single gene, the product of which is required for binding of a protein to a conserved domain of the protease and cellulase gene promoters. It is supposed that this binding is required for down-regulation of enzyme synthesis. Expression of *rpfN* probably depends on a σ^{54}-RNA polymerase and may be modulated by other, as yet unknown, genes.

In addition to further characterisation of the *rpf* genes and their interactions with each other, we are investigating the factors to which the regulatory systems respond.

Protection of plants with avirulent hrp mutants
Calliope:
Protective mutants of *P. solanacearum* have been obtained by insertion into the *hrp* genes of an Omega-KM cassette which is no longer transposable. The presence of these protective mutants within the root system, collar and lower part of the aerial stem prevents the susceptible tomato cv. Flordel from any further invasion by a virulent strain in the most favourable cases, and limits percentage of wilting in all cases.

Bacteriocins are produced *in planta* by some bacterial strains. However, selecting for bacteriocin production does not increase the protective effect of *hrp* mutants.

Inoculation procedure appears to be the crucial step for ensuring effective protection. The factors that ensure a successful root inoculation by *hrp* mutants are not yet precisely known and are under study.

The French Bio Safety Committee (Commission du Génie Biomoléculaire) recently (Feb. 1993) authorised small-scale field experiments in naturally infested fields in Guadeloupe for the coming two years.

HIGHLIGHTS

Significant similarities of predicted Hrp proteins of X.c. pv. vesicatoria to predicted Hrp proteins in *P. solanacearum* and *P.s.* pv. *phaseolicola* and to *Yersinia* and *Shigella* proteins involved in pathogenicity were found.

A *hrp*-dependent elicitor protein produced by *P. solanacearum* has been discovered and purified.

A diffusible regulator of enzyme synthesis in *X.c.* pv. *campestris* has been discovered. Two-gene avirulence specificity may be involved in non-host resistance in *P.S.* pv. *phaseolicola.*

WIDER CONSIDERATIONS

The work of the group has convincingly demonstrated the similarities in pathogenicity mechanisms of different plant pathogenic bacteria. Moreover, relatedness has been found to virulence factors of animal and human pathogens. These facts suggest that related disease control strategies may be applicable to many plant pathogens. One such approach, the protection of plants by prior infection with a disarmed pathogen, is being tested by one of the partners.

COOPERATIVE ACTIVITIES

The group held meetings on two occasions during the year at which research progress was discussed. The meetings were held in Toulouse and Norwich. The latter meeting was a joint event involving two other BRIDGE groups with interests in plant pathogens. Biomaterials and technical information have been exchanged within the group throughout the year.

EUROPEAN DIMENSION

The collaboration has been most successful and has led to the development of lines of work which would have been difficult to start without the benefit of input from other partners.

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Molecular basis of signalling in *Rhizobium meliloti-Medicago* interactions and genetic improvement of nodulation ability (BIOT CT-900159)

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OBJECTIVES

The common goal of the research projects carried out by the collaborating groups is the elucidation of the molecular basis of the symbiotic nodule development, with an emphasis on the various types of signal exchanges between the symbiotic partners.

While in the first period we focused mainly on the investigation of *nod* gene regulation and the involvement of *nod* gene products in Nod signal production, during the reporting period (with the exception of characterization of the *nfe* genes determining competitiveness of the bacteria which was continued) the major emphasis of the research was shifted more to the plant side. The experiments aimed at the better elucidation of:

— the role of lipo-oligosaccharide type molecules in plant development
— the signal transduction pathway by identifying Nod factor inducible genes including those involved in cell cycle control
— how the symbiotic nodule developmental pathway interacts with or avoids the plant defense reactions and how it affects flavonoid and phytoalexin production
— what is the role of the *nfe* region providing higher competitiveness of *Rhizobium* strains which could be used for the genetic improvement of nodulation ability.

Topics:

(i) Studies on the control of *nod* and *nfe* gene expression and genetic improvement of nodulation ability.
01: Identification of *nod* gene transacting factors (*syrM, nodD3*) and repressor structure determination (with 02).
02: Expression of *nod* genes in tobacco and alfalfa and study of *nod* gene function.
03: DNA sequence of the *nfe* region, expression of *nfe* genes in other *Rhizobium* strains.

(ii) Structural and functional analysis of signal molecules synthetised by the Nod proteins.
01: Construction of Nod factor overproducing strains of *R. meliloti*.
01 and 02: Purification of Nod factors.

(iii) Identification of *Medicago* genes activated by the *Rhizobium* signals.
01: Studies of gene expression and identification of genes.
03: Plant nodulin genes in relation to *nfe* expression.

04: Improvement of plant DNA extraction and construction of RFLP map of *Medicago*.

(iv) The effect of *Rhizobium* signals on flavonoid biosynthesis.

01: Isolation of genes of the phenylpropanoid pathway and plant defense genes in relation to the development of symbiosis.

03: Plant defense genes in relation to *nfe* expression.

RESULTS

Topics ***i.01*** and ***ii*** had been completed earlier. For the last period we report on the following projects:

(i) **Studies on the control of *nod* and *nfe* gene expression and genetic improvement of nodulation ability.**

02: Expression of nod genes in tobacco and alfalfa and study of nod gene function.

Transgenic tobacco plants transformed with the *nodA* and *nodB* genes of *Rhizobium meliloti* exhibit significant growth abnormalities, indicating that the products of these genes are somehow involved in production or modification of growth signals in the plant (Schmidt et al., 1993). To explain the effects caused by these genes in non-legume transgenic plants, and to elucidate their specific role in the pathway of Nod signal synthesis, which is not yet understood in detail, we have purified NodB protein from recombinant *E. coli* cells and determined its biochemical function by direct assays (John et al., 1993).

Overexpression of *nodB* in *E. coli* under the control of the phage T7 promoter resulted in the synthesis of high levels of NodB protein, which accumulated in the form of inclusion bodies. An efficient refolding strategy was used to reactivate inactive NodB protein from these insoluble aggregates. Gel chromatography resulted in the separation of the renatured NodB into three active fractions with different relative molecular masses, which may represent oligomeric forms of this protein. Small amounts of NodB protein were also purified by immunoaffinity chromatography. Purified NodB was incubated with N-acetylglucosamine and various chitooligosaccharides. TLC analysis of the reaction mixtures showed that chitooligosaccharides were deacetylated by NodB. The monosaccharide N-acetylglyucosamine was not affected. The NodB-treated chitooligosaccharides could be re-N-acetylated with acetic anhydride. Evidence that the enzyme deacetylates only one N-acetylglucosamine residuc of the chitooligosaccharide chain is provided by the release of equimolar amounts of acetate independent from the chain length of the substrate molecule. The site of deacetylation was determined by sequential degradation of end-labeled (^{14}C)-tetraacetyl-chitotetraitol with chitinase and exoglycosidase.

Our data suggest that during lipooligosaccharide synthesis NodB removes the acetyl group from the terminal non-reducing N-acetyl-glucosamine residue in a chitooligosaccharide precursor molecule. The free amino group at the nonreducing terminus could then be acylated with a specific long chain fatty acid. When NodB is synthesized in transgenic tobacco plants, this deacetylase causes characteristic morphological alterations (Schmidt et al., 1993), indicating that tobacco must contain substrates that can be modified by NodB to form plant growth signals. In further studies we want to use this enzyme as a tool to isolate and label the N-acetylglucosamine containing oligosaccharide signals from plants, which hopefully will lead to a better understanding of plant growth and development in general.

Transformation and regeneration of *Medicago* with the *nodABC* genes have started at ISV by 01. Analysis of the transgenic alfalfa plants will be done by 01 and 02.

03: DNA sequence of the nfe region, expression of nfe genes in other Rhizobium strains.

We have continued the molecular analysis of the *Rhizobium meliloti* GR4 *nfe* DNA region and the construction of a cloning vector to transfer the *nfe* region in a stable way to other *Rhizobium* strains/species. Previously we reported that the 9 kb *nfe* region contains several ORFs. In addition to the *ocd* gene (ORFC) and the IS*Rm3* (ORF4), ORF5 was characterized as IS*Rm4*. The products of other ORFs, *nfe1* and *nfe2*, whose expression is NifA-dependent, were identified by using *in vitro* transcription/translation and bacteriophage T7 RNA polymerase/promoter system, respectively. A high degree of homology between the amino terminal domain on Nfe1 and the NifH protein was found. *nfe1* shows also homology to the upstream non-coding DNA region of the *fixABCX* operon. No homology for *nfe2* or its predicted product has been found. 128 bp downstream from *nfe2* we found an ORF (ORFC) coding for a putative protein of 320 amino acids which was homologous to the ornithine cyclodeaminase from *Agrobacterium tumefaciens* (OCD). Although the ORFC DNA sequence is present as a single copy in the wild type GR4 genome, different biochemical studies on the conversion of ^{14}C labeled ornithine into proline have indicated the presence of an additional ocd gene in GR4. We raised antibodies against the ORFC coded product to analyze and characterize the presence of OCD in GR4 and other *Rhizobium* strains.

Competitiveness of strains GR4, Rm41 and its derivatives was tested in nodulation assays. From the data obtained we concluded that GR4 is the most competitive strain, while Rm41 and its derivatives are less competitive ones. Unlike Rm2011, the transfer of the *nfe* region on pRmNT40 to Rm41 does not modify the competitiveness of this strain. This result needs however, further support because of the low stability of plasmid pRmNT40 in Rm41 (about 50 % of the cells isolated from nodules had lost the plasmid). Competitiveness of Rm41, on the other hand, was increased by the transfer of pCK1 that carries a constitutive *nifA* gene of *Klebsiella pneumoniae* (Sanjuan and Olivares, 1991. Mol. Plant-Microbe Interact. 4, 365-369). It is still not clear why the transfer of the *nfe* genes increases the competitiveness of the recipient strain in some cases but not in others.

For stable maintenance of exogenous genes in these bacteria, cloning vectors have been constructed by using the *oriV* region of one of the resident plasmids of *R. meliloti* GR4. Analysis of the region revealed that a single gene, *repC*, homologous to that of Ri plasmid of *Agrobacterium rhizogenes* was sufficient to provide plasmid stability and replication properties. We have selected the vector pJMB45 as a possible candidate to solve the problem of the stable maintenance of exogenous genes in these bacteria as an alternative to their integration into the genome.

(iii) **Identification of Medicago genes activated by the Rhizobium signals**

01: Studies and identification of the Nod factor activated Medicago genes in M. varia and M. sativa cell cultures as well as in roots and root hairs of M. sativa and M. truncatula.

A family of Nod factors have been purified and identified from *R. meliloti* by participant 01 during the last two years (Schultze et al., 1992. Proc. Natl. Acad. Sci. USA, 89, 192-196). The signal molecules are sulfated lipo-chitooligosaccharides, beta-1,4-linked oligomers of three to five N-acetylglucosamine residues

predominanly with a C16:2 fatty acid moiety on the non-reducing end and with a sulfate group at the reducing end which is required for nodulation of *Medicago* (Roche et al., 1991. Cell, 67, 1131-1143).

The molecular basis of nodule initiation which obviously requires the activation of cell cycle control elements in the resting cortical cells and expression of early nodulin genes, specifically involved in nodule organogenesis, was investigated.

Activation of cell cycle by Nod signals was studied on *Medicago* cell cultures. First it was demonstrated that cultured *Medicago* cells responded to Nod factor. The analysis of the *in vitro* translation product by 2D-gels showed that the purified Nod factor caused both quantitative and qualitative changes in gene expression. Parameters for optimal experimental conditions (e.g. concentration of Nod factor, duration of treatment, cell types, synchronisation of the cultures) have been determined. Cell cycle progression was followed by thymidine incorporation, flow cytometry and by studying the expression of G1-S and G2-M specific marker genes. Our data indicate that Nod signals stimulate reactivation of cells for division and speed up cell cycle progression. Nod signals might stimulate cell cycle progression only at certain points, e. g. G1-S transition, or have general control on the cell cycle.

Early nodulin genes from *Medicago* have been identified based on their homology with early nodulins from pea. Two genes homologous to *enod12* have been cloned from *M. sativa*. Their sequences show strong homology and their overall structure is characteristic to hydroxyproline-rich glycoproteins. Both genes (*Msenod12A* and *B*) are highly expressed in young nodules (Allison et al., 1992). Genomic clones from both genes were isolated and promoter-*gus* fusions were introduced into *M. varia* plants. Characterization of the transgenic plants is in progress. Using reverse transcription-PCR analysis the induction of *Msenod12B* by Nod factor treatment of the roots was demonstrated. Furthermore, a gene homologous to a newly identified nodulin from soybean, *enod40*, has been cloned from *M. truncatula* *(Mtenod40)*. Temporal and spatial expression of this gene during nodulation was studied by *in situ* hybridization. Genomic clones of *Mtenod40* are currently under characterization.

04: Improvement of plant DNA extraction and construction of RFLP map of Medicago.

The complete procedure of DNA extraction has been improved. A pretreatment of the plant material has been worked out for DNA extraction. The plant material is placed into a bag, frozen in liquid nitrogen for a few seconds and then incubated with an enzymatic solution containing cellulases and pectinases at room temperature for half an hour. Then the bag is put for a few minutes into a hammer grinder for disruption of cells. After this pretreatment the content of the bag is poured into a cartridge where isolation of nuclei, nuclear lysis and DNA purification by dialysis are implemented. This process allows to have DNA in solution, ready to use, and obtain DNA of good quality ($OD_{260/280}=1.85$) and perfectly digestable by restriction enzymes. Typically 15 to 50 µg of DNA can be expected per 100 mg of material depending on the species.

The establishment of the RFLP map of *Medicago* has been delayed since it was preferred to fully define the best methodology. The preparation of the blots has been automatized and tested on a large diversity of species. They were perfectly reproducible and could be hybridized with various probes. In addition, a system suggesting further experimental steps to perform has been initiated. The optimization of the technical part will now allow us to establish the RFLP map of new species of *Medicago*.

(iv) **The effect of *Rhizobium* signals on flavonoid biosynthesis**

01: Isolation of genes of the phenylpropanoid pathway and plant defense genes in relation to the development of symbiosis.

The phenylpropanoid pathway has special importance in the *Rhizobium*-legume symbiosis. In *Medicago* certain flavonoid products are *nod* gene inducers, others are phytoalexin isoflavonoids and are involved in the plant defense reaction. Genomic and cDNA clones encoding enzymes on both branches of the phenylpropanoid pathway have been isolated from *M. sativa* and expression of these genes has been studied in symbiotic and pathogenic (compatible and incompatible) interactions. In addition, production of the flavonoid and isoflavonoid compounds was analysed in these interactions as well as under various growth conditions. Expression of genes encoding chalcone synthase, chalcone reductase and isoflavone reductase involved in the isoflavonoid synthesis was rapidly and highly stimulated in the incompatible but not in the compatible pathogenic interactions. Expression of these genes increased also by several stress conditions such as wounding or nitrogen starvation. In contrast expression of genes encoding peroxidase or pathogenesis-related proteins seems to be more specific for the defense reaction. On the flavonoid branch, we have completed the nucleotide sequence of the flavanone-3-hydroxylase of the genomic locus as well as the sequence of a PCR fragment representing a part of the dihydroflavonol-4-reductase transcript.

The flavonoid synthesis in the plant root was affected by the growth conditions: production of *nod* gene inducers was increased under conditions of limited nitrogen supply. We have isolated these compounds and determined their chemical structures. Some *nod* gene activators were identified as isoflavonoids which was in agreement with the enhanced expression of genes on the isoflavonoid branch indicating that even precursors of phytoalexins can provide signals for symbiosis.

HIGHLIGHTS/MILESTONES

It was demonstrated that purified Nod factor stimulates cell cycle progression as well as reactivation of cells for division and induces expression of early nodulin genes.

The ability of NodB to remove the acetate group at the non-reducing terminus of chitooligosaccharides can be used to isolate and radiolabel N-acetylglycosamine containing plant growth regulators.

An *oriV* region of a *Rhizobium meliloti* plasmid has been, for the first time, genetically analyzed and characterized.

A relation between symbiotic and plant defense reaction can be envisaged since certain isoflavonoids might provide a common signal for both pathways.

An automatic multisouthern/northern electrophoresis and blotting system has been developed for the establishment of RFLP maps or genetic fingerprints and for gene expression studies.

WIDER CONSIDERATIONS

The BRIDGE project entitled 'Molecular basis of signalling in *Rhizobium meliloti*-*Medicago* interactions and genetic improvement of nodulation ability' involves 4 collaborating laboratories. The research project includes both fundamental and applied interest. Studies on signal exchanges and gene activation help elucidation of root nodule organogenesis. Moreover, the rhizobial lipo-oligosaccharide Nod

signal molecules constitute a new class of plant growth regulators based on their host specificity and their special requirement for unique developmental pathway(s). These bacterial signal molecules provide in addition a useful tool, without the complications arising from the endogenous pools of the same molecules, to study cell cycle control in plant cells which, in contrast to animal cells, even in their differentiated forms can maintain their totipotency. Identification of signal molecules produced by the bacterial and plant partners, as well as studying genes activated by these signals in the other partner could contribute to increase our knowledge on the molecular and cellular mechanisms of plant differentiation and development.

Moreover, the fact that rhizobia are the only organisms that are able to convert the atmospheric nitrogen to ammonia and provide a nitrogen source for the host plant and indirectly to other organisms without the undesirable effect of artificial nitrogen fertilizers, has a direct link to agriculture. Genetic engineering of the *Rhizobium* strains (eg. with genes providing higher competitiveness or extension of host range) as well as that of the host plant might have a significant impact on plant breeding and agricultural yields.

COOPERATIVE ACTIVITIES

ISV, MPI and EEZ as well as ISV and BERTIN had regular exchange of information, plasmids, bacteria and various biological materials. In addition, A. Savouré and B. Hoffmann from BERTIN work full time at ISV.

9 and 10 March 1992:	Visit of Drs. H. Röhrig and J. Schmidt (MPI) at ISV for discussion of results, cooperation and exchange of material.
14-17 March 1992:	Meeting of all participants of the BRIDGE project at EEZ.
30 and 31 March 1992:	Visit of Drs. M. John and J. Schmidt (MPI) at ISV for discussion of joint projects and exchange of material.
8 December 1992:	Award of the Max-Planck Research Prize to Drs. A. Kondorosi (ISV) and J. Schell (MPI) by the Alexander von Humboldt Foundation in Bonn.
17-19 December 1992:	Visit of Drs. A. and E. Kondorosi (ISV) at MPI for discussion of results.
26 March 1993:	Meeting of all participants of the BRIDGE project at Bertin in Plaisir.

LIST OF JOINT PUBLICATIONS/PATENTS WITH TRANS-NATIONAL AUTHORSHIP

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The molecular basis of pathogenicity, avirulence and resistance in the interaction between the fungal pathogen *Cladosporium fulvum (Syn. Fulvia fulva)* and tomato (BIOT CT-900163)

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OBJECTIVES

The common interest of all participants is the unraveling of the molecular basis of the plant-fungus interaction tomato-*Cladosporium fulvum*. The laboratory of the coordinator has exchanged various materials with the laboratories of Oliver (probes for avirulence gene *avr9*, chromosome-specific probes), van den Broek (avirulence gene *avr9*, pathogenicity genes *ecp*1 and *ecp*2), Melchers (antibodies against several PR proteins, PR proteins, fungal isolates), Cervone (tomato DNA) and Fry (apoplastic fluids of C. *fulvum*-infected tomato).

De Wit would isolate new PR proteins and isolate cDNAs encoding those proteins. He would also isolate and characterize new avirulence and pathogenicity genes of *C. fulvum*. Oliver would produce pathogenicity mutants of *C. fulvum*, which either occurred spontaneously or were created artificially by transformation with the vector pAN8-1. In addition, he would set up genetic mapping in *C. fulvum* by protoplast fusions between races carrying different markers. His studies on retrotransposons would be continued. Van den Broek would perform in vivo directed mutagenesis of the the putative pathogenicity gene *ecp*2 and the avirulence gene *avr9* of *C. fulvum*. Also gene targeting would be initiated. Melchers would optimize the transformation and regeneration procedure for tomato and tobacco; the plants would be transformed with different cDNAs, especially those encoding β-1,3-glucanases, chitinases and the polygalacturonase inhibitor protein (PGIP). Fry would determine polysaccharide degrading activities of proteins occurring in apoplastic fluids and identify the preliminary structure of the released oligosaccharides. Cervone would purify PGIP from tomato, isolate the bean cDNA encoding PGIP, constuct a chimeric *pgip* gene and pass this to Melchers to transform tomato. Transformants would then be checked for resistance to various pathogens.

RESULTS

Lab 01, de Wit's research group. From the purified AVR4 protein, the putative product of avirulence gene *avr*4, a race-specific elicitor of the fungal pathogen *Cladosporium fulvum*, internal and N-terminal amino acid sequences were obtained and degenerated oligonucleotide probes were designed. Screening of a cDNA library, obtained from tomato genotype Cf5 infected with race 5 of *C. fulvum*, with the probe derived from the N-terminal part of the elicitor

resulted in several positive clones that contained an open reading frame (ORF) encoding a pre-pro-protein of 135 amino acids, containing 8 cysteine residues in the mature protein. Probably, after removal of the N-terminal signal peptide, the pro-protein is processed into a mature elicitor of 102 to 106 amino acids by plant and/or fungal proteases. Transformation of *C. fulvum* race 4 (virulent on Cf4 genotypes) with a genomic clone encoding the AVR4 elicitor, resulted in transformants that had become avirulent on tomato genotype Cf4. Analysis of the sequence of avirulence gene *avr*4 present in different races avirulent on Cf4 genotypes revealed that they all contain an identical ORF encoding the pre-pro-protein. All races virulent on Cf4 genotypes that were analysed so far, contain an ORF encoding a pre-pro-protein similar to AVR4 of which, however, one of the cysteine residues has been replaced by a tyrosine residue. These results clearly show that a single basepair change can alter the specificity of *C. fulvum* for Cf4 tomato genotypes.

Transformants of *C. fulvum* that contained the coding region of the *avr*9 gene fused to the constitutive *gpd*-promoter of *Aspergillus nidulans*, produced active AVR9 elicitors of 32, 33 and 34 amino acids, which are precursors of the mature 28 amino acid AVR9 peptide. Plant factors are able to process the precursor into the mature AVR9 peptide.

Lab 02, Oliver's research group. Work continues to focus on establishing means of isolating *C. fulvum* pathogenicity genes. To facilitate the mapping of mutated genes a method of genetic analysis based on an induced parasexual cycle has been developed. Eleven linkage groups have been identified. Because of the novel nature of the analysis care has been taken to extensively characterize the process. Telomeric DNA has been cloned and characterized, and telomere-linked RFLPs have been shown to map to linkage group ends. Molecular evidence for a high level of recombination during the parasexual crosses has been obtained from the analyses of transforming DNA present in one of the parents. Transformation may provide a means to simultaneously inactivate and tag pathogenicity genes. Transformation with pAN8-1 leads to integration of the plasmid into the *C. fulvum* genome with apparent randomness; the pathogenicity phenotypes of transformants is being assessed. Deletion mutagenesis and the subsequent isolation of DNA corresponding to the deletions by genomic subtraction provides a potential alternative strategy. A simple, single-cycle method of genomic subtraction is being developed. The characterization of clones isolated from an expression library following screening with antibodies raised to intercellular fluid is continuing. The nucleotide sequence of two clones has recently been determined; searches reveal no homology to database sequences; *in planta* expression is being analysed. The enzymes mannitol dehydrogenase and invertase, which may have a role in the sequestration of plant carbohydrates during infection, are being purified.

Lab 03, van den Broek's research group. The avirulence gene *avr*9 has been disrupted in two different races of *C. fulvum* (races 4 and 5). Transformants deleted of the *avr*9 coding sequence have clearly become pathogenic on Cf9 tomato genotypes and are no longer producing the active AVR9 elicitor. The pathogenicity on Cf9 genotypes was shown to be the direct result of this gene replacement. These results definitely confirm the function of the *avr*9 gene and clearly demonstrate that a single gene in a eukaryotic plant pathogen can induce a hypersensitive response through a protein elicitor by interacting with the product(s) of a resistance gene.

An alternative strategy has been developed to disrupt the putative pathogenicity gene *ecp*2. To simplify the analysis of the transformants, the recipient strain used

was a transformant with a simple insertion of the hygromycin resistance gene at the *ecp*2 locus and the marker gene on the plasmid was conferring the resistance toward phleomycin. Mutants deleted of the *ecp*2 gene (selected as phleomycin-resistant and hygromycin-sensitive) were shown not to produce the ECP2 protein. Preliminary results suggest that these mutants are still pathogenic on tomato plants and that the *ecp*2 gene cannot be considered a strong pathogenicity factor.

Attempts to disrupt the putative pathogenicity gene *ecp*1 were not successful yet, due to the fact that the coding sequence of this gene is flanked by sequences highly repeated in the genome of *C. fulvum*. These sequences decrease the probability of integration of a disrupting plasmid at the *ecp*1 locus. These experiments clearly demonstrate the power and the limits of the gene disruption methods for the analysis of the interaction between *C. fulvum* and tomato plants.

Lab 04, Melchers's research group. The collaboration between MOGEN (lab 04) and Cervone (lab 06) resulted in the construction of binary vector pMOG636 containing the bean *pgip* gene transcriptionally controlled by the constitutive CaMV 35S promoter and the nopaline synthase transcription terminator. Using the optimized *Agrobacterium*-mediated transformation procedure developed at MOGEN, construct pMOG636 was introduced into tomato cv. Moneymaker and yielded 51 transgenic lines. In collaboration with A. Desiderio, a scientist from lab 06, the PGIP transgenic tomato plants were analyzed for ploidy level and for the expression level of the *pgip* gene. Amongst 44 diploid transgenic plants, 35% showed high (1500-3500 IU/mg) or moderate (500-1500 IU/mg) PGIP expression. About 35% showed low expression levels (25-500 IU/mg). PGIP transgenic tomato plants were selected for assaying their resistance to the plant pathogen *F. oxysporum* f. sp. *lycopersici* race 1, a soil-borne fungus that infects the vascular tissue of tomato after penetration of roots through wound sites. In addition, transgenic tomato plants constitutively expressing class I and class II chitinase and ß-1,3-glucanase enzymes either simultaneously or alone, are available now for testing disease resistance against *F. oxysporum* f. sp. *lycopersici*.

Lab 05, Fry's research group. After assaying the incorporation of [^{14}C] leucine by tomato cell suspension cultures in the presence of numerous polysaccharides and related fragments, several were found to have an inhibitory effect, in particular, rhamnogalacturonan-II (RG-II). We have therefore studied the possibility that RG-II may be a biologically active fraction which could be relevant in plant-pathogen interactions. RG-II, a complex component of pectin in the plant cell wall, was previously believed to have a purely structural function although its complexity suggests otherwise. Purification of RG-II on a Bio-Gel P-60 column and subsequently on a Dionex HPLC showed that the most active fraction in the bioassay contained a mixture of oligosaccharides which were not simply oligogalacturonides. Further work is being undertaken to identify the active components. Both uptake and incorporation of [^{14}C]leucine were found to be inhibited by RG-II; even after 5 minutes uptake was substantially inhibited, showing RG-II had a rapid effect. The incorporation of various [^{14}C]-labeled amino acids was affected in different ways by RG-II. [^{14}C]Glutamate incorporation was strongly inhibited, more so than [^{14}C]leucine, whereas [^{14}C]tyrosine and [^{14}C]phenylalanine incorporation were not affected at all. This suggested that RG-II may act by disrupting membrane transport of certain amino acids. Several sugars were found to inhibit [^{14}C]leucine incorporation as well, in particular KDO and apiose, both constituents of RG-II, even though their effectiveness was at relatively high concentrations. However, this may be an indicator of the structural requirement for biological activity.

Lab 06, Cervone's research group. A chimeric *pgip* gene (35S/PGIP) has been constructed possessing the structural pgip gene and regulatory sequences of the CaMV 35S promoter. The 35S/PGIP chimeric gene will allow constitutive and high-level expression of PGIP in tomato. A *Bam*HI restriction site was added, by PCR, immediately upstream of the ATG of the coding sequence and 143 nucleotides downstream of the putative polyadenylation site. The *Bam*HI fragment (including the *pgip* gene coding sequence, the entire 3' transcribed untranslated region and the 3' flanking region) was inserted into the *Bam*HI site of the expression cassette of the intermediate plasmid vector pMOG 464, downstream or the ALMV RNA 4 leader sequence. The *Eco*RI-*Sst*I cassette was then excised from pMOG 464 and inserted into the disarmed *Agrobacterium tumefaciens* pMOG 402 Ti plasmid vector, resulting in pMOG 636. The construct was transferred to tomato plants using the *A. tumefaciens*-mediated transformation of cotyledon pieces. Forty four independent transgenic plants were obtained. At present, seeds are being collected from each transgenic plant. This part of work was made in collaboration with MOGEN.

HIGHLIGHTS/MILESTONES

(1) The second fungal avirulence gene, *avr4*, of *C. fulvum* has been cloned. A single basepair change in this gene changes the fungus from avirulent to virulent on tomato plants carrying resistance gene *Cf4*.

(2) Avirulence gene *avr9* has been successfully disrupted in avirulent wild-type races; transformants became virulent on tomato plants carrying resistance gene *Cf9*. Disruption of the *ecp2* gene revealed that this gene cannot be considered a strong pathogenicity factor for *C. fulvum*.

(3) Telomeric DNA of *C. fulvum* has been cloned and characterized and telomere-linked RFLPs have been shown to map to linkage group ends.

(4) The *pgip* gene of *Phaseolus vulgaris* has been cloned.

(5) Tomato plants constitutively producing the endopolygalacturonase inhibitor protein (PGIP) of *Phaseolus vulgaris* or class I and II β-1,3-glucanases and chitinases have been obtained and are being screened for resistance against various pathogens.

(6) A polysaccharide (rhamnogalacturonan-II) has been identified that mediates metabolic changes in tomato cell suspension cultures.

WIDER CONSIDERATIONS

Molecular characterization of gene-for-gene systems is in progress. Of the many bacterial genes and two fungal avirulence genes cloned to date, none showed homology with other characterized genes. It is likely that the primary function of avirulence genes varies considerably. In some cases an avirulence gene may be so important for the pathogen that loss of it would be detrimental. A property all avirulence genes have in common is the antigenicity of their direct or indirect products. Through these products the pathogens are recognized by the host which responds by inducing a hypersensitive response (HR).The primary function of resistance genes is still a matter of speculation, as none have yet been cloned. However, without knowing the primary function of either avirulence and resistance genes, their 'secondary' function (the induction of HR by their interacting products) could perhaps be exploited to engineer transgenic disease-resistant plants. Future experiments should tell us whether transformation of plants with

highly controlled avirulence-resistance gene cassettes indeed become resistant to various pathogens. Regardless of the results of this concept, such transgenic plants can teach us more about stimulus perception and signal transduction in gene-for-gene systems.

COOPERATIVE ACTIVITIES

All participants and coworkers gathered for a three-day workshop at the University of Rome (11-13 June, 1992). After a meeting of the group leaders, 20 lectures were presented. The book of abstacts was distributed among the participants and a number of copies was presented to Dr. Vassarotti, the coordinator of this project in Brussels. From 13 to 16 December, 1992, a sectorial meeting on molecular plant pathology was organized in Norwich by Dr. R. Hull (John Innes Institute, Norwich), Dr. M.J. Daniels (The Sainsbury Laboratory, Norwich) and Dr. R.P. Oliver (University of East Anglia, Norwich). After a plenary day, on which the BRIDGE bacteriology, mycology and virology projects were described and lectures on related subjects were presented, the three groups organized separate meetings (Dr. Vassarotti was one of the participants). It was decided that the three coordinators, working on plant pathogens in the framework of BRIDGE, would compose an EC-booklet highlighting the progress and new tendencies in molecular plant pathology and molecular resistance breeding. On the third day individual discussions were organized at the Sainsbury laboratory. The exchange of materials between labs 01, 02, 03 and 04 is most frequent. Culture filtrate from *C. fulvum* grown *in vitro* was sent from lab 02 to lab 05 for analysis of cell wall-lysing enzymes. The exchange of materials and staff between labs 04 and 06 was intensified.

EUROPEAN DIMENSION

This type of collaborative research facilitates the exchange of materials and researchers and it intensifies regular fundamental discussions. It is an excellent starting point to extend the collaboration and to include other laboratories. P.J.G.M. De Wit, the coordinator of this project has arranged a sabbatical for himself in the lab of J. Jones (Sainsbury Laboratory) and has two projects granted in the Human Capital Mobility Network Programme.

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Genetic and molecular approaches of the physiology of bacteroids in relation to the plant nodule metabolism (BIOT CT-900166)

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OBJECTIVES

Our project aims at an overall picture of the regulatory networks operative in the nodule that control nitrogen fixation by *Rhizobium* bacteroids. We concentrate on the interconnected roles of oxygen limitation, carbon and nitrogen supply on nitrogen fixation gene expression and activity.

Our objectives for the reporting period were:
1. Study of the role of the genetic components responsible for oxygen control of *nif* and *fix* gene expression in *R. meliloti*: molecular mechanisms responsible for signal perception and transduction by the FixL sensor and for target gene activation by FixJ;
2. Identification of the functionally homologous pathway which operates in *R. leguminosarum* and comparison of the two pathways;
3. Genetic analysis of dicarboxylic acids transport in symbiosis and its relationship with *nif* gene expression;
4. Regulation of bacterial glutamine synthetases expression and activity;
5. Mechanism of export of fixed nitrogen towards the plant cell;
6. Effect of the modulation of plant glutamine synthetase levels on nitrogen fixation activity.

RESULTS

1. Oxygen regulation of nitrogen fixation gene expression in *R. meliloti* and *R. leguminosarum* (objectives 1 and 2 ; Toulouse and Bielefeld)

In *R. meliloti* where the complete regulatory cascade was identified (Fig. 1, see next page), an in vitro transcription system has been developed in order to study the way by which oxygen concentration is relayed to the transcription apparatus. It has been shown that in anaerobic conditions the FixL sensor activates FixJ which is a transcriptional activator needed for *nifA* and *fixK* expression by sigma70 RNA polymerase holoenzyme. Stimulation of FixJ transcriptional activity is due to its phosphorylation which is modulated by FixL in response to oxygen concentration (Reyrat et al., manuscript in preparation).

Previous studies (see last progress report) led to the identification of a *fixK* homologue in *R. leguminosarum*, which could functionally complement a *fixK* mutant of *R. meliloti*. This gene called *fnrN*, is preferentially expressed under microaerobic conditions and, as established meanwhile, in symbiosis. Microaerobic

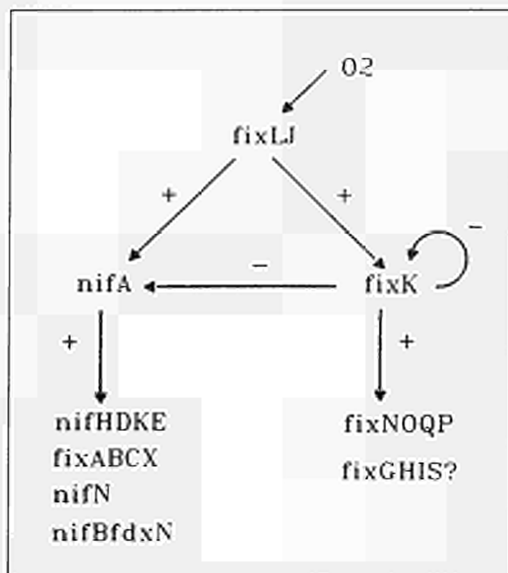

Figure 1: Model for oxygen-regulated expression of nitrogen fixation genes in R. meliloti

expression of *fnrN* in *R. meliloti* was found to be independent of *fixLJ*; in contrast, it is subject to positive autoregulation. Meanwhile, we have shown that FnrN is oxygen regulated at two levels: at the transcriptional level and at the level of protein activity (Schlüter et al. 1992).

As already indicated in the last progress report, hybridization experiments indicated the presence of two *FixN* copies in *R.leguminosarum*. In contrast to *R. meliloti*, only one copy of *fixN* is located on the symbiotic plasmid of *R.leguminosarum*; the second copy is located on plasmid pRlVF39c, which also encodes functions involved in LPS biosynthesis (Fig.2).

For the copy present on pSym, expression studies indicated that transcription of *RlfixN* is induced under microaerobic conditions; however, the overall expression of this *fixN* copy is very low. Microaerobic induction was found to be dependent on *fnrN* and, at that stage unexpectedly, on the presence of pRlVF39c. Our sequence analyses revealed the interesting fact that *fnr/fixK* and *fixL* homologous genes are present upstream of the *fixN* copy on plasmid pRlVF39c, although previous hybridization experiments using total DNA of *R.leguminosarum*, did not yield any signal (see last progress report).

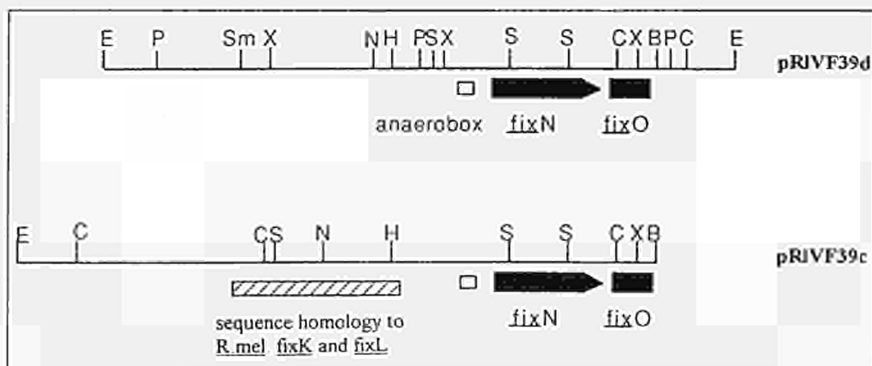

Figure 2: Physical and genetic map of the two fixN copies in R.leguminosarum

The FixK homologue shows about 32% homology to RmFixK and does not contain an N-terminal cystein motif, in contrast to FnrN. The second gene identified shows

significant homology to RmFixL, especially in the central heme binding domain and the C-terminal part of the protein. Mutational analyses are currently being carried out in order to show whether these genes are functional in symbiosis and whether/how they are involved in the oxygen control of nitrogen fixation genes in *R. leguminosarum*.

2. Carbon metabolism and nitrogen fixation (objective 3 , Cork)

The energy required for nitrogen fixation is provided to the bacteroids in the form of dicarboxylic acids (dcAs) as evidenced by the fact that mutants deficient in sugar utilization are Fix^+ whereas mutants defective in the dcAs transport gene (*dctA*) are Fix^-. Recent data indicate that the regulatory genes (*dctBD*) that control *dctA* expression in bacterial cultures do not operate in bacteroids. This pointed to the existence of an alternative symbiotic activator (ASA). As a strategy to identify ASA, we selected *R. meliloti dctD* secondary mutants that were capable of growth on dcAs as sole carbon source. Seven such mutant strains (called DAM) were selected that could be divided into two separate groups depending on their pattern of *dctA* expression. Five of these mutants constitutively express the *dctA* gene . These mutants also constitutively activate a *nifH-lacZ* gene fusion. Two DAM mutant strains were found to be inducible by dcAs. Further characterization of these mutants is currently in progress.

We previously reported a repressing effect of some *dctB* mutations on *R. meliloti nifHDK* expression. A tentative explanation could be a repressing effect of the transcriptional activator DctD in the absence of its cognate regulator DctB. Such a repressing role of DctD is supported by the following experiments. The full-length *dctD* gene has been cloned downstream of the enteric lac promoter so that *dctD* is now constitutively expressed in *E. coli* as well as in *Rhizobium* spp. We found that constitutively expressed DctD leads to the repression of the *dctA* promoter and thereby prevents the growth of *R. meliloti* on media containing dcAs as a sole carbon source. It also inhibits constitutive expression of the *dctA* gene in the *dctA* and DAM mutant strains.

The reconstituted *dct* system in *E. coli* was found to undergo regulatory effects by carbon and nitrogen sources. These effects are mediated by the CRP-cAMP complex and the NtrBC system respectively. The repressive effect on *dctA* promoter has been fully characterized and was found to be caused by competitive binding of the CRP-cAMP complex to the upstream activator (DctD) binding sites (Wang et al. 1993). The nitrogen effects are mediated by the NtrBC system and involve (a) alternative activation of the *dctA* promoter by wild-type NtrC and (b) apparent cross-talk effects of NtrB on DctD. Whether these regulations operate in *R. meliloti* remains to be determined.

3. Nitrogen metabolism and nitrogen fixation

The relationship between nitrogen fixation and nitrogen metabolism, although crucial, remains an open question. Answering it requires to evaluate the role in symbiosis of bacterial glutamine synthetases (GS) and that of the general nitrogen regulators NtrBC, to characterize the mechanisms that control $NH3/NH4^+$ export to the plant cell and to investigate whether/how the plant nitrogen status influences bacteroid nitrogen fixation activity.

3.1 Bacterial nitrogen metabolism (Objectives 4 and 5; Naples, Dublin)

R. leguminosarum possesses three genes (*glnA, glnII* and *glnT*) coding for three different GS (GSI, GSII and GSIII).

We have purified GSIII from *R. leguminosarum* biovar phaseoli and sequenced the corresponding *glnT* locus (Chiurazzi *et al.* 1992). The sequence of *glnT* reveals an open reading frame of 435 amino acids, whose first eight amino acids are identical to those determined from pure GSIII by direct sequencing, thus confirming that *glnT* indeed codes for GSIII activity. The comparison of GSIII amino acid sequence with the reported sequence of GS's from other organisms shows a sig nificant degree of homology.

GSII was purified to homogeneity from *R. leguminosarum* and characterized. The sequence of the first 26 amino acids residues showed a high similarity with the sequence of GSII from *Bradyrhizobium japonicum* or *R. meliloti*. Non-denaturing PAGE showed that GSII, either in crude extracts or in the pure state, was a mixture of an octamer and a tetramer and that, under specific conditions, the octamer/tetramer ratio could be modified in either direction. Addition of NH_4Cl to a bacterial culture derepressed for GSII caused a specific decrease in transferase activity, not paralleled by a decrease in immunoreactive material or biosynthetic activity which strongly suggests a post-translational modification.

The DNA region of *R. leguminosarum* coding for GSII was cloned and sequenced. An open reading frame encoding 326 amino acids was defined as the *glnII* gene on the basis of its similarity to other *glnII* genes and its ability to complement the glutamine auxotrophy of a *Klebsiella pneumoniae glnA* mutant. Genetic analysis of transcriptional control showed that the *glnII* promoter is dependent on positive control by NtrC protein and an upstream activator sequence (UAS). Accordingly a Tn5 insertion mutant, strain CFN2012, of *R. leguminosarum* devoid of GSII activity was shown to contain the Tn5 insertion in *ntrC*. Further analysis of strain CFN2012 indicated that this mutant has reduced levels of the PII regulatory protein and, in contrast to *ntrC* mutants of other Rhizobiaceae , grows on nitrate as sole nitrogen source.

We reported the DNA sequence of *ntrB* and its regulatory region in *R. leguminosarum* bv. *phaseoli* (Patriarca et al. 1993). The *ntrB* gene is cotranscribed with *ntrC*. The *ntrBC* promoter region was defined using *lacZ* fusion. The *in vivo* transcription initiation sites of the *ntrB* gene were mapped at -998 and -955 bp upstream of the putative *ntrB* start codon, both preceded by *E. coli* -10-35 sequences. Major aspects differentiate the *R.l. phaseoli* from the enteric nitrogen regulatory system: *ntrBC* genes are unlinked to *glnA*, are not transcribed from a -12/-24 promoter, show only negative autoregulation and different nitrogen sources do not affect the NtrC concentration.

The other relevant aspect of nitrogen metabolism that has been investigated concerns the characterization of the mechanisms that allow NH3/ $NH4^+$ transport across *R. meliloti* membrane.

When *R. meliloti* is grown in a low nitrogen medium (0.5 mM NH_4Cl) the expression of an ammonium uptake system can be detected by incorporation of ^{14}C methylammonium. We conclusively demonstrated that this ammonium uptake is an active, energy requiring system. We have previously identified outer membrane proteins, the appearance of which correlates with the onset of ammonium uptake. In an effort to isolate the corresponding genes for mutant construction, amino acid sequences of these proteins are currently sought.

The rate of methylammonium uptake was compared using different nitrogen sources. Uptake was not detected when NH_4Cl and glutamate at concentrations of 20mM were used. Uptake was detected in the presence of NH_4Cl and glutamate

at 0.5mM and KNO_3 at 20mM. The rate of uptake differed, however, when KNO_3 was the nitrogen source, being more rapid than when the other two nitrogen sources were used. The different uptake rates suggest that different uptake systems may operate.

One of the objectives of the investigation of ammonium uptake is to determine whether the system(s) that operate have any relationship to the export of fixed nitrogen from bacteroids. The isolation of uptake mutants and their testing for altered export efficiencies is a possible approach to determine the involvment of an uptake system in the reverse process of nitrogen export. To facilitate the investigation of ammonium export an assay has been developed based on the use of histidine as nitrogen source. NH3 is released in the degradation of histidine. Cells were grown in urea agar base with the inclusion of different nitrogen sources in place of urea. The presence of an indicator allows the detection of alkaline conditions resulting from NH3 production. In the presence of histidine the plate turns pink when growth of *R. meliloti* 2011 occurs. The effect is not observed in the presence of glutamate, KNO_3 or NH_4Cl. This assay may also be useful in the identification of any changes in the cell membrane that are associated with $NH3/NH4^+$ export.

3.2 Plant nitrogen metabolism and nitrogen fixation (Objective 6; Toulouse)

In order to alter the nitrogen metabolism in the nodule, experiments have been targeted to the plant glutamine synthetase (GS), which is responsible for assimilating the ammonium produced by dinitrogen fixation. An antisense RNA approach has been used in an attempt to reduce the activity of this enzyme within the nodules of transgenic *Medicago* plants. Such alterations should lead to an increase in ammonium concentration in the nodule and a decrease in glutamine. Using the leghaemoglobin promoter (to specifically alter the enzyme level only in the infected cells within the nodule), antisense RNA constructs were prepared to *MtGSa* (formally Mtgs16), the GS gene most highly expressed in *M. truncatula* nodules. However, analysis of transgenic plants containing this construct revealed no alteration in the total GS activity in the nodules nor in the pattern of GS isoenzymes. Although this first experiment has not been successful in altering the nitrogen metabolism, the plant GS genes isolated in this project and the knowledge obtained on the regulation of the plant enzyme, will be useful in further attempts to manipulate the nodule's nitrogen metabolism.

HIGHLIGHTS/MILESTONES

(1) Identification of *R. meliloti fixK* and *fixL* homologs in *R.leguminosarum*. Although their role remains to be demonstrated, this is likely a major advance in the knowledge of the regulation of nitrogen fixation genes in *R. leguminosarum*.

(2) *In vitro* reconstitution of oxygen regulated expression of *Rm nifA* and *fixK* genes. To our knowledge this is the first example where a complete signal transduction pathway of this sort could be reconstitued in vitro.

(3) The CRP-cAMP complex has a repressive effect on a *rpoN*-dependent promoter *(dctA)*. It exerts its effect by outcompeting DctD for binding to the upstream activating sequences. This is the first report of such a role for CRP in regulation of sigma 54-dependent promoters.
Points 2 and 3 illustrate the general interest of these studies.

WIDER CONSIDERATIONS

Symbiotic nitrogen fixation has emerged as a major model system in the study of plant-microbe interactions as well as in plant development.

In addition to these basic aspects, symbiotic nitrogen fixation is a matter of renewed interest because of environmental considerations, the need to reduce inputs and,in some cases, promote a more extensive agriculture.

For these reasons as well as the significant economic importance of inoculant production and the new technology of coated seeds, *Rhizobium* research should be supported as a long term project by the European Community.

COOPERATIVE ACTIVITIES

(1) The effect of the overexpressed *dctD* gene on the symbiotic efficiency of *R. meliloti* strains constructed by the Cork group is being evaluated by the Agrifutur company.

(2) Genetic materials have been exchanged particularly between Cork, Bielefeld and Toulouse.

(3) Dr D. Jörding visited Cork for a three-week period in July 1992; Dr D. Allaway visited Bielefeld for a three-week period in September 1992.

EUROPEAN DIMENSION

Symbiotic nitrogen fixation is a complex biological process whose understanding requires a long-term cooperation between laboratories addressing different, although interconnected, biological questions. The BRIDGE program allowed us to set such a cooperation which is, to our knowledge, unique in Europe as well as overseas. It contributes to the leading position of Europe in this field.

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Tomato transposon tagging: Isolation of genes involved in disease resistance, hormone biosynthesis and plant cell development (BIOT CT-900192)

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OBJECTIVES

The joint project is to develop a tomato transposon tagging system in order to enable each group to clone different genes. To this end it is aimed to jointly obtain a series of plants each carrying a transposable element insertion (either Ac or a derivative) in different positions that are equally spaced over the twelve chromosomes of tomato and to demonstrate the feasibility of this series for cloning specific genes by targeted transposon tagging. Within the second year (months 16 - 28) it was aimed (1) to map T-DNA and transposon positions in a RFLP analysis, (2) to analyze Ac/Ds transposition behaviour and (3) to initiate targeted and untargeted transposon tagging experiments.

MAJOR PROBLEMS ENCOUNTERED

In the beginning of the report period the inverted polymerase chain reaction (IPCR) technique, to obtain T-DNA or transposon flanking plant DNA for RFLP analysis, showed to be problematic in several labs. In order to improve the IPCR technique several parameters were tested again. The choice of primers and the composition of the ligation buffer turned out to be very important parameters for reliable results. Furthermore, a new procedure was developed, called supported PCR, that also avoids the problems associated with the IPCR procedure.

RESULTS

All groups have introduced transposon containing T-DNA vectors into tomato by *Agrobacterium* mediated gene transfer. Both the autonomous Ac-element and non-autonomous Ac-derivatives (Ds) have been positioned in various antibiotic resistance genes to permit selection for excision. Reintegration of Ds after excision is indicated by different marker genes inserted into the element itself. Alltogether, more than 100 locations for transposable elements have been established in tomato. On all the twelve different chromosomes of tomato inserts have been mapped.

Typical germinal excision frequencies are usually around 6 - 8%. A number of Ds containing tomato lines have been crossed to Ac lines in order to transactivate the Ds elements. Both excision of Ds from the original position and integration on new chromosomal locations could be established. For five T-DNA loci Ds transposition is mainly to closely linked sites (60-80%), however, for one T-DNA locus Ds transposition was inter-chromosomally. In order to increase the efficiency of transposon tagging a transposition / deletion system is being developed. This system extends the possibilities of insertional mutagenesis with deletion mutagenesis. Deletions

are induced by making use of a site specific recombination system (Cre/*lox*) that has been coupled to the transposition system. In this way larger regions of the genome can be screened at once for the presence of the gene of interest. For transactivation both the autonomous Ac element, stabilized Ac elements and stabilized Ac elements in which the endogenous promoter has been replaced by different tissue specific promoters, are being used.

The fact that we have tomato lines where the primary insertion site is linked to our genes of interest means that we are able to begin transposon mutagenesis experiments. First experiments, both untargeted and targeted, have been initiated in all groups.

Amsterdam

The fungus *Alternaria alternata* f. sp. *lycopersici* produces AAL toxins that are involved in the pathogenesis of the Alternaria stem canker disease in susceptible tomato cultivars. The host range of the fungus and its host selective AAL toxins is assumed to be restricted to *L. esculentum* cultivars where the disease is controlled by the *Asc*-locus positioned on chromosome 3. However, a screen within the genus *Lycopersicon* showed that species display differential responses towards AAL toxins and towards the fungus. *L. pennellii* proved to be highly insensitive to these toxins. Genetic analysis and RFLP mapping of the interspecific hybrid showed that high insensitivity to AAL toxins from *L. pennellii* is inherited as a single dominant locus allelic to the *Asc*-locus on chromosome 3.

In a targeted transposon tagging experiment a transgenic tomato line, homozygous for the *Asc* locus and a Ds element on chromosome 3 was crossed with a line also homozygous for *Asc* and containing Ac on chromosome 3. The resulting progeny in which, both, Ac and Ds can transpose, and the genotype that is homozygous for Ac and Asc, were crossed on a large scale with a sensitive male sterile tomato line (*asc, asc*). These seed populations, heterozygous for the *Asc* locus are analyzed for Alternaria susceptible mutants in order to screen for transposon induced mutations at Asc. Out of 15,000 seeds screened a number of Alternaria susceptible seedlings have been obtained that are now being further characterized.

Cologne

With the goal to isolate genes which play a role in initiation and / or function of apical and lateral meristems, transposon tagging experiments have been initiated towards the genes 'Lanceolate' (*La*) and 'lateral suppressor' (*ls*). A Ds element located on chromosome 7 and linked to both genes of interest has been activated by a cross to a line harbouring a stabilized Ac element. Progeny plants containing Ds and Ac have then been crossed to a line homozygous for the *ls* mutation. From these crosses about 100,000 seeds have been obtained which are presently being screened for *ls* and *La* mutants. Putative *La* mutants can probably be identified in the progeny resulting from this cross, because all four *La* alleles isolated so far show a dominant leaf phenotype.

In addition, untargeted transposon tagging experiments were started with the goal of isolating new developmental mutants. For this purpose several Ds containing lines were crossed by a line harbouring a stabilized Ac element. After a self pollination of F1 plants harbouring Ds and Ac the resulting F2 plants are presently being selected for transposed Ds elements. This experiment will enable us to screen about 1000 F3 families for new mutants.

Nottingham

The ultimate aim is to obtain transposon induced wilty mutants as a result of insertion into genes encoding enzymes involved in biosynthesis of the plant hormone, ABA. Transposon containing T-DNA's have been located linked to the target genes *sitiens* (chromosome 1) and also *notabilis* and *flacca* (chromosome 7). The T-DNA linked to *sitiens* is also closely linked to *aurea*, which is a marker easy to screen for due to its yellow and tall phytochrome deficient phenotype. In a targeted transposon tagging experiment plants homozygous for this T-DNA were crossed with an *aurea* mutant. The progeny of this cross were screened for transposon induced *aurea* mutants. From 30,000 seeds screened 2 *aurea* mutants were generated. One of these was a pure stable *aurea* plant the other showed a variegated phenotype. Eight IPCR fragments have been generated from both ends of the Ac copies in these two plants. If it can be confirmed that there is an Ac copy in *aurea*, it is ideally suited to allow transposon tagging of the adjacent ABA biosynthetic gene at the *sitiens* locus.

Norwich

Large scale non targeted transposon tagging experiments were carried out. Over 150 transposed Dss from a location on chromosome 4 were obtained and then selfed. One mutation arose that affects embryo development and meristem function. There are indications that this mutation is due to insertion of Ds and steps to isolate the corresponding gene are in progress. Using a line carrying Ac on chromosome 2 it was shown that this T-DNA is very closely linked to the gene dwarf *(d)*. The Ac carrying line was crossed to the dwarf line and three independent dwarf mutations were obtained. At least one revertant has been obtained in self progeny. However, cloning the gene may not be so straightforward as the T-DNA probably went in as an inverted repeat and this structure could conceivably give rise to inversions and chromosome breakage events.

A major objective is to use transposon tagging to isolate the tomato *Cf-9* gene that specifies resistance to specific races of the leaf mould pathogen, *Cladosporium fulvum*. A line was identified that carries a T-DNA (Ft 33) at a location linked to *Cf-9* and this has been back crossed into a *Cf-9* stock. Recombinants have been identified with Ft 33 in cis with *Cf-9*. This line has been crossed to a line homozygous for *Cf-9* and carrying an active stabilized Ac (sAc) to transactivate the Ds. Thus a line is available that can be crossed to *Cf-0* to look for mutations to disease sensitivity that have lost *Cf-9* function.

It was recently shown that Cf-0 plants can be engineered to express a fungally encoded avirulence peptide (*avr9*) that is recognized by *Cf-9*. When such hemizygous plants are crossed to *Cf-9* containing plants, 50% of the progeny develop a constitutive necrosis and die. Presumably, crossing a homozygous line for *avr9* to *Cf-9* would result in complete lethality. This will be used to select for Ds induced mutations in *Cf-9*, in the progeny that arise when a 35S avr9 homozygote is crossed to a line that is homozygous for *Cf-9*, heterozygous for Ft33 and heterozygous for sAc.

HIGHLIGHTS / MILESTONES

A set of tomato genotypes has been obtained with mapped transposon inserts. Stocks are available with a transposable element for each tomato chromosome.

Ds transposition is predominantly to closely linked sites in tomato.

First transposon tagging experiments in tomato have been carried out, both using targeted and non targeted transposon tagging approaches. A number of mutants has been obtained and awaits further characterization.

WIDER CONSIDERATIONS

Plants contain a large number of interesting and important genes known only by the phenotype they confer and their map position. In tomato, these include genes that affect disease resistance, fruit ripening and growth habit. Extracting such genetically defined genes from plant genomes is difficult. The two major technologies are transposon tagging and map based cloning. It is not clear yet which will prove most generally useful; both will show some successes. The BRIDGE funding to this group has established the European effort in tomato transposon tagging as more systematic, more serious and more succesful. We are now positioned to use this technology to isolate interesting tomato genes, both by non-targeted tagging and targeted tagging.

COOPERATIVE ACTIVITIES

The annual meeting exchanging the results of the participants obtained so far, was held for the fourth time, now in Amsterdam,from 17-18 december 1992.
All groups have exchanged on a very regular basis both information, plasmid constructs and mapped tomato genotypes.

EUROPEAN DIMENSION

The main advantages of operating within a European collaboration are that (1) we all know what each other is doing (2) where appropriate we can help each other or ask each other for help and (3) we have annual meetings so people in our labs can get to know each other.

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Identification of regulatory genes controlling major metabolic pathways (BIOT CT-900164)

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OBJECTIVES

The objective of this BRIDGE programme is to identify genes regulating metabolic pathways of nitrate assimilation and anthocyanin biosynthesis and to isolate and characterize clones of transcription factors involved in these processes.

RESULTS

Regulation of the nitrate assimilatory pathway

The regulation of nitrate assimilation has been studied by genetic, physiological and molecular approaches.

1) Mutant identification:

At Rothamsted, conditions have been established for the seletion of constitutive nitrate reductase-producing mutants of barley. In an attempt to identify genes regulating nitrate reductase expression, mutant plants are being selected which have significant NR activity in the absence of nitrate. Azide mutagenised barley seed (M1 generation) were grown in the field and the M2 seeds harvested. This M2 seed have been grown under nitrate-free selection conditions. Very great care is needed to produce truly nitrate-free growth conditions. The protocol involves washing of M2 seed in a flow of demineralised water for 24 hours. Sixty-well trays are used for germinations, with one seed per well — this reduces the possibility of contamination from microbial growth on non-viable seedlings. A nitrogen-free nutrient medium is used to water the plants, and at intervals the Perlite is flushed with demineralised water, again to reduce contamination. At first selection was carried out on the first or young second leaves, but inconsistent results proved to be due to slight contamination with nitrate. Better results are obtained by using the upper half of mature second leaves, when the seedlings are old enough to have used up all their seed nitrogen stores. Leaves from batches of five plants are harvested, swabbed with ethanol, and sliced. These slices are placed in 0.5 ml of phosphate-bufferred KN03 solution, vacuum infiltrated, and a little mineral oil placed on the surface. After incubation in the dark for 30 mn at 20°C, the reaction is stopped by placing the tubes in boiling water for 5 min, then on ice to cool. Nitrite accumulation is revealed by colorimetry. Any group of five plants showing activity above the background are retested individually, and any individual with greater than 5 nmol NO_2/hr/slice is replanted in compost and grown on to produce selfed M3 seeds. To date 11,750 individual plants have been tested, and 66 of these

selected for further study. One plant had an activity of > 50 nmol/hr/slice, but unfortunately it did not survive when potted into compost. Another, which on testing had an activity of 8 nmol/hr/slice produced a few seed, only two of which germinated. These plants had less activity than the parent, but still above background levels. They are being grown on to the M4 generation. The selection system as originally devised has now been shown to work successfully. Potential mutants are appearing with an encouraging frequency,low enough to suggest that 'false positives' are rare yet high enough to indicate that high NR mutants can be found. The great variation in activity of the plants selected at M2 is an encouraging sign that we may be able to detect lines with considerable variation in the extent of NR deregulation. This will help in identifying the genes responsible and examining their function.

2) Physiological analysis of consequences of a deregulated nitrate assimilation.

Nitrate reductase expression is highly integrated in the metabolic activity of higher plants. Nitrate reductase, the first step of the pathway, is regulated by light, nitrate, and N-metabolites. Tobacco plants in which one of the expressed nitrate reductases is constitutively expressed have been obtained by transformation of a nitrate reductase cDNA put under the control of transcription signals derived from the CaMV. Transgenic industrial tobaccos (White burley and PBD6 cultivars) obtained at INRA-Versailles were evaluated for their physiological characteristics by SEITA-Bergerac.The first field trial of transgenic tobacco with modified nitrate reductase activity was made in 1992 with the agreement of the French Biochemolecular engineering committee (CGB) and following the AFNOR standard (X 42 071) 'Guide of good practices in research and field testing of transgenic plants'. Compared to the non transgenic tobacco, studies were conducted to evaluate:
— Growth and agronomic characteristics
— Yield, grade index
— Chemical characteristics:

On green leaves: Nitrate reductase activity

On lyophilised leaves: Sugars, alkaloïds (nicotine and nornicotine), nitrate, nitrite, total nitrogen, organic acids, protein content.

For the transgenic tobacco expressing the coding sequence for NR from *N. sylvestris* (constuct pBCSL 16), the main results were:
— No significant leaf morphological differences.
— An earlier flowering (approx. 6 days) accompagnied with a decrease of number of leaves.
— A decrease of nitrate content (approx. 45 % in the top leaves and 15 % in the bottom leaves). This decrease of nitrate content is more important in the midrib where nitrate is generally accumulated in the non transgenic tobacco.
— An increase of total nitrogen, alkaloïd (nicotine) and of nitrite content. Accumulation of nitrite remains very low and under the toxicity level for the tobacco. These data are promising in view of controlling nitrate accumulation in vegetative tissues, a source of concern not only for tobacco, but also for spinach and lettuce. The trial will be reconducted in 1993 to check the influence of different nitrogen fertilization conditions.

3) Cloning of regulatory genes:

In higher plants as well as in fungi, nitrate metabolism is regulated at the transcription level by the pool of accumulated N-metabolites (mainly glutamine) derived from the assimilation of nitrate or other nitrogen sources. In the fungus

Neurospora crassa , *nit-2* is the major nitrogen regulatory gene involved in this regulation. The nit-2 gene encodes a regulatory protein containing a single zinc finger motif defined by the $C-X_2-C-X_{17}-C-X_2-C$ sequence. This DNA-binding domain recognizes promoter region of *N. crassa* nitrogen-related genes and fragments derived from the tomato *nia* gene promoter. The observed specificity of the binding suggests the existence of a NIT2-like homolog in higher plants. PCR approach and cross-hybridization techniques were used to isolate respectively a partial cDNA from *N. plumbaginifolia* and a full length cDNA from *N. tabacum*. These clones encode a NIT2-like protein (named NTL1 for nit-2 like), characterized by a single zinc finger domain, defined by the $C-X_2-C-X_{18}-C-X_2-C$ amino acids and associated with a basic region. The amino acid sequence of NTL1 is 60% homologous to the NIT2 sequence in the zinc finger domain. The *Ntl1* gene is present as a unique copy in the diploid *N. plumbaginifolia* species. The characteristics of the *Ntl1* gene expression are compatible with that of a regulator of the nitrate assimilation pathway, namely a faint nitrate inducibility and a regulation by light. Experiments are under way to characterize the properties of the NTL1 protein as regards DNA binding specificity.

Transcription factors involved in light regulation of plant genes

Two lines of research have been pursued in Roma:

1) *Characterization of light-regulated transcription factors binding the I box of tomato light regulated genes:*

The I box is a conserved regulatory motif which is found upstream of plant genes whose transcription is under light and circadian control (*rbcS, cab* and *nia*). Utilizing gel retardation and UV crosslinking assays two different groups of I box binding activities were detected in tomato nuclear extracts: the first group (IBF-1), comprises the IBF-1a and IBF-1b activities, recognizing the I box of the light-responsive rbcS gene ; IBF-1a also recognizes the previously described G box. The second group (IBF-2), comprises the IBF-2a and IBF-2b activities, recognizing the I box of the light and circadian clock-responsive *nia* gene: IBF-2a also recognizes the I box of a second gene showing similar regulation, the *cab* gene. Each

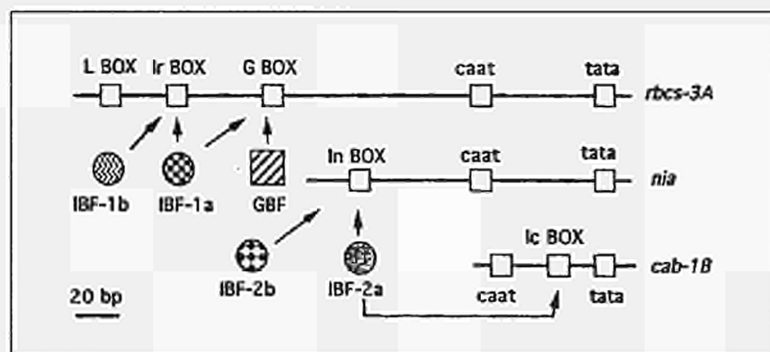

Fig. 1. Light regulated transcription factors binding to three tomato genes regulated by light

activity shows a unique combination of biochemical features and sequence-specificities. Judged by UV-crosslinking experiments, the IBF-1b and IBF-2a complexes contain a protein with an apparent molecular weight of 33 kDa.

The nucleotides recognized by the various IBF activities were determined by systematic mutagenesis. To the limit of experimental resolution, IBF-1a and GBF compete for the same nucleotides on the G box. Nevertheless, these two activities are biochemically and immunologically distinct. A different situation is found for IBF-2a and IBF-2b, which compete for binding on the *nia* I box, although they recognize different nucleotides on this sequence. The IBF-2a binding sites on the *cab* and *nia* promoters show extensive homology to a wheat circadian clock-responsive element. When studied in different light-dark regimes, IBF-1b and IBF-2b show constitutive expression, while IBF-1a levels decrease after prolonged exposure to the dark. IBF-2a is diurnally regulated and shows a dramatic induction around the onset of the light period. Transfer of the plants in continuous darkness demonstrates that this induction is under the control of a circadian clock. Interestingly *Cab* and *Nia* genes are also under the control of such a circadian clock.

2) Study of the light-regulated of carotenoid biosynthesis genes.

Transcript levels for the tomato carotenoid biosynthesis genes *PDS* and *PDY*, encoding phytoene desaturase and phytoene synthase, have been studied during de-etiolation (when carotenoid levels increase 4-fold), dark adaptation, and photooxidative stress (induced by the herbicide norflurazon). None of the two mRNAs is significantly induced during de-etiolation, while PSY mRNA decreases 10-fold during dark adaptation. Both transcripts, finally are induced during photooxidative stress. Previously described light-regulated genes (rbcS, rab, *nia*, CHS) are regulated both during de-etiolation and during dark adaptation, and are repressed by photooxidative stress. According to these data, *PSY* belongs to a novel class of light-regulated genes.

Analysis of the regulation of the anthocyan biosynthesis pathway in Antirrhinum majus

At the John Innes, the characterisation of regulatory mutants affecting anthocyanin production in *Antirrhinum majus* has been performed.

1. Characterization of regulatory mutants affecting anthocyanin production in Antirrhinum majus

(a) The *mixta* mutation affects the intensity of anthocyanin produced in the flowers. Since this mutant was somatically and germinally unstable it appeared to be due to a transposable element insertion. Hybridisation of genomic cDNA from mutant and wild-type lines to a probe of the *Tam-4* transposon revealed one particular DNA fragment present only in the mutant lines indicating a novel insertion of *Tam-4*. This fragment was cloned using *Tam-4* as a probe. Probing DNA from *mixta* plants and from independant wild-type revertants with the sequences flanking the transposon revealed that this *Tam-4* insertion was at the *mixta* locus. The flanking sequences were used to identify a genomic clone of 15 kb. Probes made from fragments of DNA from the genomic clone around the site where *Tam-4* is inserted in *mixta* lines, revealed a 1.4 kb transcript which was absent in *mixta* lines. Two cDNA clones, homologous to these DNA fragments were isolated from a cDNA library made from mRNA from petal lobes. The cDNA clones appeared to be full length and sequencing revealed that the *mixta* gene encodes a *myb*-related transcription factor. Thus,

mixta is the first example of a *myb*-related transcription factor that regulates flavonoid biosynthesis in dicotyledonous flowers.

(b) Unstable lines for another regulatory locus, *Eluta* have been obtained. Germinal revertants will be submitted to a large-scale screening programme this summer to determine whether it will be possible to isolate *Eluta* by transposon tagging.

2. Characterisation of promoter sequences of anthocyanin biosynthetic genes.

Extensive allelic series of mutations of the *CHS* gene of A. majus have been obtained by *in vivo* mutagenesis using the transposon *Tam 3*, which is inserted 63 bp upstream of the start of transcription in the *CHS* gene promoter. These mutations of the *CHS* promoter define regulatory *cis*-acting motifs. One region, -63 to -326 bp, is required for *CHS* expression in the upper tube of the flower. It also carries motifs that affect the degree of expression from the *CHS* promoter. Deletion of sequences further upstream has no effect on *CHS* expression. These cis - acting sequences need to be close to the first 63 bp of the *CHS* promoter (including the TATA box) to function. Displacement, by an unrelated insertion of 600 bp, reduces *CHS* expression, and blocks it completely in the upper tube. Reduction of this insertion to 80 bp restores the *CHS* expression in the upper tube although the level of expression is still reduced. This suggests that the spatial and quantitative signals within the 263 bp *cis*-acting region are distinct, with individual requirements for their spatial relationship to the TATA-binding transcriptional machinery. At present systems are being established to determine when and where different transcription factors regulate *CHS* expression.

3. Effects of altering expression of myb genes in transgenic plants.

A single acyanic tobacco plant was obtained using an antisense constructs of the *Anthirrhinum myb* 340 gene. While there was reasonably good evidence that in this plant there was an inhibition of anthocyanin regulation, since the later biosynthetic genes *F3H* and *DFR* were not expressed, no reduction in expression of a tobacco *myb* gene could be detected despite the isolation of three different cDNAs from tobacco flowers homologous to *Antirrhinum myb* 340. Now that an *myb* gene regulating anthocyanin production has been identified in *Antirrhinum* flowers (*mixta*), homologues will be isolated from tobacco and tested for their expression in the acyanic plant. Over expression of *myb* 308 results in a novel phenotype in transgenic tobacco, with pale leaves and greatly retarded growth rates. Leaves fail to develop normally and at maturity develop necrotic lesions due to isolated cell death. This phenotype is dominant and fully heritable. The leaf palisade fails to develop properly resulting in paler leaves. A reduction in photosynthetic potential may be the cause of the reduced growth rates. We are, at present, attempting to characterize the target genes of *myb* 308 to identify the metabolic process it controls.

4. Isolation of target genes for myb related transcription factors.

Target genes for transcription factors are being identified by two methods. Differential cDNA cloning will be used where alterations in expression of transgenic *myb* genes have resulted in clear phenotypic changes. A system in yeast using the lacZ reporter gene has been developped. The transcription factor gene can be expressed following induction with galactose. There will be expression of β-galatosidase from the lacZ with a minimal yeast promoter if the *myb* binding site is lying just upstream of the TATA box. Using this system with *myb* 305 and its cognate binding site, TAACGG, the expression of the transcription factor gives rise

to blue yeast colonies. This screen will be used for cloning fragments of target genes in yeast.

5. bZIP Transcription factors

Two genes encoding bZip transcription factors which are expressed in *Antirrhinum* flowers have been isolated. They are also expressed in other organs. They can bind to the *CHS* promoter by gel shift experiments with protein made by *in vitro* translation. It is planned to determine if and when they regulate *CHS* expression.

Identification and analysis of myb -related genes from tomato.

The work undertaken by AGC has been focused in the isolation of *myb*-related genes from the tomato, *Lycopersicon esculentum* using the *Antirrhinum myb*-related clones isolated at the JICPSR as probes.

a) Cloning myb-related cDNA clones

During the first year of the BRIDGE programme, a cDNA library was made from *Lycopersicon esculentum* cv Ailsa Craig tomato hypocotyl mRNA.Twenty-one *myb*-related clones were isolated using a mixture of degenerate oligonucleotides as probes, the sequences of which were similar to the oligos used by JICPSR. Of the 21 clones, 20 were different. Since northern blot analysis using total RNA did not give clear patterns, the analysis was repeated using polyA$^+$ RNA. Seven types of tissue were collected from Ailsa Craig plants including hypocotyl,leaf, root, green and red fruits, immature and mature flowers. As shown in the Northern blots (Figure 3), a number of the clones hybridized to multiple transcripts, some of which are tissue-specific. Other probes hybridized to double or triple transcripts, whereas some hybridized to only one transcript. The sizes of bands detected ranged from 0.4 to 5 kb in length. The multiple transcripts detected are unlikely to be due to the conserved *myb*-repeats included in the probes as other probes which also contain the repeats only hybridized to one band. The multiple transcripts could result from expression from different promoters, termination at different polyadenylation sites or the use of alternative splice sites.

b) Sequence analysis:

Having studied the expression patterns of the clones, three showing different expression patterns (pTHMO1, pTHM18 and pTHM27) were fully sequenced in both directions. The *myb* 51-53 imperfect repeats are found in all three clones confirming that the clones are indeed derived from *myb*-related genes. Clone pTHM27 is similar to the *Antirrhinum myb* gene 308 (AM308) in that the first 22 amino acid residues of pTHM27 are identical. Two conserved regions outside the *myb* repeats have also been found in both clones. The first region is GIDPTTHRSIND, which is identical in both clones, and the related sequence GIDPXXH is also found in two other *Antirrhinum* clones (AM315 and AM330) and in *myb* genes from barley (Hv1 and Hv33) and from maize (Zm38). The second region, CPDLNLDLKISPP (AM308) is similar to CLPDLNLELRISPP (pTHM27) and the related sequence CPDLNLDLXISPP is also found in *myb* genes Hv1 and Zm38.

c) Antisense constructs

Antisense constructs were made from pTHMO1, pTHM18 and pTHM27 using the variable regions of the clones. To make antisense construct from pTHM01, a 531 bp fragment was cloned between the CaMV 35S promoter and NOS terminator carried by a pUC-based vector. The antisense cassette was cloned into a pRK290

based plant transformation vector containing GUS and NPTII genes to give pATM012. To make the antisense construct from pTHM18, a fragment of 303 bp covering the sequence from 377 to 680 bp was cloned into the vectors as above to yield pATM182. Similarly for the pTHM27 antisense construct, a fragment of 466 bp (nucleotides 569 to 1035) was cloned into the vectors as above to give pATM272. To investigate the effects of the antisense constructs described above, *Agrobacterium* mediated plant transformations have been carried out using seedling cotyledons, hypocotyl sections and stem segments of tomato c.v. Ailsa Craig. A total of 1182 explants have been inoculated with pATMO12, 1194 with pATM182 and 1179 with pATM272. Shoots have started to emerge from the inoculated explants. Transformants will be characterized next year.

Regulation of anthocyan biosynthesis in *Petunia*

In Madrid, *Petunia* has been used as a source of material to identify and characterize *myb*genes presumed to be involved in the control of anthocyan biosynthesis. DNA binding studies have been performed to establish whether or not the consensus binding sequence is the same for different *myb*Ph proteins.

a) DNA-binding studies:

Using a binding site selection technique from a mixture of random oligonucleotides, it was shown previously that *myb.* Ph3 binds the sequence CACGTGTAG. During this year, further rounds of selection have allowed to establish a more optimal binding sequence for *myb.* Ph3: AaaCAGCGTTA. Hydroxy-radical footprinting (the missing nucleoside technique) has demonstrated the importance of this sequence in binding by *myb.* Ph3 protein. This more tightly defined sequence is not so well recognized by other *Petunia myb* proteins (*myb.* Ph1 and *myb.* Ph2), indicating that different *myb* proteins have non identical, although similar, DNA-binding properties. Work is in progress to evaluate the effect of changing each nucleotide of the *myb.* Ph3 optimal binding sequence in binding by *myb.* Ph3 as well as by *myb. Ph1* and *myb.* Ph2. The above defined *myb.* Ph3 optimal sequence is an imperfect palindrom, a class of sequences usually bound by dimers. Binding studies with co-translated wild type and truncated versions of *myb.* Ph3 protein have shown that this protein binds as a monomer like its animal counterparts c-*myb* and *v-myb*. The imperfect palindromic structure of *myb.* Ph3 DNA binding site suggests that the DNA-binding domain of *myb* proteins has a double domain. Genetic and molecular evidence from other laboratories suggest a functional interaction between *myb-* and *myc*-type proteins in plants (i.e between the maize C1 (*myb*) and R (*myc*) proteins and possibly between the *Arabidopsis* GL1 (*myb*) and TTG (*myc*) proteins). However, binding studies with co-translated *myb.* Ph3 and the *Antirrhinum* DELILA *myc* protein did not provide evidence of any physical interaction between these two proteins.

b) Transient expression studies:

The effect in transcription of *myb.* Ph3 is being studied in transient expression experiments. Two *myb.* Ph3 binding sites have been fused to the chimaeric -90 minimal 35S promoter: GUS genes. Preliminary results with cotransfection of this reporter gene and *myb.* Ph3 cDNA (driven by 2 x 35S promoter) or control plasmid, indicate that *myb.* Ph3 protein inhibits by 10-fold the activity of the above promoter. Therefore it appears as if, although this protein contains amino acids regions predicted to have the structural characteristics of activator domains, *myb.* Ph3 protein can act as a transcriptional repressor on some target genes. Experiments are in progress to further confirm this finding and to study the effect

of *myb* Ph3 when its binding site is placed in a different promoter context. In addition, constructs are being prepared to perform a similar study in yeast.

Regulation of anthocyanin accumulation in maize

The regulation of anthocyanin accumulation is studied in maize by the Milano group. The genetic system under investigation is defined by two major components: *Sn*, and *R*. The R gene family determines the timing, distribution and amount of anthocyanin pigmentation in maize. This family comprises a set of regulatory genes, consisting of a cluster of several elements at the R locus, on chromosome 10, the *Lc* and *Sn* gene lying about two units *R* distal and *B* on chromosome 2. Each gene determines a tissue specific pigmentation of different parts of the seed and plant. The proposed duplicated function of *R, Sn; Lc* and *B* loci is reflected in cDNA sequence similarity.

1. Characterization of the promoter sequence of the transcriptional activator Sn and other related R alleles.

Last year, the sequence of the *Sn*::bol3 promoter was established. Genomic clones containing promoter sequences of different *Sn* and *R* alleles were obtained and sequenced (1.5 kb of the region upstream the transcription start site). The promoter analyzed belong to the *r-r, rch-hopi* and *Rsc* genes, all showing different tissue specific expression. The comparison of these sequences with that previously reported of Sn highlights that *r-r* promoter is identical to that of *Sn*, while that of *rch-hopi* shows several polymorphisms. The *Rsc* promoter shows from -1 till — 800 a high degree of homology and a complete divergence from — 800 on. By comparison and further characterization of these sequences it should be possible to identify the basis of tissue specificity.

2. Cell specific expression of regulatory and structural genes of anthocyanin pathways in maize.

The presence of *R* or *Sn* transcritps correlated precisely with the induction of A1 and C2 messengers. Additional proof that the cell specific localization of *Sn* and *R* mRNA correlates with that of structural genes (C2 and A1) transcripts and with the accumulation of the anthocyanin pigment was obtained by *in situ* hybridization experiments.

3. Interaction among members of the R family

An intriguing phenomenon concerning this system comes from genetic data pointing to an interaction between members of the *R* family. *R* genes extracted from R/Sn heterozygotes, exhibit a *Sn* phenotype in their mesocotyls, as if in the heterozygous association *Sn* had imposed an imprinting of the *R* gene leading to its expression in an additional tissue. This imprinting is negatively affected by chromosomal rearrangements and varies in its frequency (10-40%) according to the *R* alleles. Molecular analysis (RACE) points to the activation at the transcriptional level of the imprinted gene in a tissue where it is normally repressed. Recent studies on transgenic plants have disclosed similar events taking place between ectopic genes and the resident homologous genes in ways that lead to changes in gene expression at either one or both locations. It might be that these two types of interactions have a common basis and that their study will disclose new levels of gene regulation.

HIGHLIGHTS / MILESTONES

Identification of deregulated mutant of nitrate assimilation under way (Rothamsted). Transcription factors expressed under the control of a circadian rythm and new types of light regulated genes (Rome). Transcription factors of the zinc finger type identified and cloned (Versailles). Deregulated expression of nitrate reductase activity leads to decreased nitrate storage (Bergerac). *Mixta*, member of the *myb* family identified and cloned by tagging with *Tam4* in *A. majus*. Full characterization of *CHS* promoter mutations. Establishment of tobacco phenotype following overexpression of *myb* 308 and development of a yeast selection system for target motifs of *myb* in Norwich. Evidence for different DNA-binding selectivity of *myb* genes from *Petunia* in Madrid. *Myb* genes may not only act as transcription activators. Myb- related genes from tomato identified at AGC, with different patterns of expression. Three of them fully sequenced and used in antisense strategies. Several promoters of maize transcrition factors (*Sn* and *R*) sequenced and compared and the tissue specificity of their expression studied in Milano.

WIDER CONSIDERATIONS

The identification of new plant regulatory genes has been succesfully achieved in several of the contributing laboratories involved in this BRIDGE program. Our efforts will now be put on the identification of their targets at the molecular level, and we are expecting major breakthrough with respect to the identification of their function during the coming years.

COOPERATIVE ACTIVITIES

All project leaders met at ENEA in Roma last April to contribute to a meeting organized by Giovani Giuliano, and will meet next April in Versailles for a two days-meeting. Versailles and Roma are exchanging materials for the transcripition factor binding studies. The Norwich and Madrid groups have collaborated on the characterization of plant transcription factors in yeast. Francesca Sparvoli from the Milano group has isolated and sequenced 7 anthocyanins biosynthetic enzymes from grape using maize and *Antirrhinum* clones as heterologous probes in collaboration with the group of C. Martin.The collaboration between INRA and SEITA has now started and been very promising in terms of industrial perspectives.The industrial-academic collaboration between AGC and the JICPSR group has continued. AGC has now mastered the tomato transformation techniques with the help of the John Innes Institute.

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The plasma membrane and the tonoplast of plant cells as targets to increase plant productivity (BIOT CT-900175)

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OBJECTIVES

Using the techniques that have been designed during the first period of the contract (January 1991-March 1992), the different groups have further characterized different transporters involved in the uptake of sugar, amino acids, organic acids and ions across the plant membranes. This characterization was made at the physiological level, at the biochemical level and at the gene level.

RESULTS

I. Plasma membrane

1. The sucrose carrier

(a) Cloning and expression of the sucrose transporter cDNA from spinach and potato
The Berlin group has isolated two sucrose transporter cDNAs from spinach (S21) and potato(p62) by complementation of a yeast strain that is unable to grow on sucrose due to the absence of an endogenous sucrose uptake system and the lack of a secreted invertase. The deduced amino acid sequence of the cDNA p62 is highly hydrophobic and is 68% identical to the spinach sucrose transporter S21. Sensitivity to protonophores and pH dependence of sucrose uptake into yeast are consistent with active transport. Substrate specificity and inhibition by protein modifiers are similar to results obtained for sucrose transport into protoplasts and PMV and the spinach transporter S21 with exception of a reducted maltose affinity. Under stringent hybridization conditions, only one gene is detectable. The gene is highly expressed in mature leaves, whereas stem and sink tissues show only low expression on the RNA level. Both the properties and the expression are consistent with a function of the sucrose transporter P62 in phloem loading.

Using the yeast strain designed in Berlin for complementation, the group in Poitiers is currently developing a similar approach to isolate cDNA encoding sucrose transporter from a Sorghum cDNA library. Several clones are currently investigated.

(b) Antibodies directed against the sucrose transporters
To obtain antibodies directed against the sucrose transporter, two strategies were followed. Synthetic peptides derived from the putative central cytoplasmic loop behind the membrane spanning region 6 were used to immunize rabbits (Berlin). Synthetic peptides directed against the N and C terminal parts of the spinach sucrose transporters are also used for immunization (Berlin, Poitiers).

In order to produce antisera against several other hydrophilic regions, the respective regions were PCR-amplified and cloned into a maltose-binding protein fusion vector. The fusion proteins were affinity purified and also used to immunize rabbits. The antisera are currently under investigation in Berlin.

The antibodies directed against a 42 kD putative sucrose transporter were used in Poitiers to test cross-reaction between these antibodies and the spinach sucrose transporter cDNA expressed in the yeast. The anti-42 kD antibodies did not react with the yeast plasma membrane in Western blot, and they were unable to inhibit selectively sucrose uptake by intact yeasts or spheroplasts. The data may suggest that S21 encode for a protein different of the 42 kD protein previously identified as a putative sucrose transporter in sugar beet, but they may also be interpreted by species differences, or by the fact that the transporters expressed in a yeast and in a plant cell are processed in a different way. Further insight into this problem will await the cloning of the sucrose transporter cDNA from sugar beet leaf.

(c) Epitope tagging of the spinach sucrose transporter
To circumvent the problems associated with the generation of antisera directed against integral membrane proteins, epitopes for M-encephalin and a c-myc domain were fused to the sucrose transporter. The alterations at the C-terminus do not affect the transport activity of the spinach transporter S21, arguing that the C-terminus is not involved in substrate recognition. The tagged protein can be identified in transgenic yeast using commercially available antisera. The molecular mass of the tagged protein on minigels with a constant 10% acrylamide concentration is in the range of 46-50 kDa. Potato and tobacco plants expressing the tagged proteins will be produced.

(d) Overexpression of the sucrose transporter in transgenic plants
Potato and tobacco plants were transformed with constructs expressing S21 under control of the CaMV 35S and patatin B33 promoter and are currently under investigation. Northern studies indicate that overexpression on the RNA level can be detected. Preliminary analysis of the transgenic plants may indicate a slight reduction of the apical dominance in the transformants. Direct analysis of the effects of overexpression on sucrose transporter mRNA levels and transport activity are ongoing.

(e) Antisense repression of the sucrose transporter
The potato sucrose transporter cDNA was cloned in antisense orientation behind the CaMV 35S promoter and transgenic potato plants were produced. Seventy transformants were analysed for reduced P62-RNA levels and 5 plants with reduced transporter RNA were further analysed. Phenotypically the plants have related defects though to different extents. The leaves are curled, locally bleached and have increased deposition of anthocyanins. Both root growth and tuber development are strongly reduced. The leaves accumulate increased levels of sucrose, hexoses and starch. These effects are consistent with the hypothesis that the sucrose transporter P62 plays an essential role in phloem loading. The antisense plants prepared in Berlin will be used in Poitiers to characterize sucrose transport in plasma membrane vesicles (PMV).

(f) Isolation of genomic clones for the sucrose transporter of potato
Southern blot analysis under stringent conditions indicates that the sucrose transporter genes constitute a small gene family of at least two loci. Using the potato cDNA P62, four clones were isolated from a genomic potato lambda library. The clones are currently under investigation in Berlin.

(g) Regulation of sucrose transport(Poitiers)

The uptake of sucrose, 3-O-methylglucose (3-O-MeG) and valine were studied in discs and in purified PMV prepared from mature sugar beet (*Beta vulgaris L.*) leaves. The uptake capacities of leaf discs freshly excised were compared with the uptake in discs that had been floated for 12 h on a simple medium (ageing) and with discs excised from leaves that had been cut from the plant 12 h before the experiments (cutting). After cutting, sucrose uptake was doubled compared to fresh tissues, while the uptake of 3-O-MeG and valine remained unaffected. After ageing, there was a general stimulation of uptake, which represented 400, 300 and 400% of the uptake measured in fresh discs for sucrose, 3-O-MeG and valine respectively. Sucrose uptake in fresh discs was inhibited by N-ethylmaleimide (NEM), p-chloromercuribenzenesulfonic acid (PCMBS) and mersalyl acid (MA). Cutting induced the apparition of a sucrose uptake system that is not sensitive to NEM, but sensitive to PCMBS and MA. In contrast, ageing induced the development of an uptake system that is sensitive to NEM, but poorly sensitive to PCMBS and MA. Autoradiographs of discs fed with [14C]sucrose show that cutting resulted in an increase of vein labeling with little effect in the mesophyll while ageing induced an increase of labeling located mainly in the mesophyll. pH sensitivity of sucrose uptake after ageing is much stronger than in fresh or cut tissues. The data show that cutting is sufficient to induce dramatic and selective changes in the uptake properties of leaf tissues, and that the effects of cutting and ageing on the uptake of organic solutes are clearly different. This provides an interesting model to study the regulation of transport.

Parallel experiments were run with purified PMV prepared from fresh and cut leaves. The comparison of the uptake properties of PMV with that of the corresponding leaf tissues indicate that the sucrose uptake system was recovered in PMV better than the hexose and the valine uptake systems. As observed with the leaf discs, cutting induced a twofold increase of the initial rate of sucrose uptake in PMV but it did not affect the uptake of valine and 3-O-MeG. Cutting induced an increase of both *Vmax* and *Km* of the sucrose transport system in PMV. Measurements of the pmf imposed to the vesicles indicated that the increase of sucrose uptake induced by cutting was not due to a better sealingness of the vesicles. Hexoses did not compete with sucrose for uptake in PMV from fresh and cut leaves, while maltose was an inhibitor of sucrose uptake stronger in PMV from cut leaves than in PMV from fresh leaves. The sensitivity of sucrose uptake to NEM, PCMBS and MA in PMV from fresh and cut leaves paralleled that described above for the corresponding leaf discs. These data show that

- the changes induced by cutting on sucrose uptake by leaf discs are due to membrane phenomena, and not to the metabolism of sucrose
- the study of sucrose uptake with PMV give a good account of the physiological situation
- the specific effects induced by cutting on the sucrose uptake system are not lost during the preparation of the PMV.

Mono and bidimensional electrophoresis patterns of polypeptides from fresh PMV and from PMV obtained after cutting shows that cutting is accompanied by the appearance of 5 different 45 kD polypeptides, using a constant acrylamide concentration of 10% for the second dimension. The possibility that these polypeptides may correspond to the 42 kD putative sucrose transporter (previously identified in 10-22% acrylamide linear gradients) is currently investigated.

Further work will be done to undertand the reasons of the increased uptake of sucrose after cutting and ageing: expression and activity of the plasma membrane

ATPase , expression and activity of the transporters, using the heterologous probes available in Berlin (S21 and P62, AAP1 and AAP2).

(h) Biochemical identification of the sucrose transporter (Poitiers)
Methods to purify sucrose-binding proteins have been developed. Sucrose was bound to a sepharose epoxy-activated column, and solubilized membrane polypeptides were analyzed for their ability to be retained on this column. About 1% of the polypeptides were retained on the column, eluted by sucrose, but not by lactose (used as a negative control). Pretreatment of the membrane polypeptides with NEM or PCMBS before chromatography prevented the appearance of the sucrose-eluted peak. Use of lactose as a ligand on the column does not allow the appearance of this peak. After reconstitution in proteoliposomes, this peak exhibits sucrose transport activity. Analysis by SDS-PAGE shows several polypeptide bands, including the 42 kD polypeptide previously identified as a sucrose transporter. The peak eluted by sucrose is further separated into two main peaks (A and B) after MonoQ chromatography. Peaks A and B were differentially labeled by NEM in the presence of sucrose, maltose or palatinose, to identify polypeptides that were specifically protected by sucrose. Peak C yields a differentially labeled area at about 55 kD, and peak D yields a differentially labeled area at about 38-40 kD. After silver staining, both peak C and D still shows several bands, although the number of bands is very limited. The data indicate either that there are several sucrose-binding proteins (among which sucrose transporters) or that the sucrose-binding proteins (sucrose transporters?) are associated with other polypeptides in the membrane.

2. *Amino acid transporters*

(a) Molecular biology
In Berlin, complementation studies using a gap- yeast strain have allowed the identification and characterization of several cDNA clones mediating amino acid transport, starting from an *Arabidopsis* cDNA library. Two of these clones (AAP1 and AAP2) are being fully characterized.

In Poitiers, a complementation test based on the use of a bap$^-$ yeast strain and on the resistance to sulfometuron has been designed to clone cDNA encoding transporters of branched amino acids (Leu, Ileu, Val). Two clones exhibiting the phenotype expected for growth and for amino acid uptake are being sequenced. A search in the data bank indicates that one of these clones exhibits 98.5% homology at the DNA level with a catalase from *Arabidopsis*, while preliminary data suggest that the other clone exhibits sequences expected for a membrane protein. The latter clone will be further sequenced. The former one has been selected presumably because it metabolizes sulfometuron and thus allows the yeast to grow in the presence of this xenobiotic.

(b) Physiology and biochemistry (Poitiers)
Although amino acid uptake has been studied in detail with PMV from sugar beet leaf tissues, the kinetics of uptake by these tissues have not yet been characterized. This has now been done by studying the concentration dependence of valine uptake into peeled leaf discs from sugar beet. Two apparent saturable phases (K_{m1} = 0.21 mM; K_{m2} = 9.7mM; V_{max1} and V_{max2} = 190 and 4500 pmol valine.min^{-1}.cm^{-2} leaf area respectively) are superimposed by a diffusional phase. The effects of ageing on the kinetics of uptake are presently investigated.

Studies with PMV showed that imposition of an electrical gradient at an external pH of 5.5 was able to drive active uptake of valine, while the same electrical

gradient at pH 7.5 did not induce valine accumulation. This indicates that the amino acid/amino acid transporter complex possesses a positive charge at pH 5.5 but is neutral at pH 7.5. Because the amino acid remains zwitterionic within this pH range, it is assumed that the charge protein of the protein is modified between pH 5.5 and pH 7.5. These studies may be extended to precise the functional pK of the amino acid transporter.

3. *Ion transporters*

(a) Molecular biology (Rothamsted)
The possibility of using a yeast expression system to clone nitrate transporter genes from barley has been investigated. As a prelude to this, attempts were made to express and characterize the *crnA* nitrate transporter from *Aspergillus nidulans* in *Saccharomyces cerevisiae.*

The polymerase chain reaction (PCR) was used to obtain a DNA fragment containing the *crnA* coding region with minimal 5' and 3' untranslated regions. The resulting fragment was inserted, in the correct orientation, into the yeast expression vector pYES2 (Invitrogen) so that transcription was under the control of the inducible *GAL*1 promoter. Expression of the *crnA* gene was induced by growing the yeast cells in glucose-free medium containing D-galactose as the carbon source and several alternative procedures were then used to assay for functional expression of the *crnA* gene in the yeast plasma membrane. These included:
- nitrate depletion from the medium;
- nitrate accumulation in the yeast cells, and
- changes in membrane potential on introducting nitrate into the medium. However, no evidence for functional expression was obtained.

Inspection of the published *crnA* sequence indicated that sequences flanking the ATG translation initiation codon in the *crnA* mRNA may form a hairpin loop in vivo. It was thought possible that this hairpin was hindering or preventing translation of the mRNA in yeast. The 5' untranslated region, and the first 5 codons of *crnA* were thus replaced with a custom-made olignonucleotide. This not only removed the potential for hairpin formation, but also presented us with an opportunity to change the region 5' to the ATG codon to a consensus sequence preferred by *S. cerevisiae* for translation initiation. However, subsequent nitrate transport assays with yeast cells carrying the modified construct still failed to demonstrate functional expression.

In parallel with the above work, the *cnrA* fragment was also sequenced to ensure that no point mutations had occurred during the PCR amplification. When compared to the published *cnrA* sequence (Unkles *et al.* PNAS 88, 204-208, 1991), the sequence of the PCR fragment showed an apparent 2 bp deletion located 109 bp from the termination codon and coinciding with the location of an intron in the *cnrA* gene. Several other independently isolated clones of *cnrA* were sequenced (including the original partial cDNA clone isolated by Unkles *et al.*) and also found to possess this 2 bp 'deletion', suggesting that the published sequence may be incorrect. The 2 bp 'deletion' changes the reading frame of the C-terminus and predicts a protein product 24 amino acids larger than previously thought. Unfortunately, as the 3' primer used in the PCR amplification was designed using the published sequence, the PCR-amplified *cnrA* fragment used for the yeast expression experiments lacks the last ca 70 bp of the coding region and would specify a *cnrA* polypeptide in which the C-terminal 22 residues are replaced by 30 spurious residues encoded by the polylinker.

We are now attempting to reconstruct the complete *cnrA* coding sequence by replacing the 3' region with the correct sequence obtained from another *cnrA* clone. We will then assay this construct for its ability to confer galactose-inducible nitrate transport activity in yeast.

(b) Physiology (Milan)
b1. Physiology of ion transport in Arabidopsis. Arabidopsis thaliana having been chosen for the identification of the molecular nature of the systems involved in ion transport and its regulation, the continuation of the study of the main physiological features has led in 1992-93 to the following results:

(1) Cs^+ is a powerful, rather specific and apparently competitive inhibitor of K^+ uptake and of K^+-induced E_m hyperpolarization (a result important for the characterization of a putative mutant in K^+-transport, partially resistant to Cs^+, isolated by us, see mutagenesis).

(2) H^+-ATPase-mediated H^+ extrusion in *Arabidopsis* is strongly activated by weak acids permeating in the uncharged form, and acidifying the cytosol. Thus changes in cytosolic pH may be responsible for marked differences in the activity of H^+ and of related solute transport systems.

(3) The stimulation of the H^+ pump by K^+ or FC + K^+ is associated with an increase of cytosolic pH and of malate level. Opposite effects are induced by the H^+ pump inhibitor erythrosin B. Vanadate, considered as a satisfactory inhibitor of the pump in sub-cellular systems, induces in *Arabidopsis* tissues *in vivo* a side-effect due to its capacity to inhibit PEP carboxylase, thus influencing malate synthesis, intracellular pH and thus, secundarily, the H^+ pump.

(4) The study of ion channels of *A. thaliana* cells was extended from the plasma membrane to the vacuolar membrane. We have observed the presence of hyperpolarization-actived, Ca^{2+}-regulated cation channels with a slow kinetics of action, the activity of which is stimulated by increasing the temperature between 14 and 20°C.

(5) In cultured cells of *Arabidopsis*, the stimulation of H^+ extrusion by low water potential (mannitol or PEG) is rapidly reversible, is associated with an increase of K^+ uptake and of malate accumulation, and is only partially inhibited by protein synthesis inhibitors. The stimulating effects of high medium osmolarity and of fusicoccin are clearly additive, suggesting different modes of action.

(6) In plasma membrane preparations the treatment with trypsin in controlled conditions removes a C-terminal region of the two proteins and activates both the PM H^+-ATPase and the Ca^{2+}-ATPase. Following this treatment the two enzymes become no longer sensitive to the activating action of either fusicoccin (the H^+-ATPase) or calmodulin (Ca^{2+}-ATPase), thus suggesting that the activation by these factors depends on conformational changes such to eliminate the regulatory function of the C-terminal part of the two enzymes.

b2. Characterization of Arabidopsis thaliana transport mutants. Research has continued on the characterization of two mutants previously isolated: the 5-2 mutants partially insensitive to fusicoccin and the Cs-113 mutant resistant to cesium when heterozygote. Exposed to fusiccoccin, stomata of 5-2 mutant open only partially, area of leaf discs poorly enlarges, proton extrusion, K^+ uptake and Em hyperpolarization are reduced. The results indicate that the lower capacity of the 5-2 mutants to extrude protons (much more evident when the pump is stimulated by fusicoccin) depends on some lesion of the H^+ extrusion system, or of its regula-

tion, rather than on some deficiency of the K^+ uptake system, or of its effect on Em.

The homozygote Cs-113 mutant, when grown in liquid media containing NO_3- or NH_4^+ and K^+ in the concentration range 0-5 mM shows a reduced growth (in respect to wt) especially in NH^{4+} media with K^+ between 0-0.25 mM. These results, when integrated with the aforementioned data on the strong and apparently competitive inhibition of K^+ uptake by Cs^+ in this material, suggest that the mutation may involve the K^+ uptake system, thus opening the way to further research in this direction.

II. The V-type ATPase and the malate transporters of the tonoplast

1. Malate transport and CAM-related transport proteins of the tonoplast (Oxford)

(a) Monoclonal antibody characterization

In a further attempt at producing monoclonal antibodies against the tonoplast membrane of *Kalanchoë daigremontiana*, one cell line was identified that initially produced antibodies, and inhibited malate uptake by about 15%. However, this cell line was unstable and ceased producing antibodies on subcloning. With the small amount of antibody produced, it was possible to develop some Western blots using a secondary antibody conjugated to gold. The monoclonal antibody recognized a protein band of about 43 kDa.

It is hoped to obtain more antibody against this protein by excising the 43 kDa region from a number of polyacrylamide gels, and injecting the proteins into a mouse in order to raise polyclonal antibodies. If this is successful, the injected mouse could then be used to raise more monoclonal antibodies. If we can obtain a specific antibody against the malate transport protein, this could be used in characterization of the protein identification of the gene by screening an appropriate cDNA expression library.

(b) Molecular cloning and characterization of PCR-generated clones for the 16 kDa and 70 kDa subunits of the V-type H^+-ATPase

Previously, two sets each of polymerase chain reaction (PCR) primers were designed to aligned amino-acid sequences from various plant and non-plant sources for the 16 kDa and 70 kDa subunits of the Vacuolar-type H^+-ATPase. For each subunit, one set of external degenerate primers was utilized to generate PCR fragment. PCR reactions containing *K. daigremontiana* cDNA yielded a 350 bp and a 517 bp fragment for the 16 kDa and 70 kDa subunits, respectively, as predicted. Internal primers also yielded PCR products of the expected size.

Several steps have been taken for the identification and characterization of each of the PCR products obtained from the experiments utilizing the external primers. The 517 bp PCR product generated by the 70 kDa primers was sequenced directly and was found to have high identity at the DNA and protein level to the 70 kDa subunit of the vacuolar H^+-ATPase of other plant and non-plant sequences. Initial sequencing efforts of the 350 bp fragment obtained with the 16 kDa PCR primers were inconclusive. However, with further attempts at direct sequencing of the 350 bp PCR product and hybridization analysis utilizing the oat 16 kDa cDNA (kindly provided by Dr H. Sze), the cDNA fragment was confirmed to be that for the 16 kDa subunit gene of the vacuolar H^+-ATPase. To facilitate further characterization of these cDNAs, the fragments were blunt-end cloned into pBluescript. Restriction digests followed by hybridization analysis identified two different clones for the 16 kDa subunit and two for the 70 kDa subunit. Forty-one percent of the entire 70 kDa PCR clones representing 11% of the expected full length cDNA and

80% of the entire 16 kDa PCR clones representing 35% of the expected full length cDNA have been sequenced.

For each subunit, the individual clones show divergence in codon usage. The 16 kDa clones (BL35 and BL36) are only 88% identical and are more divergent than the 70 kDa clones (BL60 and BL61). However, the deduced amino-acid sequences for the two 16 kDa clones are 100% identical, and those for the two 70 kDa clones are 99% identical. The percent identity of the deduced amino acid sequence for the 70 kDa clones ranged from 85% to 100% to other plant kDa subunit sequences, with the highest similarity being observed to the carrot sequence (95-100%). The deduced amino acid sequence from the 10 kDa clones showed protein identities ranging from 57 to 97% to other sequences for the 16 kDa subunits. The *Kalanchoë* sequence had the greatest identity to the oat sequence (97%). The size of the *Kalanchoë daigremontiana* and *K. blossfeldiana* mRNAs for the 16 kDa subunit as determined by Northern analysis is approximately 1 kb, and is 2.4 kb for the 70 kDa subunit.

Currently, attainment of full-length clones of the 16 kDa isoforms is in progress utilizing the RACE-(rapid amplification of cDNA ends) PCR method. Fragments have been generated for both the 5' and 3' ends of the 16 kDa gene as confirmed by hybridization analysis. Four discrete fragment bands were observed for the 5' end of the 16 kDa gene and were sized at 222, 300, 396, and 500 bp. In contrast, 5 individual bands sized at 222, 396, 460, 517, and 700 bp were generated in the 3' and 5' PCR fragments is presently being conducted.

Concurrently, a *Mesembryanthemum* CAM-induced library has been screened via PCR with the 16 kDa and 70 kDa vacuolar H^+-ATPase and NOD26 homolog family primers. Positive clones were identified only to the 16 kDa gene. PCR experiments utilizing primers designed to the ABC transport protein superfamily show large effects of magnesium concentration, with one to several fragments being produced with the same *Kalanchoë daigremontiana* cDNA. Of all the fragments, a consistently prominent band of 300 bp is produced at several different concentrations. PCR of the *Kalanchoë* cDNA with the NOD26 homolog primers has yielded a prominent band at 70 bp and a faint band in the expected 300 bp region. Cloning of these fragments is in progress.

Future work will entail the sequence analysis of the 3' and 5' ends of the 16 kDa clones. Northern analysis will be conducted to determine if the vacuolar H^+-ATPase gene expression in *Kalanchoë daigremontiana* is correlated with the upregulation of crassulacean acid metabolism (CAM), and a suitable marker for determining the time of upregulation for CAM-related tonoplast proteins. Sequencing of the clones of the NOD26 and ABC PCR-fragments will be pursued and their expression studied and compared to that of the H^+-ATPase subunits with Northern analysis. Such studies may help to determine the time at which the malate transport system in CAM plants is synthesized and when maximal expression occurs.

2. *The malate transporter of the tonoplast in C3 plants (Toulouse)*

Two distinct approaches were previously developed in order to identify the protein responsible for malate transport activity at the tonoplast of *Catharanthus roseus* cells:

- reconstitution of the malate transport activity into liposomes,
- photoaffinity labeling of the protein transporter with a radioactive analog of malate.

These approaches have been used in 'routine' for the last six months.

The malate transport activity measured with proteoliposomes depends on the build up of an inside positive membrane potential (created by potassium gluconate and valinomycin to form an inwardly directed potassium diffusion potential). Addition of valinomycin induced a stimulation of malate uptake. This uptake was competed with excess unlabeled malate and was diethylpyrocarbonate-sensitive demonstrating that the process is carrier-mediated.

However, depending on the experiment, the stimulatory effect of valinomycin appeared at different times of the uptake (maximum after two to eight minutes after addition). As a consequence, this method is tedious (consumption of membrane protein, lack of control, etc...). Even though the reconstitution system seems efficient to test the malate transport activity of purified protein fractions, it appears difficult to employ it for studying the uptake characteristics of a purified protein transporter.

Using the photolyzable malate analog, N-(4-azido-salicyly) aspartic acid, the labeling of a 40 kDa polypeptide was confirmed: in the course of the identification of the malate transporter, different batches of probe (distinct synthesis and iodination) were used as well as different lines of *Catharanthus roseus* cells. In particular, with solubilized tonoplast proteins, photolysis of the iodinated analog led to the incorporation of label in a polypeptide pattern that was very similar to that of native tonoplast vesicles. Again, a 40 kDa polypeptide was totally protected from labeling by the inclusion of 100 mM malate in the photolysis medium and also by the inclusion of 2 mM DEPC. This result allows us to start the purification of the 40 kDa polypeptide.

For this purpose, different chromatography steps are currently developped:

(1) Hydroxyapatite columns were successfully used for the purification of mitochondrial anion exchangers as well as for the partial purification of the malate transporter of barley vacuoles.
(2) Two different affinity chromatography supports were prepared: the ligands, aspartic acid and DL-threo-hydroxy aspartic acid, were bound to an activated CH Sepharose 4 B. When linked to the matrix, these ligands mimic the structure of malic acid.
(3) Finally, a High Performance Electrophoresis Chromatography (HPEC) separation is also available for the denaturated tonoplast proteins of *Catharanthus roseus* cells: the complexity of the protein pattern confirms the large number of tonoplast proteins as previously shown by 2-D gel analysis, specially in the range of 20 to 60 kDa.

HIGHTLIGHTS/MILESTONES

Several cDNA encoding sucrose transporters (spinach, potato) and amino acid transporters (*Arabidopsis*) of the plasma membrane have been cloned and characterized, and other cDNA from other species may be expected to be cloned in this programm during the next months. Transgenic plants overexpressing the sucrose transporter, and antisense plants for the sucrose transporter are already available.

WIDER CONSIDERATIONS

The manipulation of plant morphogenesis and plant yield via the expression of major membrane transporters, which still belonged to a remote future at the beginnning of the project, is now a reality, within two years. The techniques designed in the project are general, and will allow the cloning of numerous trans-

porters from various plants, followed by transgenesis, according to specific needs required by agronomical constraints. The transgenic plants thus obtained may be manipulated for their morphogenesis, for their sugar or nitrogen content, or for other features whose expression is controlled indirectly by the nutritional status of the cell.

COOPERATIVE ACTIVITIES

A meeting gathering all participants, and external reviewers (Dr. Hedrich, Hanover; Dr. Kaback, Los Angeles; Dr. Sauer, Regensburg; Dr. Sentenac, Montpellier) was held in Oxford, U.K., June 25-26, 1992. Another meeting of the participants has been held in Berlin, March 29 and 30, 1993. cDNA clones, and mutant yeasts are shared between Berlin, Poitiers and Rothamsted. Transgenic plants prepared in Berlin are being transferred in Poitiers for further analysis.

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Construction of artificial chromosomes for higher eucaryotic cells (BIOT CT-910259)

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OBJECTIVES

The general objective of the proposed project is the construction of artificial chromosomes for mammalian cells by assembling biologically relevant DNA sequences from chromosomes and naturally occuring linear plasmids. The reason for creating such constructs is that so far no satisfactory autonomously replicating vectors exist for higher eucaryotic cells and organisms and it may well be that the ideal eucaryotic vector may have to mimic a chromosome. This kind of construct will allow directed genetic engineering of higher eucaryotes, will help to isolate other biologically important sequences, will be useful in the production of medically important molecules, will help to understand differentiation processes and may prove to be a safe and reproducible method for the somatic therapy of some genetic diseases. Intermediate objectives are the isolation of the different chromosomal sequences that will be required for the construction of an artificial mammalian chromosome. These will include origins of replication and telomeric sequences, which are essential even for the construction of linear plasmids, intermediate structures on the way to artificial chromosomes. In the final stages of the project it will be necessary for functional centromeric or other stabilizing sequences to become available for the actual construction of the chromosome. Telomeric sequences have been isolated from a number of organisms including man; the mechanisms by which they function are still obscure to a great extent. Much is being learned about replication origins, but a functional, non viral, origin still awaits isolation. Centromeres have been isolated in yeast, but not in higher organisms. The attainment of these intermediate steps will constitute important achievements in basic cell biology.

RESULTS

Of the three functional elements necessary for the construction of an artificial chromosome, replication origins, centromere and telomeres, the localization in the human chromosome of two, centromere and telomeres, are shown in the Figure (see next page).

The major effort was made towards a structural and functional analysis of these three elements from various biological systems. Further work concentrated on structural studies on defined DNA-sequences and on the construction of new eucaryotic circular vectors (number in paranthesis indicates the groups performing the experiment).

The photograph shows an in situ hybridization to a centromere and to telomeric sequences (3)

TELOMERES: Attempts have been made to introduce YACs (yeast artificial chromosomes) into mammalian cells. However, these constructs become integrated into the host genome. It has to be assumed that yeast telomeric sequences do not function in mammalian cells. Therefore YAC vectors have been designed to be used as shuttle vectors between yeast and mammalian cells. These vectors carry selectable markers for yeast and mammalian cells, but the principal innovation is that a human telomeric sequence is included at a location inside the telomere. After growth in yeast the human telomeres can be revealed by I-Sce1 cleavage (3). An important prerequisite for using such shuttle vectors is the ability to manipulate such large DNA constructs. Methods have been developed which will allow to introduce these constructs in relatively intact form into mammalian cells (3). Another approach to test the telomeric requirements for replication as a linear molecule in higher eucaryotes was made using *Xenopus* unfertilized eggs as biological system. In these cells injected DNA becomes replicated regardless whether it contains an origin or replication or not. Constructs carrying either no telomeric sequence or *Tetrahymena* (a ciliated protozoan) or human telomeres were injected in *Xenopus* eggs and their stability and replication tested after various time intervals. While the constructs carrying either no or *Tetrahymena* telomeres are rapidly degraded, the construct with the human telomeres (which are identical to the *Xenopus* telomeres) is effiently replicated as a linear DNA-molecule (1, 3). In other constructs human telomeric sequences were ligated to SV40 or BPV, permissive cells will be transfected and the physical stability and the replication of the linear vector tested (1, 2, 5). Another question raised was how telomeres are modified in an immortilized cell line, these experiments have implications on cell senescence and oncology. A human B-lymphocyte cell line was infected with Epstein-Barr virus and the changes in telomere length and in the subtelomeric regions analyzed in the course of cell proliferation (5). In addition to the terminal telomeric sequences internal telomeric sequences are found in many eucaryotic chromosomes. The organization of such internal telomeric sequences was studied in the polytene chromosomes of a ciliated protozoan. The results obtained indicate that all internal telomeric sequences are contained in a conserved element sharing some structural features with transposable elements (1). The sequence and organization of the telomeric sequence was analyzed in another protozoan, the coccidial parasite *Eimeria tenella* (6). Telomeric sequences adopt special DNA

structures and it is believed that the structure of telomeric DNA is an important determinant of telomere function. Therefore, the structure of various telomeric DNAs has been investigated when contained in negatively supercoiled DNA (2). The ends of a linear DNA molecule which is stably maintained in *Xenopus* oocytes is analyzed (1, 2).

While many informations concerning telomere structure and function are now available, much less is known about mammalian REPLICATION ORIGINS and CENTROMERES. However, the amplification promoting sequences derived from the non transcribed spacer region of murine rDNA (4) show striking sequence homologies to the first well mapped and analyzed origin of bidirectional replication, the origin of the DHFR locus from CHO cells. In the appropriate test system this origin acts as a very efficient amplification promoting sequence (4). Experiments are in progress to make a functional test whether these amplification promoting sequences act in vivo as replication origins (1, 4). No tests for centromeric function in mammalian cells are available up to now. Work is in progress to define the minimal mammalian centromere and to manipulate it within the cell (3). In addition, structural studies on mammalian centromeric sequences were made (2).

FURTHER WORK concentrated on structural studies related to recombination and the construction of new mammalian circular expression vectors. It could be shown that the simple alternating repeat sequence GA.CT has a significant influence on homologous recombination. At present it is investigated whether this type of repeated DNA could be used to target SV40 integration in both permissive and non-permissive cells (2). A versatile expression vector for heterologous proteins in mammalian cells on the basis of a high copy situation in the recipient cell was constructed. This vector exhibits a broad host range for recipient mammalian cells (4). Another vector, also based on the amplification promoting sequences, was used to transfect human T-lymphocytes. This vector amplifies spontaneously to high copy number. When HIV-1 DNA sequences were inserted into this vector in antisense orientation, HIV-1 replication could be efficiently inhibited. This type of vector may prove useful for the somatic therapy of some human diseases where large amounts of transcript are required (1, 4).

HIGHLIGHTS/MILESTONES

All the experiments described are important prerequisites for the construction of a mammalian artificial chromosome. In fact, from the results obtained in recent time it seems very likely that such an artificial chromosome will be available in the near future. Furthermore, many of the results obtained, like the work on replication origins, centromeres, recombination or DNA structures adopted by specific sequences, are of major interest for basic biological questions. In the course of constructing linear vectors new circular expression vectors have been designed which prove useful for biotechnology of eucaryotic cells.

WIDER CONSIDERATIONS

An artificial chromosome provides the chance for safe and specific manipulation of higher organisms. Since these constructs will not integrate into the host genome and not disturb gene balance in the organism they may be the ideal vectors for somatic gene therapy. In addition, linear constructs can not be propagated in procaryotic cells. Thus, uncontroled spreading of these vectors in nature is extremely unlikely.

COOPERATIVE ACTIVITIES

There are strong interactions between all participating groups as well as exchange of material and staff between the different laboratories. In addition to the meetings organized in the frame of the T-project on animal cell biotechnology regular group meetings take place.

EUROPEAN DIMENSIONS

In our project six european laboratories interested in different aspects of the eucaryotic chromosome have joined together in an effort to construct an artificial mammalian chromosome. It is obvious, that this type of research can only be performed in the frame of an EC-project and the experience made within the last two years showed that this type of interaction proved extremely useful not only for our group but also for other groups funded by the EC.

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Improved techniques for establishing a high expression production system for recombinant proteins from animal cells (BIOT CT-900185)

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OBJECTIVES

In the second year of the project the genetic work was aimed at establishing the gene targeting methodology with reporter genes and mouse chromosomal flanking sequences. Furthermore, the screening for higher expressing cell clones with single copy transferants and the isolation of the flanking chromosomal sequences of the corresponding inserts was planned. Cell biology work intended to establish base line data of the cell clones found by the screening of efficiently expressing cells should begin. This includes measuring both reporter gene expression stability and reporter gene expression in relation to physiological parameters in aggregated culture systems and macroporous supports. Evaluation of these parameters should enable us to choose the best constructs for further study.

MAJOR PROBLEMS ENCOUNTERED

Not all cell lines are able to grow in the same bioreactor, so direct comparison of physiological parameters is not appropriate.

The efficiency of specific targeting of the designed expression vectors to the the dedicated chromosomal sites is extremely low.

RESULTS

Genetic work:

After establishment of a screening system for high expression of single genes (retroviral vectors) in mouse Sp2/0 cells, single cell clones with long term stability of high expression of the reporter gene have been isolated. Flanking sequences of retroviral inserts were isolated by inverse PCR, vectors for gene targeting including convenient reporter genes, and selectable markers for ± screening have been developed. In a model targeting experiment these were tested with chromosomal DNA fragments from mouse cells.

Bioreaction:

Phenomenon of different cell lines exhibiting different relationships between growth and production kinetics was examined further. Analysis was extended to a second product and additional culture systems. The results show that the major factors affecting productivity and relationship of growth and production kinetics are the choice of cell line and culture system. The effect of culture system is

postulated to be related to differences in cell morphology observed in the different culture systems.
These findings question the validity of the use of just one cell line to elucidate general principles for the expression of recombinant proteins from mammalian cells.

Aggregation:

Three-dimensional structures can be an advantageous method of cell growth, using the natural aggregative properties of anchorage dependent cells. It has further been proven that aggregate size can be hydrodynamically controlled, that aggregates can be perfused by simple sedimentation and that under controlled size ranges of aggregates growth, viability and productivity of cells remain unchanged or are even improved. For the non-anchorage dependent cells, highly performing three-dimensional structures by using macroporous supports are being created.
Specialized supports: A technique has been developed to bind proteins in a well defined way to the inorganic glass surface. The target is to provide an optimized surface for the growth and viability of cells by using proteins of the 'extracellular matrix' such as elastin and fibronectin.

HIGHLIGHTS / MILESTONES

The perfection of the screening for high alkaline phosphatase secreting cell lines and the use of this system for the finding of various high producers.

The three-dimensional matrices obtained in the hydrodynamically size controlled aggregates yield higher protein productivities than two-dimensional confluent cultures.

The discovery that the relationship of growth and production kinetics is dependent upon the choice of culture system, while cell specific productivity is also dependent upon the choice of cell line.

The development of a technique to bind cellular matrix proteins directly to porous glass beads.

COOPERATIVE ACTIVITIES

A member of the Portuguese group visited CAMR for two weeks to learn the operation of the high density culture systems and later also went to the German partner for the collection of cell lines and exchange of information.
A member of the British group visited the Portuguese partner for analysis of results.

Biochemical analysis of samples of one group are done by other partners.
A meeting for the whole T project was coordinated by the Portuguese group (Sintra, Portugal, February 14-17, 1993).

Two joint meetings within the T-project on 'Animal Cell Biotechnology' were held in Ireland on May, 1992, and in Portugal on February, 1993. In addition, two sub group meetings of the geneticist members of the project took place.

WIDER CONSIDERATIONS

In this project the relationship of genetic and technological parameters for the production of recombinant gene products in mammalian cell lines is to be evaluated. The parameters include the chromosomal localization of the transfected

genes and the methods of cultivation. The characteristics of these parameters such as the long-term stability, strength of gene expression, product accumulation and cell physiology were determined. The final goal is a protocol for the construction and cultivation of cell lines with which to achieve optimal conditions for recombinant protein production at the technical scale. So far, our results let us conclude that gene expression stability is related to the chromosomal localization of the transferred gene, that the characteristics of product accumulation depend on the method of cultivation but that gene expression is determined by both.

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Structural and functional analysis of regulatory genes controlling liver-specific proteins (BIOT CT-910260)

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OBJECTIVES

The overall objective of the Bridge Project is to study the regulatory proteins and genes which control the synthesis of some representative liver-specific proteins.

The specific aims of the proposed research are:

1) To isolate and characterize several genes involved in the transcriptional regulation of liver-specific proteins including genes involved in hepatocyte differentiation.

2) To study the mechanism of transcriptional regulation and the functions of the regulatory genes by reverse genetics (site directed mutagenesis *in vitro* transcription, homologous recombination, anti-sense RNA, ectopic and unregulated expression in transgenic animals).

3) To identify and characterize the promoter elements and trans-acting factors involved in the transcriptional control of the regulatory genes in an attempt to define putative regulatory cascades in gene regulation.

4) To study the role of extracellular signals (matrix components and growth factors) in the regulation of liver-specific genes in advanced tissue culture systems employing different extracellular matrices.

RESULTS

01. V. Zannis and I. Talianidis

I. Transcriptional Regulation of the Human ApoCIII Gene. We have continued the systematic characterization of the promoter elements and the factors required for the transcription of the human apolipoprotein CIII gene. The distal regulatory element I binds five activities designated CIII-I 1, 2, 3, 4 and 5. Proteins CIII-I 1, 2, 4 recognize the regulatory region H proteins CIII I,3 recognize the regulatory region J. Elements F, H, I also bind the ubiquitous transcription factor Sp-1. Another regulatory protein designated CIII-F3 recognizes the elements F and J. Cotransfection with pRSV-Sp1 resulted in only a moderate 2-3 fold increase in the apoCII promoter activity in HepG2 cells probably due to the high endogenous levels of Sp-1 in this cell line. Mutations which affected the binding of the

regulatory proteins CIII 1, 2 and 4 and Sp-1 to element H reduced hepatic transcription to 15% of control, whereas mutations in elements J and F reduced transcription to 60% of control. Previous studies have established that HNF-4 is a major activator of the apoCIII promoter. This factor binds to proximal element B (-72 to -87 nt). Cotransfection experiments have shown that the upstream regulatory elements are also important for the HNF-4 dependent activation of the apoCIII gene. Mutations in elements F, G, H, I and J which affected the binding of specific transcription factors to these sites reduced the HNF-4 dependent activation of the apoCIII gene. The finding suggests that the factors binding to these sites synergize among themselves to promote HNF-4 dependent activation of the apoCIII gene.

II. Identification of an Indirect Autoregulatory Mechanism Involved in HNF-1 Gene Regulation. Recent studies have shown that HNF-4 is an essential positive regulator of another liver-enriched transcription factor HNF-1, defining a transcriptional hierarchy between the two factors operating in hepatocytes. To assess the possible autoregulation of the HNF-1 gene we have examined the effect of HNF-1/LFB1 on its own transcription. In transient transfection assays, HNF-1 strongly downregulated transcription driven by its own promoter in HepG2 cells. In addition, HNF-1 also repressed the activity of HNF-4 dependent apoCIII and apoAI promoters. The same effect was observed in experiments employing vHNF-1/LFB3, a distinct but highly related protein to HNF-1. Both HNF-1 and VHNF-1 downregulated HNF-4 activated transcription from intact and chimeric promoter constructs carrying various HNF-4 binding sites implying that they act by impeding HNF-4 binding or activity. DNA binding and cell free transcription experiments however failed to demonstrate any direct or indirect interaction of HNF-1 and vHNF-1 with the above regulatory regions. Both factors repressed HNF-4 induced transcription of the apoCIII and HNF-1 genes in HeLa cells, arguing against the requirement of a hepatocyte specific function. These findings define an indirect negative autoregulatory mechanisms involved in HNF-1 gene expression, which in turn may affect HNF-4 dependent transcription of other liver specific genes such as apoCIII and apoA-I.

02. V. De Simone

I. Analysis of the Regulatory Elements and Factors Controlling the Transcription of LFB3/vHNF1. We have isolated several mouse genomic clones covering the 5' portion of the LFB3/vHNF1 gene. Fusions of the LFB3/vHNF-1 5' flanking proximal region to a reporter gene combined with deletions and site-specific mutagenesis and DNA binding assays allowed us to identify the cis-acting elements and the factors required for the transcription of LFB3/vHNF1 gene. This analysis showed that the transcription of LFB3/vHNF1 depends on the binding of three transcription factors:

1) The OCT protein which has a binding site adjacent to the TATA box.
2) The liver-specific HNF-4/LFA1 factor, which has a binding site in the -260 nt region.
3) A RARE factor which has a binding site partially overlapping with that of HNF-4/LFA1.

II. Functional Analysis of LFB3/vHNF1. The transcriptional activation domain of the LFB3/vHNF1 has been mapped by deletion and nucleotide substitution mutagenesis of the protein coding region. This analysis has shown that the protein region spanning amino acids 350-450 is crucial for trans-activation.
Finally, a gene targeting project aimed at blocking the expression of LFB3/vHNF1 both by dominant negative mutation and by homologous recombination is in

progress. These experiments are being carried out in organoid *in vitro* cultures, and will address the question of the role of LFB3/vHNF1 during the morphogenesis of nephrotic tissue.

03. R. Cortese

A POU-A Related Region Dictates DNA Binding Specificity of LFB1/HNF1 by Orienting the Two XL-Homeodomains in the Dimer. LFB1/HNF1 regulates the hepatocyte-specific transcription of several genes by binding as a dimer to cis-acting elements that match the inverted palindrome GTTAATNATTAAC. The DNA binding domain of LFB1/HNF1 is characterized by a unique tripartite structure that includes an unusually long homeodomain (domain C), a region related to the POU-specific A-box (domain B) and a short N-terminal dimerization domain (domain A). A recombinant peptide corresponding to the isolated homeodomain of LFB1/HNF1 binds as a monomer to a half-palindrome binding site, but shows diminished sequence specificity. Domain B, in addition to the homeodomain, is required and is sufficient for proper recognition of LFB1/HNF1-responsive sites. A protein consisting of only these latter two domains is a monomer in solution, but forms dimers upon DNA binding. The protein-protein contacts established within the bound dimer restrain the orientation of the two homeodomains with respect to one another, thus contributing in a critical fashion to the recognition of the dyad symmetry-related LFB1/HNF1 sites. The DNA-dependent dimerization domain (domain A) is required to increase the affinity of DNA binding, but does not influence the dimer geometry.

04. G. Bressan

I. Targeted Disruption of the Type VI Collagen Gene. The extracellular matrix is part of a signalling system involved in the maintenance of tissue structure and its remodelling in pathological conditions. In liver, our attention has been focused on type VI collagen, a pericellular protein with wide distribution in the organ, including the space of Disse. In order to determine the function of type VI collagen in liver, we have expressed, *in vivo*, mutated forms of one of the three chains which make up the protein and then look for abnormalities detectable under control and pathological conditions such as liver regeneration and experimental fibrosis. Several types of mutations are under investigation. One gives rise to inactivation of the gene for the alpha-1 chain and has been obtained by homologous recombination in mouse ES cells. Four targeted clones have been identified and three of them have been used to produce chimeras. None of the chimeras tested so far have transmitted the mutation through the germline. New clones are now being isolated and tested for germline transmission. In addition, the whole procedure of gene targeting will be carried out with a different type of ES cell lines (R1 instead of D3). The second type of α1 chain mutant has been constructed by fusing a fragment of the gene containing about 7 kb of the 5' flanking region, the first exon, the first intron and part of the second exon with a cDNA fragment containing the rest of the exonic sequences, except for a short stretch of collagen coding sequence and will be used to generate transgenic mice.

II. Regulatory Sequences Controlling Tissue Specific Expression of the α1 Chain of Type VI Collagen During Development. It is known that in sinusoids the protein starts to be produced at detectable levels just before birth and persists during the morphogenetic process with establishes the mature liver structure. To study the regulation of the α1 gene we have generated reporter plasmids where the CAT gene is placed under the control of the 5' regulatory region of the α1 gene. The

location of sequences responsible for spatial and temporal regulation of transcription in developing liver will be determined by analysis of expression of the reporter gene in cell cultures and in transgenic animals for different CAT constructs.

05. J. Chambaz and P. Cardot

We have used DNA binding and competition assays and protein fractionation to identify the hepatic nuclear activities which are involved in the regulation of the human apoA-II gene. Such activities may be of general importance for the regulation of liver specific genes.

This analysis has shown that the regulatory elements M, D and F bind new activities which have not been identified in apolipoprotein or other liver specific promoters. These activities have been designated AIIM1 and AIIM2 for element M, AIID1 and AIID2 for element D and AIIF2 for element F. The activity AIIM2 is present in liver, but absent in CaCo-2 cells. We have purified to near homogeneity the factor AIID2 from rat liver nuclear extracts using ion exchange and affinity chromatography. The protein has a molecular weight in the range of 60 kDa and will be microsequenced in order to clone the corresponding cDNA. A set of regulatory elements binds activities which resemble liver-enriched or ubiquitous factors shown previously to play important roles in the regulation of their target genes. Thus element I binds to activities related to NF1 and elements L, C, D, G, AB and F bind to C/EBPα as well as other heat stable activities. The affinity of the bacterially expressed C/EBPα for the various apoA-II regulatory regions follows the order AIIL ~ AIIC > AIID > AIIF > AIIF > AIIAB. Protein fractionation showed that element J binds at least three hepatic nuclear activities and is also recognized by members of the nuclear receptor family HNF-4, EAR-2, EAR-3 and ARP-1. Another liver enriched factor, HNF-1 was shown previously to bind to element H. Despite the importance of HNF1, HNF4, NF1 and C/EBPα in the regulation of numerous other target genes, deletion of the HNF1, NF1 and C/EBP binding sites did not affect drastically the hepatic transcription of the apoA-II gene. Rather, the hepatic and intestinal transcription is affected severely by deletion of elements A, B, K, L and N. In addition, the intestinal transcription is affected by deletion of elements C, J and M.

This analysis establishes the organization of several nuclear activities on the human apoA-II promoter. Our findings suggest that the expression of a liver specific gene such as apoA-II is controlled by a combination of factors and that the relative importance of individual factors is determined by the promoter context. Maximum expression of the apoA-II gene requires the synergism of factors bound to the proximal and distal regulatory elements.

06. M. Yaniv

I. Functional Analysis of HNF1/LFB1 and vHNF1/LFB3. We have pursued the study of homeodomain family transcription factors HNF1/LFB1, vHNF1/LFB3 involved in liver specific gene expression. In a first series of experiments we attempted to map the regions (residues) of HNF1 protein involved in transcriptional activation. Deletion of C-terminal residues resulted in a gradual decrease in the normalized transcriptional activity. The activity was totally lost by deletion of 236 C-terminal amino acids. In the course of these studies we have observed that C-terminal deletions increase the stability of the protein and exposed a domain that retains the protein in the cytoplasm. Further deletion that removes the 330 C-terminal residues restores nuclear transport (the DNA binding domain contains

a clear nuclear localization signal). The findings indicate that the cytoplasmic retention is probably an active process.

In another series of experiments we have studied two alternatively spliced species of vHNF1. We have previously isolated two cDNA clones that differed by the presence or absence of an extra 26 amino acids encoding exon located between the homeo and POU domains. We showed that vHNF1-A containing this extra exon is a more potent transcriptional activator than vHNF1-B lacking the exon. Analysis of the distribution of mRNA coding for both species in different tissues did not reveal strong variations. We found both vHNF1 mRNA in liver, kidney, intestine, lung, thymus and ovary with the highest concentration in liver and kidney.

II. Inactivation of vHNF1 Gene in Transgenic Mice. In an attempt to determine the role of HNF1 and vHNF1 in the control of cell differentiation and organogenesis we initiated a program of gene disruption. After isolation of genomic clones for HNF1, the first exon containing the dimerization domain and the N-terminal part of the POU domain was replaced with a β-gal gene. The chimeric gene was introduced in a recombination vector containing PGK neo and tk cassettes. After electroporation, several ES clones that have undergone homologous recombination were isolated. Cells from one of them were introduced into blastocysts and implanted into competent pseudo pregnant mothers. We obtained several dozen of chimeric descendants. Several of them transmitted the disrupted gene to their progeny. Analysis β-gal expression in one of these mice revealed clear staining in liver, kidney and intestine as could be expected. Male and female heterozygous mice will be mated soon to analyze the phenotype of a null mutation. In parallel, genomic clones of the vHNF1 gene were isolated and a recombination vector is being constructed for the inactivation of this gene.

07. G. Ciliberto and V. Poli

I. Analysis of the Mechanisms of Transcriptional Activation by IL-6 in Hepatic Cells. One of the major target tissues for IL-6 in higher organisms is the liver, where this cytokine is responsible for major changes in gene expression and metabolism following stress, injury and inflammation. This process, known as acute phase response, is characterized by the increased transcription of a set of genes, including C-reactive protein, haptoglobin, hemopexin, α2-macroglobulin, etc. The upregulation of these target genes follows the interactions of IL-6 with two receptor molecules on the surface of hepatocytes: One is the IL-6 specific receptor (Type A receptor) and the other is a signal transducer transmembrane (Type B receptor) which is shared with the other closely related cytokines LIF, Oncostatin M, CNTF and IL-11.

Transcriptional upregulation of target genes by IL-6 in the liver is due to increased binding of transcription factors to target promoter sequences. We had previously suggested that a transcription factor of the C/EBP family, namely IL-6/DBP (C/EBPβ) is functionally activated by IL-6 via a post-translational mechanism.

Analysis of several IL-6DBP (C/EBPβ) deletions and point mutations and generation of chimeras with other b-zip proteins indicates that information essential for post-translational activation by IL-6 derives from cross-talk between two domains. The first domain is located in the amino-terminal region and the second in the leucine zipper regions. Such cross-talk is achieved through changes in protein phosphorylation. Experiments are underway to localize the site(s) of phosphorylation.

II. Study of Structure-Function Relationships in IL-6, Aiming at the Future Development of IL-6 Antagonists. IL-6 is a 184 amino acid polypeptide postulated

to belong to the class of helical cytokines. We have built a three-dimensional model of human interleukin B based on the similarity of its hydrophobicity pattern with that of other cytokines and on the knowledge of the x-ray structure of growth hormone, Interleukin 2, Interleukin 4, interferon beta and granulocyte macrophage colony stimulating factor. The resulting model is a bundle of four α-helices and suggests possible alternative conformations for the nine carboxy-terminal amino acid. In this region important information for biological activity is located in residues 182 to 184. We have generated a collection of single amino acid variants in residues 176-181. Analysis of their biological activity in two systems (murine hybridoma IL-6 dependent cells, and human hepatoma cells) and of receptor binding properties of a subset of them indicates that the entire region is involved in forming the receptor binding surface. Remarkably, we also found a mutant with receptor affinity and biological activity 3- to 5-fold higher than wild-type: this molecule is of practical interest, given the potential of using IL-6 in the treatment of neoplastic diseases and thrombocytopenia. This approach, which combines IL-6 molecular modeling with site-directed mutagenesis, provided us important information pertinent to the structure IL-6 in the absence of NMR or x-ray crystallography. In the future we will investigate with a higher level of confidence the possibility to dissect receptor binding from signal transduction domains of IL-6. In other words, taking into account the available knowledge on the binding of helical cytokines to dimeric receptors we will try to generate IL-6 variants with intact or improved binding to type A receptor and impaired binding to type B receptor (gp130), which can be used as antagonist.

Recently, in collaboration with Columbia University, Dr. V. Poli has generated by gene targeting mice with homozygous deficiency in the IL-6 or IL6-DBP C/EBPβ. Both mutations are not lethal and we will soon start studies at IRBM to assess the impact of these mutations on the development of the immune system and liver physiology.

08. M.C. Weiss

I. HNF4 and HNF1 As Well As a Panel of Hepatic Functions Are Extinguished and Reexpressed in Parallel in Chromosomally Reduced Rat Hepatoma-Human Fibroblast Hybrids. Rat hepatoma-human fibroblast hybrids of two independent lineages containing only 8-11 human chromosomes show pleiotropic extinction of thirteen out of fifteen hepatic functions examined. Reexpression of the entire group of functions most often occurs in a single step, and except for one discordant subclone, correlates with loss of human chromosome 2. The extinguished cells and their reexpressing derivatives have been examined for the expression of seven liver-enriched transcription factors. C/EBP, LAP, DBP, HNF3 and vHNF1/LFB3 expression are not systematically extinguished in parallel with the hepatic functions. However, HNF1 and HNF4 show a perfect correlation with phenotype: these factors are expressed only in the cells showing pleiotropic reexpression. Since recent evidence indicates that HNF4 controls HNF1 expression, it can be proposed that the HNF4 gene is the primary target of the pleiotropic extinguisher. Study of the pleiotropic extinguisher described here provides an avenue towards investigation of the factors and of the pathway implicated in regulation of the HNF4 gene.

II. Expression Patterns of vHNF1 and HNF1 Homeoproteins in Early Post-Implantation Embryos Suggest Distinct and Sequential Developmental Roles. The homeoproteins HNF1 (LFB1/HNF1-A) and vHNF1 (LFB3/HNF1B) interact with an essential control element of a group of liver-specific genes. During development, these putative target genes are initially expressed in the visceral endoderm

of the yolk sac and subsequently in fetal liver. To assess the possible involvement of HNF1 and/or vHNF1 as transcriptional regulators in the early steps of visceral endoderm differentiation we have analyzed the expression pattern of both factors both *in vitro* during differentiation murine F9 embryonal carcinoma cells and *in vivo* during early post-implantation mouse development. The differentiation of F9 cells into either visceral or parietal endoderm is accompanied by a sharp induction in vHNF1 mRNA and protein. By contrast, only low levels of aberrantly sized HNF1 transcripts, but not DNA binding protein, are found in F9 cells and its differentiated derivatives. At 6-7.5 days of gestation high levels of vHNF1 mRNA are present in the visceral extra-embryonic endoderm, which co-localize with transcripts of the transthyretin gene. HNF1 transcripts are first detected in the yolk sac roughly two embryonic days later, after the developmental onset of transcription of target genes. In addition, two alternative spliced isoforms of vHNF1 mRNA, vHNF-A and vHNF1-B, are expressed in embryonic and adult tissues. Taken together, these data suggest that vHNF1 participates as a regulatory factor in the initial transcriptional activation of the target genes in the visceral endoderm of the yolk sac, whereas the later appearance of HNF1 could be required for maintenance of their expression.

III. Regulation of Albumin Gene Expression in Hepatoma Cells of Fetal Phenotype.

See description under participant 11.

09. G. Mignot

The task of TM innovation remains the large scale growth of genetically altered hepatocyte cultures for expression of hepatic proteins of medical importance such as factor IX.

10. G. Brownlee

Characterization of the Clotting Factor IX Promoter. A knowledge of the mechanism by which the clotting factor IX promoter is controlled has implications for the identification of new liver-specific transcription factors, the elucidation of how regulation is perturbed in Haemophilia B patients with naturally occurring promoter mutations. Such knowledge may lead to large-scale cultures of suitably modified liver cells for the production of biologically active factor IX for the treatment of patients.
A particular advantage in the study of the factor IX promoter is the fact that there are a large number of naturally occurring promoter mutations; 21 kindreds with promoter mutations are now known, defining 13 molecularly unique point mutants, clustered over a short region of the promoter from nucleotides -26 to +13 close to the major site of mRNA initiation. Previously we had identified a binding site for a C/EBP-like protein, which recognizes the region +1 to +18 of the promoter and have described the molecular defect underlying Haemophilia B Brandenburg (a G→C mutation at nucleotides -26). A study of this patient and others including the classic promoter mutant at -20 (Haemophilia B Leyden) allowed us to define an HNF4 site (-25 to -15) overlapping with a androgen responsive element (-36 to -22). This androgen response element is responsive to testosterone --- albeit somewhat weakly. We believe that this element explains, at least in part, the increase in factor IX observed in most promoter mutants at puberty. We have recently turned our attention to a detailed study of a new transcription factor binding to the -5, -6 region of the factor IX promoter. This region is defined by several haemophilia B mutants. A specific gel shift assay has been set up for this factor which is detectable in rat nuclear liver extracts. The properties and sequence

recognition motif of this factor differ from all other known liver specific factors and thus it may represent a new transcription factor. Screening a λgt11 expression library with oligonucleotides containing the binding site of this factor failed to produce cDNA clones. Currently, we employ classical purification methods, including ion-exchange chromatography and sequence specific affinity chromatography to purify the transcription factor to homogeneity from rat liver nuclear extracts as a first step towards cloning and characterization of this new factor.

11. A. Rollier

Regulation of Albumin Gene Expression in Hepatoma Cells of Fetal Phenotype: Dominant Inhibition of HNF1 Function and Role of Ubiquitous Transcription Factors. (Trans-national collaboration involving Participants 08 and 13). Two widely used hepatoma cells lines, mouse BW1J and human HepG2, express gene products characteristic of fetal hepatocytes, including serum albumin, while reporter genes driven by the albumin promoter are expressed at very low levels compared to highly differentiated hepatoma cells. We have investigated the low albumin promoter activity in BW1J cells to understand the differences in liver gene regulation between fetal and adult cells. Addition of the albumin upstream enhancer, or any other fragment of the albumin gene, failed to modify expression of the transfected promoter in BW1J cells. Analysis of cis elements of the albumin promoter showed that, in contrast to highly differentiated II4II cells, in BW1J cells the activity depends largely on ubiquitous transcription factors. Both BW1J and HepG2 cells produce the liver-enriched transcription factor HNF1; dimerization and DNA binding properties are identical to those of liver HNF1, yet the protein fails to show the anticipated transcriptional stimulatory activity. A transfected HNF1 expression vector strongly transactivates the albumin promoter in HepG2, but only weakly in BW1J cells, and in hybrids (BW1J x Fao), indicating that the inefficient HNF1 function is dominant. We conclude that hepatoma cells of the fetal phenotype are deficient in the use of HNF1 to drive transcription of the albumin gene, and that they harbor a dominant modulator of HNF1 function.

HIGHLIGHTS/MILESTONES

1) Further insight into the regulatory proteins which control the transcription of the apoA-II, apoCIII albumin, factor IX, HNF-1, vHNF-1 and HNF-4 genes. b) Cloning of new members of the HNF-1 family and their role of vHNF-1 forms in the early transcriptional activation of target genes in the visceral endoderm of the yolk sac. c) potential interactions between HNF-1 and HNF-4 may lead to negative autoregulation of HNF-1 and other HNF-4 responsive genes. d) Demonstration of dominant suppression of HNF-1 functions in hepatocytes of fetal phenotype. e) Potential role of HNF-4 as the target of a pleiotropic extinguisher of liver specific functions.

2) Elucidation of the significance of the homeodomain, dimerization domain, and POU-specific A-box for the binding specificity of the liver enriched factor LFB1/HNF-1. b) Localization of domains or residues required for transcriptional activation and possibly cytoplasmic retention of HNF-1.

3) Utilization of gene targeting techniques to generate mice with homozygous deficiencies in HNF-1, IL-6DBP (C/EBPβ), or IL-6. Experiments are also in progress to generate mice deficient in vHNF-1 forms and in α1 subunit of the collagen IV gene.

4) Evidence that the IL-6 mediated activation of C/EBPβ results from post-translational phosphorylation.

5) Derivation of a model for the most probable secondary ad tertiary structure of IL-6. Analysis of the functional domains of IL-6 by *in vitro* mutagenesis based on this model may lead to the generation of IL-6 antagonist for treatment of neoplastic diseases.

WIDER CONSIDERATIONS

The trans-national BRIDGE project entitled "Structural and Functional Analysis of Regulatory Genes Controlling Liver-Specific Expression" involves 12 laboratories from 4 countries. The liver is the site of synthesis of many proteins that are crucial for normal body functions as well as in diverse pathological states. The project involves the study of the regulatory proteins and genes which control the synthesis of some representative liver-specific proteins, such as albumin, apolipoproteins, clotting factors, and acute phase proteins that are produced in response to inflammation. The broader goal of this project is to understand what are the molecular mechanisms which permit the genes encoding for these proteins to switch on and off in normal and pathological states. The basic knowledge that will emerge from this biotechnology project will have numerous industrial and pharmaceutical applications as follows:

1) Generation of improved hepatocyte cultures which produce large quantities of liver specific proteins of pharmacological importance such as factor IX.

2) Design of new types of drugs which are based on the three-dimensional structure of a protein and its interaction with DNA or other proteins or activating ligands. Such information has been obtained in this project for HNF-1/LFB1 and IL-6.

3) Generation of laboratory animals with partial or total deficiency in regulatory proteins which control the synthesis of liver specific proteins. This approach may provide experimental animal models of specific liver diseases which can be used to explore new modalities of treatment of such diseases.

4) Improved hepatocyte cultures can also be used for toxicology tests that are currently performed using livers of animals. This approach will save animal lives and will allow cost-effective and rapid screening of toxic substances.

5) Some of the hepatic regulatory proteins may play a role in liver differentiation and regeneration. Drugs which modify the action of these proteins may be used to control liver regeneration.

COOPERATIVE ACTIVITIES

1) The participants of the project and two guests with background in toxicology had a three-day conference at Iraklion Crete on September 10-12, 1992. The participants had formal presentations of their work, as well as numerous informal discussions and exchange of ideas. This meeting reinforced the scientific ties of the participants and helped in focusing further the overall directions of the project. Another meeting has been scheduled for September 9-11, 1993. Several of the participants will also meet informally at the meeting entitled 'Regulation of Liver Gene Expression in Health and Disease' at Cold Spring Harbor Laboratories, New York, May 5-9, 1993.

2) Drs. Zannis, Chambaz and Cardot met in Brussels on August 30, 1992 and again on November 16-19, 1992 at the American Heart Association Meetings in New Orleans, Louisiana.

3) Dr. Zannis visited the laboratories of Drs. Cortese and Ciliberto at IRBM on June 13, 1992.

4) Various biological reagents such as clones for NF1 forms, mutant C/EBP NFY A and B, antibodies to liver specific factors and hepatocyte cultures have been given freely by Drs. Cortese, Yaniv, Ciliberto and Weiss to the other participants.

EUROPEAN DIMENSION

The project brings together several leading European laboratories from four countries with common interest in hepatic gene regulation but with diverse research expertise. Several participating laboratories have made pioneering contributions in the field of eukaryotic gene regulation. The expertise of the laboratories are also complementary, thus providing strengths in the fields of molecular biology, cellular biology, and protein chemistry.

The project fosters interactions, not merely among the project leaders, but also among the younger scientists of the groups and thus ensures continuity of interaction among the European scientists. The participation of IRBM in this project both strengthens the overall science and provides a forum for industrial utilization of the research findings.

The collaboration of the European laboratories avoids duplication of efforts, facilitates exchange of scientists, allows the unrestricted transfer of materials and expertise, and promotes the integration and upgrading of the European academic institutions.

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The development of a genetic and physical map of the porcine genome (Pig Gene Mapping Project — PiGMaP) (BIOT CT-900187)

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OBJECTIVES

The project objectives are as follows:

(1) To produce a genetic map with markers spaced at approximately 20 centiMorgan intervals over at least 90% of the pig genome.

(2) To produce a physical map with at least one distal and one proximal landmark locus mapped on each porcine chromosome arm and also genetically mapped.

(3) To develop a flow karyotype for the pig based on FACS sorted chromosomes.

(4) To develop PCR based techniques to enable rapid genotyping for polymorphic markers.

(5) To evaluate synteny conservation between pigs, man, mice and cattle.

(6) To develop and evaluate the statistical techniques required to analyse data from QTL mapping experiments and to plan and initiate the mapping of QTLs in the pig.

Specific targets for the second twelve month period include:

(a) Study to map QTLs designed and additional crosses established.

(b) All reference families established and DNA samples stored.

(c) Panels of hybrid cell lines established.

(d) 80 porcine coding sequences and polymorphic markers physically assigned chromosomal locations.

(e) 200 markers of PIC > 0.3 identified and their monogenic segregation confirmed.

(f) Porcine flow karyotype using FACS established.

RESULTS

The overall objectives of PiGMaP are to develop a 20 centiMorgan genetic (linkage) map covering 90% of the genome; to produce a physical map with at least one distal and one proximal landmark locus mapped on each chromosome arm and to plan experiments to map the so-called quantitative trait loci (or QTLs) [refs 18, 19, 20, 21, 35, 36, 39]. As the generation of the maps is an iterative process, elements of last year's report [ref 2] are retained with new data and observations inserted where appropriate.

Genetic (linkage) mapping

The genetic (linkage) map is being developed by a group of ten laboratories within the collaboration. Reference pedigrees have been established in five centres (Edinburgh, France, Germany, the Netherlands, Sweden). These pedigrees take the form of three-generation families in which grandparents from genetically divergent breeds have been crossed to produce the parental (F_1) generation which have subsequently been intercrossed. In the Scottish, French and Dutch pedigrees the founder grandparental breeds are the Chinese Meishan and the European Large White (Yorkshire) [ref. 34]. The Swedish and German pedigrees have European Wild Boar and European improved breeds as their grandparents. The pedigrees were established ahead of schedule and DNA from 110 F_2 pigs plus their respective parents and grandparents has been distributed to all ten laboratories for genotyping. Representative pedigrees from the five centres are included in this shared panel of reference families. Distribution of DNA to laboratories in Australia and the United States has commenced. Thus the objective (b) of establishing reference families and isolating DNA has been achieved.

Three classes of molecular genetic markers are being employed in the genetic mapping studies. First, expressed sequences are being used in Southern blot analyses to detect diallelic RFLP loci. The diverse origins of the founding breeding stock means that many loci screened in this manner do indeed prove to be polymorphic. Both homologous (mainly cDNA) and heterologous (human, rodent, and other mammalian) probes are being used to develop these RFLP markers. During the past twelve months further RFLP markers of this type have been developed by the Edinburgh, Bologna, Foulum, Oslo and Uppsala groups and include polymorphisms at the *ADA, BNP1, C9, CHAT, CHGA, CRC, CR2, EDN, FUCA1, GBA, GCT, GHR , GPI, HSP, IL2, INSR, ITIH, 12-LO, MUC, ORM, PEPN, SPP1, TNP2, TYR,* and *UBB* loci [refs. 5, 6, 28, 49, 50, 56, 57]. These expressed sequences will provide the means of aligning the pig gene map with the maps of other species, in particular with those of the 'map rich' species — man and mouse.

Hypervariable markers based upon both minisatellite and microsatellite loci are also being developed [refs. 60]. Over 300 microsatellite loci have been sequenced by groups in Copenhagen, Edinburgh, Hohenheim, Merelbeke, Toulouse, Uppsala and Wageningen. Primers for polymerase chain reaction amplification and genotyping of such loci have been designed and the highly polymorphic nature of the loci confirmed [refs. 7, 13, 14, 24, 26, 27, 40, 62, 63]. Markers with the combined benefits for comparative mapping of expressed sequences and high polymorphic information content (PIC) as provided by microsatellite repeats have been developed by scanning the EMBL and GenBank databases for sequences that have both these attributes. Amongst the loci for which such markers have been developed are *CGA, DAGK, IGF1,* and *SPP1*. In conclusion, the objective (e) of identifying 200 markers of PIC > 0.3 and determining the degree of polymorphism in the reference pedigrees has been fulfilled.

Genotypic data on animals in the shared panel of reference families are sent to the Edinburgh laboratory by the typing laboratories and entered into a central relational database built with INGRES software. Once a month all the data are examined for linkage relationships using the LINKAGE package. The results of the two point linkage analyses are distributed to collaborating laboratories on a confidential basis. Data for 150 loci were included in the most recent (March, 1993) analysis. Linkage relationships were established for 110 of these loci. Linkage groups have been assigned to 13 of the 18 porcine autosomes [ref. 1, 8, 15, 41, 42]. The 40 loci for which linkage relationships have not yet been identified indicate that we have isolated markers in, as yet, poorly mapped regions of the porcine genome.

Physical mapping

Participants in Copenhagen, Toulouse, Uppsala and Utrecht are applying *in situ* hybridisation techniques to the assignment of genes to chromosomes. The past year has seen a continuing shift from radioactive to non-radioactive methods of *in situ* hybridization, and specifically to fluorescent *in situ* hybridization (FISH). Amongst the loci which have been mapped by this approach since the previous report are — *ALB* (chr 8), *CRC* (chr 6), *DAO* (chr 14), *GPI* (chr 6), *GPI* (psuedo) (chr 1), *GRP78* (chr 1), four *HSP 70* loci (chr 7 and chr 14), *IFNG* (chr 5), *LIPE* (chr 6), *MHC(SLA)* (chr 7), *MUC* (chr 5), *PGA* (chr 2), *PI* (chr 14), *PLAU* (chr 14), *PRKAR1* (chr 12), and > 100 anonymous DNA segments (containing microsatellites, and plamids containing minisatellite sequences) [refs. 4, 11, 46, 51, 55, 61, 65]. Thus the objective (d) of identifying the chromosomal location of 80 porcine coding sequences and polymorphic markers has been achieved. The physical mapping of functional genes to chromosomes is essential to the alignment of the porcine gene map with the maps of other species, in particular with those of humans and mice.

Synteny mapping can be effected by analysis of somatic hybrid cell lines. However, analysis of the new pig-rodent somatic hybrid cell lines created for the project, indicate that fragmentation and rearrangement of the porcine chromosomes in the hybrid lines is a problem. The use of alternative fusion partners is being explored.

The polydisperse nature of the porcine karyotype allows the chromosomes to be sorted effectively by the use of a dual laser FACS machine. The DNA content of the haploid porcine genome (~ 2770 Mb) has been estimated from the flow karyotype [ref. 58]. The DNA content of individual chromosomes ranging from 295 Mb for chromosome 1 to 68 Mb for chromosome 18 (the Y chromosome is only 47 Mb) has also been estimated.

The groups in France and Cambridge, that established flow cytometry of pig chromosomes have now determined the chromosomal identity of the flow sorted peaks [refs. 23, 29, 31, 59]. Material corresponding to a single peak isolated by preparative flow cytometry is amplified by the polymerase chain reaction (PCR) using degenerate primers and fluorescent label incorporated into the PCR product.

This fluorescently labelled DNA is then used to probe or paint metaphase chromosomes and thus identify the chromosomal origin of the flow sorted peak. The identity of all twenty peaks, corresponding to the eighteen autosomes plus the X and Y sex chromosomes has been determined by both groups [refs. 47, 48, 52, 53, 64]. The resulting fully characterised and confirmed flow karyotype will be useful for future studies directed at particular chromosomes.

Already the flow sorted material has been used to develop chromosome specific libraries for chromosomes 1, 6, 7, 13 and 16, again well ahead of schedule (original target 30 months) [ref. 7, 13, 14, 16]. One subchromosomal region specific library for 6p1.1-q1.2 has been established by coincident cloning using DNA isolated from flow sorted chromosome 6 and from a somatic hybrid cell line containing, amongst other porcine chromosome fragments, 6p1.1-q1.2 [ref. 10].

Status of the pig gene map

The number of loci assigned to chromosomes either directly or through linkage to or synteny with direct assignments has doubled again during the second year of the collaboration and now approaches 200 [ref. 30].

Quantitative trait loci (QTL) mapping

A limited amount of QTL mapping is being undertaken at each of the five centres that have established mapping populations. The design of effective QTL mapping experiments have been discussed. New statistical methods for locating quantitative trait loci have been developed [refs. 3, 33, 37, 38, 43, 44, 45]. Thus, the objective (a) of designing studies to map QTL has been met.

HIGHLIGHTS/MILESTONES

The second year of PiGMaP has been marked by the continued friendly and open collaboration between the participants. Several targets have been reached ahead of schedule. The development of a fully characterised flow karyotype for the pig is a particular highlight of this year's efforts. The contribution of the PiGMaP collaboration to the mapping of the porcine genome was evident at the 1st Pig gene mapping workshop (PGM1) held at the 23rd International Conference on Animal Genetics, Interlaken, Switzerland, on 7th August 1992. Seventy-five percent of the presentations on pig gene mapping at this international conference were by laboratories participating in the PiGMaP collaboration.

WIDER CONSIDERATIONS

The pig gene mapping project (PiGMaP) has brought together key laboratories in six EC countries and two EFTA countries in a coordinated and collaborative programme to develop a porcine gene map. In the second year of activity the resources required to develop the gene map have been assembled. The pig pedigrees and polymorphic genetic markers necessary for genetic (or linkage) mapping established during the first year are now being used to elaborate the genetic map. The number of genes assigned (or mapped) to chromosomes has almost doubled again and approaches 200. Techniques for physically sorting the pig chromosomes have been exploited to develop chromosome specific libraries (or

gene banks). Consideration has also been given to the future exploitation of the gene map in locating the genes responsible for controlling traits such as growth and reproductive performance. The initial grouping of fifteen laboratories has been extended to seventeen in the context of a European Laboratory Without Walls. Laboratories in Australia and the United States are now collaborating in the genetic (linkage) mapping studies. The Genome Mapping Informatic Infrastructure (GEMINI) project initiated in early 1993 under the auspices of the EC Biotech programme will provide enhanced computing facilities for the PiGMaP collaboration and make the results of PiGMaP more readily accessible to the wider scientific community.

COOPERATIVE ACTIVITIES

The collaboration between the participating laboratories has continued to operate at several levels. The reference animals necessary for the genetic (or linkage) mapping are provided by five groups. DNA from these animals is shared by a wider grouping of ten laboratories, plus additional laboratories in Australia and the United States. Linkage studies of this shared pool of animals is proceeding in a fully collaborative and cooperative manner — data are entered into a central database, linkage analyses performed and the results distributed. Further chromosome specific libraries have been created by combining chromosome sorting in some laboratories with PCR amplification and cloning in other laboratories and the libraries have subsequently been distributed to other participants. Groups isolating markers have collaborated with those using *in situ* hybridization to assign marker loci to chromosomes. In particular, lambda and cosmid genomic clones, which have been shown to contain polymorphic microsatellite loci are being mapped to chromosomes.

One project meeting has been held in this second reporting period — organised by the Copenhagen laboratory from 27th to 29th November, 1992. In addition to the original collaborating laboratories, representatives of a further German laboratory, two US groups and one Australian group attended, three with the support of EC funds. An on-line link to the Edinburgh laboratory's computing databases was established and entry of new genotypic data effected.

EUROPEAN DIMENSION

The PiGMaP collaboration has placed Europe in the forefront of pig gene mapping worldwide. The pooling of resources and expertise across Europe means that it has been possible to develop a fully integrated view of the porcine genome. The evident benefits of this coordinated approach to the analysis of complex genomes have been recognised by other livestock gene mappers. A similar coordinated European project to map the bovine genome has been initiated with EC BIOTECH funding.

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A study of fish genes and the regulation of their expression (BIOT CT-900188)

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OBJECTIVES

The following objectives were set during the second year of the project:
(1) Isolation of trout metallothionein promoter (Univ. Würzburg)
(2) Cloning of cDNA of the pineal gland of catfish (Univ. Liège)
(3) Isolation of cDNAs of cod immunoglobulins (Univ. Uppsala)
(4) Optimisation of DNA microinjection of fish eggs (Univ. Leuven)
(5) Cloning of first gene of the catfish pineal gland (Univ. Liège)
(6) Isolation of cDNAs of monoclonals (INRA and Univ. Uppsala)
(7) *In vitro* test of specific promoter of the catfish pineal gland (Univ. Liège)
(8) Assay of tiPRLI promoter-reporter gene constructs *in vitro* (Univ. Liège) and *in vivo* (Univ. Leuven)
(9) Ploidy manipulation of sea bass gametes (IATS) and catfish gametes (Univ. Leuven)
(10) Cloning of sea bass prolactin gene (Univ. Padova)
(11) Isolation of herpes virus promoter (INRA)
(12) Anti-VHS strategies using the N gene (INRA)
(13) Isolation of salmon promoters (Univ. College Galway)
(14) Isolation of *Xiphophorus* oncogenes (Univ. Würzburg)
(15) Isolation of cDNAs of trout immunoglobulins (Univ. Uppsala)

Objective 2, 5 and 7 have not been initiated because no funds for a postdoc were available. Objective 6 has been dropped. INRA has shifted some of its interests to genes involved in the development of the central nervous and immune system by insertional mutagenesis and enhancer trapping. The new initiative has been coordinated with several other BRIDGE partners.

MAJOR PROBLEMS ENCOUNTERED

The partners participating in the project have not encountered serious problems. Progress is substantial, according to schedule and extends beyond the original plans. Satisfaction with the research effort looks good. Smaller problems vary from financial (the Univ. di Padova faced serious delays in payment; the Univ. Würzburg didn't spend funds according to schedule; the Univ. Uppsala faced major cutbacks; funding is not sufficient to influence long term orientation of research — INRA), staff problems (lack of funding for post-doc to start project

— Univ. de Liège; premature departure of post doc — Univ. Uppsala), scientific tangles (incorrectly differentiated hepatocyte cell line and difficult isolation of the serum albumin promoter — Univ. College Galway; mosaicism in transgenic fish — several partners; complexity of the immunoglobulin light chain — Univ. Uppsala) to experimental accidents (chlorine pollution — INRA).

RESULTS

It is a pleasure to report the scientific results of the BRIDGE partners. Growing interactions and the appearance of important results have positively influenced progress.

The **Univ. of Leuven** has continued testing of reporter gene constructs provided by the Univ. de Liège to optimise the *in vivo* transfer of genes in zebrafish and African catfish. A strong transient replication and expression of the constitutive viral promoter Cytomegalovirus has been observed within days after fertilisation. Stable integration of fusion genes is being assayed in African catfish. A first deletion mutant of the tilapia prolactin I promoter (tiPRLI3.4) has been brought to expression transiently *in vivo*. The expression of four other mutants (prepared by the Univ. de Liège) is being tested *in vivo* (as transient and stable transgenes), immunohistochemically and kinetically. Ploidy manipulation of African catfish (gynogenesis) resulted in the identification of the critical periods for combined heat, cold and hydrostatic pressure shocks and the verification of paternity by means of multiple-locus DNA fingerprinting.

The **Univ. de Liège** has cloned, characterised and determined the complete nucleotide sequence of the tilapia growth hormone (GH) gene and 3.3 kb of 5' flanking sequences. Several potential binding sites for the mammalian pituitary factor Pit-1 (responsible for tissue specific expression of PRL and GH in mammals) have been effectively found by deoxyribonuclease I protection experiments. A fusion gene between the 5' flanking sequences of the tilapia PRL I gene (3.4 kb fragment) and the luciferase reporter gene (tiPRL3.4-Luc) has been constructed and tested *in vitro*. It is active when introduced into rat pituitary GC cells and in non-pituitary carp cells (EPC) cotransfected with the RSVratPIT-1 fusion gene. Several deletion mutants of tiPRL3.4-Luc have been built; they will be assayed *in vitro* by transient expression in order to locate regulatory elements. Cloning of the cDNA encoding tilapia Pit-1 is in progress.

The industrial partner **Eurogentec s.a.** continued its support to the cell culture of carp cpithclial cclls (EPC) and the transfection experiments of cells with electroporation to the Univ. de Liège.

The partner at the **Univ. di Padova** has studied the transcriptional regulation of the rainbow trout growth hormone (GH) gene (in collaboration with the Université de Liège) (see above). The rat Pit-1 transcription factor binds to and activates the rtGH promoter, which suggests the conservation of the basic mechanisms regulating GH transcription in fish and mammals. Moreover, the rainbow trout Pit-1 transcription factor has been cloned by PCR and sequenced. A high degree of homology with chum salmon (11 point mutations and 2 insertions) and rat (69%) was observed. Thirdly, the sea bass prolactin gene (PRL) was cloned and sequenced. It shares 81% identity at the cDNA level and 73% at the protein level with tilapia PRLI. Fourthly, sea bass cDNA GH was cloned into an inducible expression vector and expressed as a protein of 22 kDA identical to native sbGH.

The **C.S.I.C.** (Torre de la Sal) has strenghtened its research on the identification of the labile period for phenotypic sex induction in sea bass. The production of

populations with skewed phenotypic sex ratios was achieved successfully with 17α-methyldihydroxytestosterone (all male) and 17α-ethynylestradiol (majority female). The treated populations were bred for progeny testing and transferred for grow-out to a fish farm of Sepia International. In a second project, the optimal conditions for ploidy manipulation of sea bass (triploidy and gynogenesis) through heat shock and hydrostatic pressure shock were further explored. The ploidy level was verified on karyotypes and with flow cytometry. Multiple-locus DNA fingerprinting is being used for paternity testing.

The industrial partner **Sepia International** provides logistic support by supplying sea bass eggs and by providing perfectly controlled facilities for the rearing of sea bass. Several families (and their replicates) of sea bass have been transferred from Spain (C.S.I.C.) to the facilities of S.I. in Gravelines (France) for further growth under optimal and standardised conditions.

The contribution of **I.N.R.A.** (Jouy-en-Josas) involved four aspects. First, special attention was paid to the improvement of transformation methods. Two fish lines carried multimers of foreign DNA which showed some evidence of extrachromosomal inheritance. It turned out that they were integrated as long palindromes with abnormal secondary structures (such as hairpin loops). Second, several vectors — prepared to work in mammals and containing promoters of mammalian and viral origin — linked to CAT and bovine GH were optimised for expression in fish cells by adding selected introns and terminators. New improved constructs tested *in vitro* showed that only part of the mammalian introns are correctly processed by fish cells and that both SV40 terminators work efficiently in fish cells. Third, the potential of various constructs for expression *in vivo* was tested. The mouse immunoglobulin enhancer/promoter linked to CAT (from L. Pilström) was expressed in Ig positive cells (tissue-specific expression). The 'accidental' expression of RSV-bGH construct was explained by favourable position effects and the construct CMVTk-*Xmrk* (from M. Schartl) is being tested. Fourth, the nucleocapsid protein gene of viral hemorrhagic septicemia virus has been cloned, sequenced, and expressed transiently *in vitro* with the CMV promoter. The OMV Herpes virus was screened for strong promoters for gene manipulation.

The **Univ. College of Galway** has cloned five genes (whose expression in salmon liver is increased during smoltification) to elucidate their molecular mechanisms. Clone 1 has been identified as salmon transferrin and clone 2 has been identified as a mitochondrial gene encoding subunit III of cytochrome C oxidase. Another aim has been the isolation and characterisation of promoters of fish genes. Seven other genes expressed at high levels in salmon liver were cloned and sequenced to characterise their promoters. They include: salmon apolipoprotein A-1 (most abundant in a cDNA bank), salmon serum albumin and salmon globin genes (including α and β cDNAs). The expression of serum albumin in salmon seems regulated by the transcription factor 'hepatocyte nuclear factor 1' (HNF1) which contains a potential binding site for HNF4. Also salmon nuclear extracts were prepared to show with gel shift experiments that proteins in the salmon liver nucleus bind to the rat albumin promoter.

The **Univ. of Würzburg** has been studying the melanoma-inducing locus Tu of *Xiphophorus* which contains a gene (*Xmrk*) that encodes a novel receptor tyrosine kinase. The *Xmrk* proto-oncogene is differentially expressed during organogenesis of the medaka. Expression could be shown very early in selected tissues. Duplication of axial organs (neural tube and notochord) was observed in injected embryos, which might reflect interference of the ectopically expressed *Xmrk* oncogene with

a normal function of *Xmrk*. In a second more technical and methodological part of the project, expression vectors containing sequences of fish origin were constructed. This included the isolation of fish promoters (rainbow trout metallothionein A gene (tMTa)), the isolation of a fish structural gene (silver carp growth hormone (scGH)) and the fusion of the promoters with the structural gene and testing of the all-fish constructs (tMTa-scGH) for expression in fish cell culture. The arrangement of exons and introns of the scGH gene is very similar to other carps and mammals, but not to tilapia and salmonids. The fish gene scGH was efficiently expressed in fish cells in a metal-inducible manner by a homologous fish MT promoter and the primary transcript was correctly expressed.

The **Univ. of Uppsala** continued research on the immunoglobulins (Ig) of fish. A cDNA library of the rainbow trout Ig heavy chain was constructed and screened with an antiserum against Ig from brown trout. One of the longest clones (clone 15) was sequenced and used to screen a gDNA library of erythrocyte DNA. One genomic clone contained the most 3'J-segment, an intron and the beginning of the CH1. Second, the Ig light chain of rainbow trout and Atlantic cod were isolated from cDNA libraries and sequenced. Their organisation is of the clustered type (the cluster measuring about 4 kb) which opposes current theory that the loci of the two Ig chains have the same type of organisation within the vertebrate taxa. Third, the Ig promoter/enhancer combination from mouse linked to CAT has been expressed tissue-specifically *in vivo* in rainbow trout (see above) which demonstrates the conservation of these regulatory motifs in vertebrates.

This summarises the scientific progress of research on fish genes and the regulation of their expression by 8 academic and 2 industrial partners.

HIGHLIGHTS/MILESTONES

The above summarised research has resulted in numerous highlights such as:

- the evolution of the melanoma oncogene system in *Xiphophorus* involves the suppression of a dominant acting oncogene by an accidentally acquired promoter.
- correct expression of a fish growth hormone gene using a fish promoter in fish contrasts with the negative results of human GH and hMT.
- the potential evolutionary conservation of a 'Pit-1-like' protein and binding site on the prolactin gene.
- the evolutionary conservation of cis-elements and trans-factors needed for pituitary-specific expression in fish and mammals.
- the tilapia GH gene contains (similar to salmonids but unlike carp) 5 introns.
- the identification of stable integration patterns of multimers in the fish genome.
- the construction of efficient *in vitro* expression vectors.
- the evolutionary significance of several fish genes under study (*Ig, GH, PRL, Xmrk*).
- the tissue-specific expression of a murine immunoglobulin promoter/enhancer *in vivo*.
- the clustered type of organisation of the locus of Ig light chain in teleosts.
- a reliable method for the spawning, fertilisation and sex manipulation of sea bass.
- the first isolation of the promoter of a transcription factor (HNF1) in salmon and several other promoters.
- the suitability of the firefly luciferase gene as very sensitive reporter gene.
- the expression *in vitro* and *in vivo* of fusion genes under control of the enhancer/promoter of prolactin I.

— the isolation of the genes of two important metabolic proteins (salmon cytochrome C oxidase and transferrin) activated during smoltification.
— the convergence of genetic research on fish towards a few model fish: rainbow trout (population genetics, ploidy manipulation, stable integration), medaka (gene regulation, stable integration), zebrafish (developmental genetics) and *Xiphophorus* (quantitative genetics and oncogenetics).
— the successful manipulation of the phenotypic sex ratio in sea bass.
— the correct expression of sea bass recombinant growth hormone.

WIDER CONSIDERATIONS

Fish are very suitable vertebrate models for the study of genes and the nature of their control.

The European Community (BRIDGE programme) is funding a significant research project on fish genes and the nature of the elements controlling their expression since January 1991. Although a single fish carries in each cell the same set of genes, the expression of these genes is highly specific. For example, the hormone prolactin is nearly exclusively produced in the pituitary gland and salmon transferrins are induced when the smolt (Atlantic salmon) enters the sea. Vertebrate and biomedical research has depended on mammalian models (such as mice) for molecular biological research. This project proves that fish are scientifically comparable and economically more reasonable models to study. Eight laboratories and two industrial partners from six EC countries and one EFTA member country have the common goal to integrate fish gene research. Applications of this research have already surfaced in the field of cancer research and mid term applications are expected in the fields of disease control, smoltification (of Atlantic salmon), sex control and selection. Spin off applications include the domestication of sea bass, Atlantic salmon and African catfish.

COOPERATIVE ACTIVITIES

Two coordination meetings were organised. Dr. D. Chourrout organised at INRA in Jouy-en-Josas (Paris, France) a well attented meeting on 11-12 June 1992. The programme included a progress report by each partner followed by a discussion, a visit to the laboratories on fish genetics of INRA at Jouy-en-Josas and a plenary discussion on scientific progress, common business and participation in new research programmes. All partners met again at a fall coordination meeting organised by Dr. C. Lecomte (Eurogentec s.a., Liège, Belgium) and Dr. A. Belayew (Univ. de Liège, Sart Tilman, Belgium) at Eurogentec and the Univ. de Liège on 11-12 December 1992. The meeting focussed on research progress of each partner, a seminar on 'Research at the "Centre d'Ingéniérie des protéines", Univ. de Liège' by Prof. J.M. Frère (Univ. de Liège), a visit to the new laboratories of Eurogentec s.a. and the 'Laboratoire de Génie Génétique et de Biologie Moléculaire' and a plenary discussion on common business, including scientific matters. Partners exchanged documents and biological material, and discussed extensively and informally research and papers in progress.

Short term staff exchanges occurred among I.N.R.A. and Univ. Würzburg (Dr. D. Chourrout worked for 1 week in November 1992 in Würzburg), Univ. de Liège and Katholieke Universiteit Leuven (several one day visits of several researchers), Univ. College of Galway and Univ. de Liège/Eurogentec (visit of F. Gannon). Long term exchanges occurred among Univ. Leuven and Instituto de Acuicultura de Torre de la Sal (Drs. M. Blazquez) and Univ. College of Galway and Univ. Würzburg (Dr. De Ryckere). The I.A.T.S. (Spain) has moved several families of

sea bass to the fish farm of Sepia International in Gravelines. Exchange of scientific material (cDNA and gDNA banks, reporter genes, medaka, sea bass eggs, specific gene constructs and cell lines) and consultations on technical aspects (fish care, DNA techniques and experimental protocols) occurred among all partners .

EUROPEAN DIMENSION

The dispersed geographical distribution of researchers working on fish genes makes European and trans-continental collaboration a necessity. Few partners find sufficient scientific support in their country. Over the past 2 years a gradual and spontaneous coordination of research has surfaced within the framework of 'Fish genes and the regulation of their expression'. This proves that time and people (i.e. grants) are the essential factors to make collaboration an effective tool in high quality research.

Unfortunately, since there is no perspective for further formal collaboration within the BRIDGE programme after 31 December 1993, centrifugal efforts are already surfacing to continue research within other national and international (AIR 2, BIOTECH, HUMAN CAPITAL AND MOBILITY) programmes. Research on the regulation of the expression of fish genes can not be accommodated in these programmes which represents a serious handicap in the near future.

JOINT PUBLICATIONS WITH TRANS-NATIONAL AUTHORSHIP

Argenton F., Vianello S., Bernardini S., Jacquemin P., Belayew A., Colombo L. and Bortolussi M. (submitted) The transcriptional regulation of growth hormone gene is conserved in vertebrate evolution. *Biochim. Biophys. Res. Comm.*

Bearzotti M., Perrot E., Michard-Vanhee C., Jolivet G., Attal J., Théron M.-C., Puissant C., Grabowski H., Dréano M., Kopchick J.J., Powell R., Gannon F., Houdebine L.-M. and Chourrout D. (1992) The expression of various gene constructs transfected into fish cells. *J. Biotechnology* **26**: 315-325

Cavari B., Hong Y., Funkenstein B., Moav B. and Schartl M. (submitted) All-fish gene constructs for growth hormone gene transfer in fish

Michard-Vanhée C., Chourrout D., Thuvander A. and Pilström L. (submitted) Immunoglobulin promoter/enhancer from mouse induces a tissue-specific expression in transgenic rainbow trout (*Oncorhynchus mykiss* Walbaum)

Nakayama I., Foresti F., Tewari R., Schartl M. and Chourrout D. (submitted) Sex chromosome polymorphism and heterogametic males revealed by two cloned DNA probes in the ZW/ZZ fish *Leporinus elongatus*

OTHER PUBLICATIONS:

Adam D., Dimitrijevic N. and Schartl M. (1993) Tumor suppression in *Xiphophorus* by an accidentally acquired promoter. *Science* **259**: 816-819

Altschmied J. and Schartl M. (in press) Genetics and molecular biology of tumor formation in *Xiphophorus*

Bearzotti-Le Berre M., Cissay L. and Bernard J. (submitted) Expression of recombinant N gene from viral hemorrhagic septicemia (VHS) virus in epithelioma papulosum cyprini (EPC) cell line

Bernard J. and Mercier A. (submitted) Sequence of two EcoRI fragments from *Oncorhynchus masou* herpes virus

Blazquez M., Piferrer F., Zanuy Z. and Carillo M. (1992) Identification of the labile period for sex steroid-induced gonadal differentiation in sea bass (*Dicentrarchus labrax L.*). 2nd Int. Symp. Fish Endocrin. June 1-4, Saint-Malo (France)

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Daggfeldt A., Bengtén E. and Pilström L. (in press) A cluster type organisation of the immunoglobulin light chain in Atlantic cod (*Gadus morhua L.*) and rainbow trout (*Oncorhynchus mykiss* Walbaum) indicated by nucleotide sequences of cDNA and hybridisation analysis. *Immunogenetics*

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Study of the avian herpesvirus genome of Marek's disease virus (BIOT CT-900173)

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OBJECTIVES:

The objectives for the period ending in March 1993 (19 months) were as follows:
(1) Complete sequencing of MDV gH and gD genes.
(2) Partial sequencing of HVT homologues of gD and gH.
(3) Complete sequencing of at least one non-essential gene of HVT other than TK.
(4) Produce antisera against MDV gD and gH.
(5) Construct HVT vector.

RESULTS:

gD and gH genes of MDV and HVT.

The sequence of gH and gD genes of MDV and HVT have been determined at IAH. The results (Scott *et al.* 1993, Zelnik *et al.* 1993) demonstrate that the homologous genes in MDV and HVT are closely related on the basis of their amino acid sequence but that there were significant differences. The % amino acid identity between the MDV and HVT homologues being 56 and 49 for gH and gD respectively. The cloned genes are now available for insertion and expression in suitable vectors.

The MDV gD and gH genes have been expressed as fusion proteins using the prokaryotic expression vector pGEX-3X and antisera against the fusion proteins have been raised in rabbits. An anti-peptide serum against a predicted antigenic region of gD has also been raised. Both antisera reacted with the fusion proteins in immunoblots but failed to react with MDV-infected chick embryo fibroblasts (CEF). The apparent lack of expression of gD and gH in MDV-infected CEF will be investigated in the future.

Sequencing of HVT U_S region

A number of genes mapping in the short unique region (U_S) of several alphaherpesviruses have been shown to be non-essential for virus replication *in vitro* and potentially useful as insertion sites. In order to identify homologous sites in HVT U_S and to gain information about the organisation of HVT genes and their relationship to MDV genes mapping in U_S, we decided to sequence the entire U_S region (8.6 kbp) of HVT. This was a collaborative effort involving IAH and Rhône Mérieux laboratories (Zelnik et al, 1993).
Eight potential open reading frames (ORFs) were identified, 7 of which had counterparts in herpes simplex virus (HSV). The homologous proteins include U_S 1, U_S 2, U_S 10, protein kinase (U_S 3) and the glycoproteins gI, gD and gE. In addition, HVT contains one ORF which has a counterpart in MDV but is not homologous to any other known herpesvirus gene. It appears to be unique to Gallid herpesvirus

2 and 3. The % of amino acid identity between proteins encoded by the U_s of HVT and MDV ranged from 35 to 65, the most conserved protein being U_s 2. Most of the genes were collinear with those of HSV except for U_s10 which is transposed in MDV and HVT. A characteristic feature of HVT is the fact that approximately two thirds of the gE gene is located in the inverted repeats flanking U_s.

Construction and properties of HVT recombinants

(1) ***Expression of MDV gB at the TK locus of HVT.***

We reported previously (Ross *et al.* 1993) the construction of an HVT recombinant expressing the MDV gB gene at the TK locus under the control of the MDV gB promoter. Subsequent experiments at IAH have shown that the growth of the recombinant *in vivo* was impaired compared to wild type HVT, as shown by lower titres of antibodies to HVT antigens and lower numbers of infected lymphocytes. However, vaccination of a susceptible line of Rhode Island Red chickens (HPRS RIR) with 5000 p.f.u., a dose commonly used for vaccination in the field, provided total protection against challenge with the highly virulent strain RB1B of MDV. Moreover, we have also shown that vaccination with the recombinant was more effective ($P < 0.01$) than vaccination with a TK^- mutant of HVT which does not contain the MDV gB gene.

These results demonstrate that HVT has potential as a vector and that MDV gB is an important immunogen which can improve the protective immune response conferred by HVT alone. However, because the wild-type HVT vaccine also provided total protection against RB1B, we have been unable to determine whether the recombinant virus is more effective than the conventional HVT vaccine. We believe that the genotype of the chickens used in our experiments is an important factor in determining their response to vaccination and might explain the unexpected efficacy of wild-type HVT against challenge with the highly virulent RB1B virus. Further experiments using different lines of chickens are planned.

Additional evidence for the importance of MDV gB as an immunogen has been obtained using a fowlpox virus recombinant (provided by Rhône Mérieux) which expressed MDV gB. Rhode Island Red chickens vaccinated with the fowlpox recombinant at IAH were partially protected (50% surviving) whereas White Leghorns and a commercial line of chickens were better protected (75% survived, results obtained by Rhône Mérieux). These differences in the degree of protection could also be attributable to differences in the genotype of the chickens.

(2) ***Expression of infectious bursal disease virus (IBDV) VP2 gene at the ribonucleotide reductase locus of HVT.***

Experiments were carried out at Rhône Mérieux Laboratories to determine whether the small subunit of the ribonucleotide reductase (RR2) gene of HVT is a suitable locus for expression of foreign genes and whether sequences upstream of RR2 could function as a promoter to drive the expression of the IBDV VP2 gene. A transfer plasmid was constructed so that the entire RR2 open reading frame (ORF) was deleted and replaced by the VP2 ORF which started at the initiation codon of RR2 and ended at the RR2 stop codon. Co-transfection of the plasmid and infectious HVT DNA resulted in homologous recombination and led to the isolation of virus recombinants which were identified by screening for expression of the VP2 gene using a monoclonal antibody. One recombinant (vHVT1) has been purified by 4 successive cycles of selection. It grows normally in chick embryo fibroblasts(CEF). Analysis of vHVT1 DNA by the polymerase

chain reaction and Southern blotting confirmed the absence of RR2 sequences and showed that IBDV VP2 sequences were integrated at the RR2 locus of the HVT recombinant.

The results show that the RR2 gene is non-essential for *in vitro* replication of HVT in CEF and that sequences upstream of the RR2 initiation codon are able to drive the expression of genes inserted into the RR2 locus. In addition, the IBDV VP2 gene expressed by the HVT recombinant appeared to be structurally identical to the native protein since it was recognised by the monoclonal antibody which is specific for conformational epitopes of VP2. Protection experiments are in progress to evaluate the efficacy of the recombinant against IBDV challenge.

HIGHLIGHTS/MILESTONES

The potential of HVT as a vector has been established. Both the TK and RR2 loci of HVT may be used for insertion and expression of foreign genes. TK may not be an ideal insertion site since TK- virus does not replicate as well as wild-type HVT *in vivo*. Nonetheless, vaccination with 5000 p.f.u of TK- recombinant expressing MDV gB protected against MDV. Both the MDV gB and IBDV VP2 proteins synthesised by HVT recombinants were authentic. More work is required to identify insertion sites compatible with normal replication of the recombinants *in vivo* before the system can be exploited commercially.

The complete sequence of the U_s region of HVT has been determined. The results have advanced our knowledge of the genes mapping in the U_s of HVT, their relationship to the genes of MDV and other herpesviruses and have provided a solid basis for the exploitation of this region of the HVT genome for the expression of foreign genes and the construction of recombinant vaccines in the future.

WIDER CONSIDERATIONS

Much progress has been made in identifying the genes of HVT, a vaccine widely used for the contol of Marek's disease in chickens. The work done so far has also demonstrated that HVT can be used as a vector for expressing genes of other avian pathogens and that the prospects for developing multivalent vaccines and improved Marek's disease vaccines are encouraging. The results are not only of interest from a commercial viewpoint but are also of fundamental interest and have advanced our knowledge of the evolutionary relationship between the genes of avian and mammalian herpesviruses. The knowledge gained in the HVT/MDV system could be useful for the development of vaccines against other herpesviruses.

COOPERATIVE ACTIVITIES

The IAH and Rhône Mérieux Laboratories have continued to work very closely and have exchanged immunological reagents, cloned DNAs, virus recombinants and sequence data. A notable achievement of the joint effort has been the rapid sequencing of the entire U_s region of HVT. Both laboratories have benefited from the exchange of technical expertise. Meetings between IAH and Rhône Mérieux laboratories took place in Compton in June 1992 and in Lyon in February 1993. In addition, extensive discussions took place during the ELWW meeting in Denmark in November 1992. On this occasion, Professor Kaleta and his colleague from Giessen were also present and their contribution to the programme which is due to begin in August 1993 was discussed.

EUROPEAN DIMENSION

Research on Marek's disease is supported only at IAH in the United Kingdom. The BRIDGE programme on Marek's disease offers an opportunity to collaborate with other laboratories in Europe and is consequently invaluable to our research. Moreover, the opportunity to meet other groups working on similar and related topics during ELWW meetings has generated new ideas for ongoing research and new areas of research for the future.

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Development of second generation vaccines against parvoviruses (BIOT CT-910256)

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OBJECTIVES

Finc cpitopc mapping of Porcine Parvovirus (PPV) and Canine Parvovirus (CPV). Recombinant polypeptide expression. Optimization of the recombinant protein purification. Field trials.

RESULTS

INGENASA, Madrid

Epitope mapping of CPV.

The first objective was to make a topographical analysis of CPV virions and recombinant VP2 capsids. More precisely, to study the differences, if any, between natural and recombinant capsids. These differences could be important regarding to the vaccine development. The experiment was done using immunoelectron microscopy, labelling the particles with colloidal gold.

Those MAbs not previously recognized by PEPSCAN or immunoblotting, 4EA8,4B8 and 5F8 react positively with both types of capsids giving a clear staining. All the particles are surrounded by gold , about 3-4 particles of gold per capsid. These MAbs neutralize the virus very well and they are heavily positive with both types of capsids. These epitopes are obviously non linear and probably are formed due to the ensembling of several chains of VP2. They represent 50% of the neutralizing MAbs that we obtained. Preliminary data obtained in PPV indicate that in this virus the proportion of neutralizing MAbs that recognize non linear epitopes can be even bigger.

There are other MAbs, i.e. 4AG6 and 3C10 that gave some staining, possibly indicating that the epitope recognized by these two MAbs is partially hidden. This observation is in agreement with the position of the epitope on the edge of the canyon, which is surrounding the fivefold axis of the capsid.

Finally, there is another group of MAbs which are completely negative, they do not yield any labelling. The most appealing result is the presence of 3C9, also a neutralizing antibody, in this group. The location of this epitope had been previously identified by immunoblotting and PEPSCAN to be in the amino terminus of the VP2, also it was apparently absorbed by the capsids. Therefore, this result is somewhat contradictory with the absorption experiment. The conclusion of this experiment could be that the amino terminus is not fully exposed on the surface of the capsid.

The major conclusion of these experiments could be that the antigenicity, as the immunogenicity, of viral and recombinant capsids is almost equivalent, which gives more confidence on the usefulness of these recombinant capsids as subunit vaccines.

Recombinant polypeptide expression. Effect of different constructions on the level of VP2 expression

In the first construction reported, the level of VP2 expression was estimated to be around 5-10 μg per 10^6 cells. In this construction the gene was placed under the control of the polyhedrin promoter in the vector pJVP10Z where it is in competition with the strong p10 promoter. Since this modest level of expression, we have tried different alternatives to improve the production using two different strategies. We have made two new constructions. In one of them, we placed the VP2 gene in the vector pACdZ1, where the β-galactosidase is constitutively expressed under the control of the *hsp* promoter of *Drosophila*, much weaker than the p10 promoter. In the second one, we placed the VP2 gene in the vector pAcYM1, which does not use the β-galactosidase as a marker. Theoretically, the expression could be improved due to less competition between promoters. Finally, we have proved that the level of VP2 expression under the control of the polyhedrin promoter was quite irrelevant of the construction employed. The results indicate again that pJVP10Z and PAcYM1 give the same quantity of capsids , about twice the amount obtained with pAcDZ1.

The second strategy used to enhance the expression was to remove the 5'-nucleotides upstream the initiation codon and keep them at a minimum. This was done by synthesizing the gene by PCR amplification. Once synthesized by PCR, the VP2 gene was cloned also in the vector pAcDZ1. With this experiment , we aimed two objectives: 1. To demonstrate that a gene obtained by PCR was able to be used for the correct expression of VP2 and 2. If this proved to be correct, to study the effect of the PCR amplification on the VP2 expression.
The expression seems to be quite independent of the competition between promoters or of the presence or absence of 85 extra nucleotides at the 5'-end. On the other hand PCR amplification in combination with the Vent DNA polymerase has proved to be a good alternative for the synthesis of genes to be used in expression. These data suggest that other factors such as cellular toxicity of the expressed product may play a major role in the level of expression.

Downstream processing

Regarding to the protein purification procedure, we have developed an straightforward procedure, that can be easily scaled up to manufacturing levels. Basically, is to remove some time and labour consuming steps as sonication, using instead a lysis buffer, which also gives more reproducible results. From a regulatory point of view, a crucial step in this purification should be the removal of nucleic acids to guarantee that no manipulated, recombinant genetic material could be in the final sample.

Finally, we have started the scaling up of the insect cell cultures, from the shaken flask to a bioreactor. Basically, we have used a bioreactor equipped with a microsparger for aeration. We have done some experiments that show a good performance with doubling times of 24h, up to a cellular density of $3x10^6$ cells/ml in TNMFH-5% FCS in the presence of 0.2% pluronic poliol. Beyond this limit a perfusion or another continuous equipment seem to be necessary to guarantee an optimal feeding of the cells. The capsids production seem to be highly satisfactory.

Centre Veterinary Inst., Lelystad

Epitope mapping. Animal experiments.

A synthetic peptide vaccine was discovered that upon immunization gives solid protection in the host again a challenge with canine parvovirus. The selection of the peptide was a result of experiments with a range of different pentadecapeptides representing sequences that appeared antigenic in epitope mapping studies (PEPSCAN method) of the canine parvovirus capsid protein (Langeveld et al. 1993). The studies indicate, that a careful selection of the sequence for immunization is required to obtain neutralizing antibodies in rabbits. This knowledge will enable us to design peptides from the sequence of the capsid protein of the highly homologous virus: porcine parvovirus. Such synthetic peptides are currently under investigation to prepare virus neutralizing antibodies.

The findings of this synthetic vaccine method are being layed out in a manuscript entitled: 'Solid protection in dogs against infection with canine parvoviru after vaccination with synthetic peptides from a select region of the N-terminus of VP2'.

Epitope mapping studies by PEPSCAN with polyclonal antisera from pigs and rabbits yielded 6 antigenic sites in the VP2 molecule of PPV, one of which was only found in pig sera; another one only ocurred in rabbit sera in a domain homologous in position (but not in sequence) to a neutralization site of the canine parvovirus. The resulting domains are under investigation with respect to their immunogenicity and potency to induce neutralizing antibodies when injected as synthetic peptides in experimental animals.

SVIV, Lindholm

Field trials. Animal experiments.

Prior to artificial insemination, 6 gilts were vaccinated (twice with 3 weeks interval) with a vaccine based on the recombinant protein VP2 from porcine parvovirus (PPV) expressed in the baculovirus/insect cell system. Four non-vaccinated pregnant gilts served as controls. The ten gilts were challenged by intravenous inoculation of a virulent strain ('839') at about 40 days of gestation and sacrificied approximately 4 weeks later. At autopsy, the fetuses from the vaccinated gilts appeared clinically normal whereas the fetuses from the non-vaccinated gilts showed severe gross-pathological lesions, typical for intrauterine infections with PPV.

In order to determine wether transplacental infection with PPV had ocurred, all the fetuses were subjected to virological and serological examination. PPV was detected in all fetuses of the control gilts. While PPV-infection could not be demonstrated in the fetuses of 3 of the vaccinated gilts, PPV was detected in almost all fetuses of the remaining 3 vaccinated gilts by passage of the samples on cell culture, but not by standard ELISA techniques. Further challenge experiments in pigs are in progress to evaluate the relevance of these differences doing a dose-response experiment.

Conformational flexibility of Porcine parvovirus and empty capsids.

In order to use recombinant proteins for vaccination purposes, it is of great importance that the antigens used have the right conformation in order to elicit an adequate immune response. Several assays are used for this purpose, and the use of a panel of MAbs has been essential: ELISA, PLA, Western blot, Dot blot, immunogold labelling have been most commonly applied in our laboratories.

Furthermore, we are using an indirect affinity assay, based on the use of a chaotropic ion (SCN-) to break the binding between MAbs and the antigen. The main idea is that the stronger the Ab binds, the more SCN- is needed to break this binding. We have used this assay to characterize virions and empty capsids from naturally ocurring virus. Although only one MAb is able to differentiate between these two in conventional assays, several of the MAbs showed different reactivity profiles in the SCN- assay. Thereby this assay has shown information on differences not obtainable using other methods. Our results show that the epitope or the neighbouring structures are influenced differently in virions and capsids, perhaps revealing differences in conformational flexibility between virions and capsids. Such influence could be due to the presence of DNA in virions. We are currently investigating the recombinant VP2, and preliminary data indicate a similarity between VP2 and empty capsids.

HIGHLIGHTS / MILESTONES

1. Demonstration of the similar antigenicity of virions and recombinant empty capsids.
2. The efficacy of the recombinant capsids in the protection of gilts against the virus challenge was established.
3. For the first time, a synthetic vaccine has been formulated able to confer full protection to the host animals.
4. A patent application has been filed out.

WIDER CONSIDERATIONS

The combination of efforts from different laboratories with different expertises has allowed the succesful obtention of two putative vaccines, one recombinant and other synthetic. The three partners agreed to continue the development stage of the vaccines towards the registration and commercialization of these products.

COOPERATIVE ACTIVITIES

A joint meeting was held in Lindholm (Nov.92) during the ELWW meeting on veterinary viral vaccines.
Exchange of materials: Sera from dogs and rabbits immunized with syntetic peptidcs were sent from CDI to INGENASA for ELISA and neutralization tests. Purified virions, MAbs and polyclonal antivirus antisera were sent from INGENASA and SVIV to CDI for PEPSCAN and competition experiments. Recombinant capsids were sent from INGENASA to SVIV for immunization of gilts.

EUROPEAN DIMENSION

The collaboration of three different laboratories facilitates the good progres of the project and gives a better perspective of the whole situation of parvo vaccines in Europe.

PUBLICATIONS

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Engineering and immunogenicity of foot and mouth disease virus procapsids produced in insect cells (BIOT CT-900190)

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OBJECTIVES

(a) delimit sequences of FMDV which can be efficiently expressed within the baculovirus expression system

(b) determine requirement for myristoylation for capsid processing and assembly

(c) antigenic characterization of FMDV proteins expressed in insect cells

(d) preliminary immunogenic characterization of FMDV proteins expressed from cDNA

MAJOR PROBLEMS ENCOUNTERED

Although we have demonstrated previously that cDNA cassettes containing the P1-2A + 3C cassettes can be recombined into baculovirus the expression level has always been low. Analysis of a number of different recombinants has now established that the expression of the 3C protease, which is required for processing of the P1-2A precursor, severely inhibits late gene expression from the baculovirus. Thus in recombinants containing 3C the expression of the late protein P10 is low and the level of transcription of its mRNA is greatly reduced. Strategies to reduce the expression level of 3C have been devised and are being tested. The results section also shows that useful products can be synthesized in the absence of the activity of 3C.

RESULTS

A recombinant baculovirus (AcMM53) containing the type C FMDV capsid precursor P1-2A cDNA has been isolated and shown to express the expected product at high yield. This construct has had the FMDV L protein sequence precisely deleted and a new initiation codon introduced next to the P1-2A coding sequence. The protein can be readily observed both by ^{35}S methionine labelling of cells and immunoprecipitation with anti-FMDV antisera or by coomassie blue staining of total cell extracts.

It is estimated that 3-5% of the total cell protein is the expressed P1-2A product. The protein is also recognized in Western blot analyses using anti-FMDV monoclonal antibodies (MAbs) which recognize continuous epitopes. Further characterization of this FMDV product has been performed using an immunodot procedure. Polyclonal anti-FMDV serum and several Mabs known to recognize continuous epitopes on FMDV reacted with the P1-2A in this assay. Surprisingly, however Mabs that have been characterized as recognizing discontinuous epitopes on VP1 (1D) and VP2 (1B) also reacted with the uncleaved P1-2A. These results suggest that the P1-2A capsid precursor is folded in a manner which permits the

discontinuous epitopes to be presented. Control experiments have indicated that these discontinuous epitopes are lost if the samples are boiled prior to assay whereas the continuous epitopes are maintained following this treatment. The serotype specificity of the P1-2A product was also maintained. Mabs raised against FMDV serotype A (both A5 and A12) failed to recognize either the intact type C FMDV or the baculovirus expressed P1-2A product.

A truncated version of the type C P1-2A precursor has also been expressed from a second recombinant baculovirus (AcMM55), the expression level was about 1-3% of cell protein. This product is expected not to be processable *in trans* by 3C and probably adopts an unfavourable conformation. It may be a suitable control for the antigenic characterization of the P1-2A molecule within insect cell extracts. Medium scale (1L) cultures of insect cells infected with the AcMM53 recombinant baculovirus have been prepared to allow purification of the product for further antigenic and immunogenic characterization.

Mutations have been introduced into the sequence encoding the amino-terminus of the P1-2A, the aim of these modifications was to block the myristoylation of the capsid precursor. Analysis of these mutants within constructs expressing P1-2A+3C using vaccinia virus vectors has shown that normal processing of the capsid precursor occurs. However sucrose gradient analysis revealed that the empty capsid components failed to assemble into material sedimenting at 70S in contrast to the wild type sequences. Furthermore it was found that such constructs could be constitutively expressed within vaccinia virus. This contrasts with all previous attempts to express cassettes capable of producing authentic empty capsid components. Each cassette has proved to be incompatible with constitutive vaccinia virus expression. It appears that the formation of FMDV empty capsid particles is inhibitory to the vaccinia virus replication. Antigenic characterization of extracts prepared from cells infected with the myristoylation negative mutants suggested that the unassembled FMDV proteins had poor antigenicity. Furthermore these vaccinia viruses, despite expressing all the capsid proteins failed to elicit a protective response in guinea pigs against a challenge with FMDV.

HIGHLIGHTS/MILESTONES

(a) Efficient expression of FMDV capsid precursor P1-2A.

(b) Demonstration that the P1-2A product displays both linear and conformation dependent epitopes.

(c) Demonstration that myristoylation of the FMDV P1-2A is required for empty capsid formation and antigenicity.

WIDER CONSIDERATIONS

Foot-and-mouth disease virus continues to be a threat to the EC and indeed the first outbreaks of the disease since the cessation of vaccination in Europe (in 1991) have occurred in Italy in spring 1993. This amply demonstrates the importance of developing a vaccine based on non-infectious material as a control measure when sporadic outbreaks of disease do occur. The demonstration of efficient expression of the virus capsid precursor in the baculovirus expression system will be important in determining the utility of this system for the production of useful alternative vaccines.

COOPERATIVE ACTIVITIES

Participants met for a two day discussion meeting in Madrid in June '92 and also were able to present the results of our work and discuss future directions at the ELWW meeting on recombinant veterinary viral vaccines near Copenhagen in November '92. Extensive transfer of plasmids and recombinant baculoviruses occurs between the laboratories and the use of the baculovirus expression system is established in Pirbright and Madrid by transfer from Wageningen. Regular contact is maintained between the laboratories. As an example, the characterization of the properties of the FMDV P1-2A is being performed in three different laboratories using the expertise available. The recombinant virus was produced in Wageningen and the production of the molecule demonstrated there. The antigenic characterization has been performed in Madird using a panel of Mabs produced and characterized there. Immunoaffinity purification will also be performed in Madrid. Studies on the processability and myristoylation of the protein are being performed in Pirbright as will the initial studies on the immunogenicity of the product.

EUROPEAN DIMENSION

As indicated the project has involved the integration of diverse experience and quite distinct technologies to achieve the progress achieved. These studies would have progressed much more slowly in any one of the labs individually. A further useful result of the close contact between the laboratories is increased collaboration on activites outside of those identified within the BRIDGE programme, thus strengthening the science base of the EC.

JOINT PUBLICATIONS/PATENTS WITH TRANS-NATIONAL AUTHORSHIP

Medina M, Domingo E, Brangwyn JK & Belsham GJ (1993) The two species of the foot-and-mouth disease virus leader protein, expressed individually, exhibit the same activites. Virology **67**, 356-359.

Martinez-Salas E, Saiz J-C, Davila M, Belsham GJ & Domingo E (1993) A single nucleotide substitution in the internal ribosome entry site of foot-and-mouth disease virus leads to enhanced cap-independent translation *in vivo*. J. Virol. **67**, 3748-3755.

Towards a second generation vaccine against bovine herpesvirus type 1 (BHV-1): Immunological characterization of herpesviral glycoproteins and construction of BHV-1 deletion mutants (BIOT CT-900191

COORDINATOR:
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PARTICIPANTS:
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U. H. KOSZINOWSKI, Univ. of Ulm, Ulm, D

OBJECTIVES

Objectives were
(1) to further analyze genes coding for nonessential glycoproteins of BHV-1:
(2) glycoprotein B and glycoprotein H of murine cytomegalovirus and to express the respective genes using recombinant vaccinia viruses for the biochemical and immunological characterization of the glycoproteins;
(3) the construction and molecular and biological characterization of a BHV-1 mutant lacking the gene for the nonessential glycoprotein gE and
(4) to evaluate the activity of glycoprotein E specicific natural killer cells, T helper lymohocytes, cytotoxic T-lymphocytes and the proliferative response of lymphocytes isolated from mutant- and wtBHV-1 immunized cattle.

RESULTS

BFAV Tübingen: Work in this laboratory concentrated on the construction of recombinant vaccinia viruses expressing nonessential glycoproteins. In contrast to the other known nonessential glycoproteins of BHV-1, the open reading frame coding for gE resisted integration into vaccinia virus when constitutively active promoters were used for expression. None of four different promoter constructs including a synthetic vaccinia promoter resulted in the isolation of recombinants. This indicated that expression of gE is toxic for vaccinia. Therefore the gE ORF was cloned downstream the phage T7 promoter which is not active in normal eucaryotic cells. This construct could be integrated into vaccinia virus and the gE ORF is expressed after double infection of cells with a phage T7 RNA-polymerase expressing virus. Demonstration of the protein failed so far, because anti gE monoclonal antibodies obtained from Lelystad react with gE only if it is complexed with gI (see below). Because simultaneous infection of cells with three different vaccinia viruses gave unclear results, a T7' RNA-polymerase expressing cell line was isolated and will be used in future experiments to demonstrate expression of gE by the vaccinia recombinant.

The virological characterization of BHV-1 mutants lacking the genes for glycoproteins G or I proved that these glycoproteins are nonessential for BHV-1 and that the absence of gE, gG and gI does not significantly influence adsorption, penetration and reproduction of BHV-1.

CDI Lelystad: A mutant of BHV-1 that is deleted for the gene encoding glycoprotein E (gE) was constructed. This deletion mutant has been evaluated for virulence and immunogenicity in calves. Monoclonal antibodies (MAbs) were

produced, characterized and used in an epitope-specific ELISA to detect antibodies to gE.

Construction of the gE-deletion mutant: The position of the gE gene has been determined to be located in the unique short region of the genome. With the use of the cloned DNA fragments which flank the gE-gene, a gE-deletion mutant fragment was constructed. By using standard transfection procedures this deletion fragment was recombined in the genome of wild-type BHV-1 strain Lam, which resulted in three putative gE-deletion mutants. These mutants were further examined with the Southern blot technique, which showed that two of these mutants had exactly the desired deletion. One of these mutants was evaluated for virulence and immunogenicity.

Virulence and immunogenicity of the gE-deletion mutant: Three groups of six specific-pathogen-free calves were used. One group was intranasally inoculated with the parent strain, the second group with the gE-deletion mutant and the third was sham inoculated and served as unvaccinated control. The gE-deletion mutant hardly induced any disease sign and was excreted at a lower level than the parent strain. After challenge inoculation with the virulent Iowa strain the controls developed typical signs of BHV-1 infection, whereas the other two groups were clinically well protected. The gE-negative deletion mutant markedly reduced the excretion of challenge virus, although not to the same degree as the parent strain (Fig 1). In addition, calves inoculated with the gE-deletion mutant had a markedly lower virus excretion frequency than control calves after dexamethasone treatment. The results indicate that gE plays a role in the expression of virulence, and that gE-negative deletion mutants are highly immunogenic and promising as vaccine candidates.

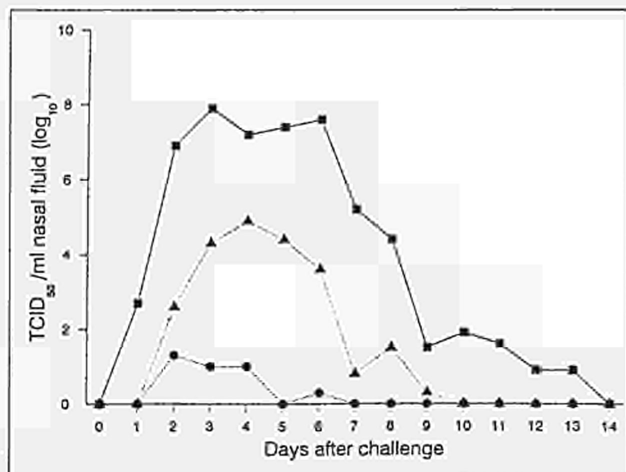

Figure 1. The nasal virus excretion after challenge exposure in calves (mean of six calves) that were, five weeks earlier, inoculated with the BHV-1 parent strain Lam (circles), with the gE-negative deletion mutant (triangles) or were sham inoculated (squares). Whereas the control calves shed virus for 13 days, the calves given the gE-negative deletion mutant shed virus for 8 days at approximately 1000-fold lower levels. Also the calves given strain Lam shed virus after challenge, indicating that even a previous infection could not prevent reinfection.

Prokaryotic and eukaryotic expression of gE: Fragments of the gE gene have been cloned in a prokaryotic expression vector, resulting in fusion proteins of the GST-protein and portions of the N-terminus of the gE. The entire gE gene has been cloned in a eukaryotic expression vector behind the HCMV ie-promoter. Transfection of this vector into Balb/c3T3 cells eventually lead to cells stably expressing gE.

Production and characterization of MAbs: MAbs against BHV1 were produced and characterized as being directed against gE with the use of gE-expressing eukaryotic and prokaryotic cells. When the gE-expressing cells were superinfected with a gE-negative mutant of BHV1 MAbs were identified that are likely to be directed against the gE/gI complex. Two of the MAbs were selected for developing an epitope-specific ELISA for the detection of antibodies against gE (gE-ELISA).

The antibody response against gE: The antibodies against gE were first detectable in calves eleven days after experimental infection and these persisted for at least three years.

Conventional vaccines: A gE-negative mutant of BHV1 was developed along conventional routes that was used for the production of a live and killed marker vaccine. It is expected that these vaccines along with the companion diagnostic test (gE-ELISA) will be marketed in 1994.

Conclusion: The gE-deletion mutants of BHV-1 are virtually avirulent and highly immunogenic for cattle. They therefore are suitable for inclusion in a vaccine. The greatest advantage of such a vaccine is that infected cattle can be distinghuised from vaccinated cattle.

University of Liège: The role of glycoprotein E (gE) in the cellular immune response to BHV-1 was studied using wtBHV-1 and a gE-deleted mutant.

Natural killer (NK) cell activity: In vitro cytotoxicity assays were performed to determine whether NK cells were able to lyse BHV-1gE^{-} as well as wtBHV-1 infected cells. The results indicate that the same frequencies of NK cells recognize the respective targets, suggesting that gE might not be involved in the NK target formation. However the kinetics of expression of the viral proteins is delayed in cells infected with the gE^{-} mutant and recognition by NK cells occurs later.

Helper T (Th) lymphocyte activity: Th lymphocyte activity was assessed by proliferative assays under limiting dilution conditions which provide quantitative results for the evaluation of a specific response against BHV-1. To test whether gE is an antigen involved in the proliferative response to BHV-1, the frequencies of Th cells specific for gE^{-}- and wtBHV-1 from immune cattle were compared. It appeared that the frequency of gE- specific Th cells is lower than the frequency of BHV-1 specific TH cells, suggesting a role for gE in the cellular immune response. Comparison of the ability of wtBHV-1 and the gE deletion mutant to raise a proliferative T cell response showed no differences in the frequencies of proliferating T cells after vaccination of cattle with the respective viruses.

Cytotoxic T lymphocyte (CTL) activity: Limiting dilution assays were also used to study the CTL response of BHV-1 immune cattle to gE^{-}-BHV-1 infected target cells. The current results indicate that gE-BHV-1 infected target cells are lysed at a slightly lower frequency than wtBHV-1 infected cultures. Further experiments involving gE expressing recombinant vaccinia virus are necessary to confirm the role of this glycoprotein in the CTL response to BHV-1.

University of Ulm: For the preparation of experimental vaccines not only the complete gB gene of the murine cytomegalovirus (MCMV) sequence but also a fragment lacking the part of the gene encoding the transmembranal region were expressed by vaccinia virus. Biochemical analysis of the expressed proteins revealed a difference between proteins expressed by the recombinant viruses and the authentic gB protein: whereas the native protein is cleaved when expressed at the membrane, the two vaccinia based constructs do not show this precursor-

product relationship. Moreover, the noncleaved precursor is not detectable at the cell membrane. So far it was not possible to define a difference at the sequence level that could explain this different fate of the proteins. Despite of the different protein maturation properties the constructs were used as vaccines to protect mice against the subsequent challenge with a lethal dose of MCMV. Both constructs had protective effects but the protective capacity of the full-length gB was superior to that of the N-terminal fragment. Remarkably, the protective capacity was limited and was not active when more than about four LD 50 were applied. The protective effector function could be clearly identified as the production of neutralizing antibodies, since the serum of the vaccinated mice had the same effect whereas the transfer of T-lymphocytes led to inconsistent results. The recombinant vaccinia viruses were used to produce monoclonal antibodies with specificity for gB. Remarkably, and diffent to usual vaccination protocols, mainly monoclonals of the IgM isotype were obtained which raises the suspicion that vaccinia itself has some general effects on the immune system which suppresses Ig class switching. The monoclonals that were obtained could be used for immunohistology, they were not active in neutralization. To obtain information on potential major linear epitopes for B cells in the gB sequence a cooperation with Dr.R. Meloen, Lelystad, was initiated. The whole sequence of gB was expressed as short overlapping peptides. So far, monoclonal antibodies and antisera from mice did not led to the clear identification of a dominant linear epitope. A second glycoprotein, gH, is also common between the family of herpesviruses and is also considered to represent a major target of the humoral neutralizing immune response. The respective gene in MCMV was located, the gene was almost completely sequenced, and, what is more important, the gene was inserted into vaccinia virus. As the natural gH gene represents a heterodimer, composed of gH and gL, it did not come as a surprise that also this protein did not acquire its natural configuration. Because the gB example taught that natural glycoprotein configuration was not essential for the induction of neutralizing antibodies also the vac-gH construct was tested and found positive in this respect.

HIGHLIGHTS/MILESTONES

The in vivo properties of one of the nonessential glycoprotein deletion mutants — the gE^{-}BHV-1 — were analyzed along with the immunologcal characteristics. The mutant is virtually apathogenic and highly immunogenic for cattle. It is concluded that such mutants are suitable for inclusion in a vaccine. The major advantage of such a vaccine is that infected cattle can be distinguished from vaccinated animals. It is expected that such a vaccine together with the companion diagnostic test (gE-ELISA) will be marketed in 1994.

Protection experiments with recombinant vaccina viruses expressing the glycoproteins gB and gH of murine cytomegalovirus showed that both glycoproteins confered protection against MCMV infection in mice by induction of virus neutralizing antibodies. Both glycoproteins have homologous counterparts in BHV-1 and it is reasonable to assume that these glycoproteins are important components of a vaccine against BHV-1.

WIDER CONSIDERATIONS

Second generation vaccines against BHV-1 should be save, efficient and allow differentiation between vaccinated and field virus infected cattle. The importance of essential herpesviral proteins like gB or gH for the induction of a virus neutralizing antibody response along with the biological and immunological properties of BHV-

1 mutants with deletion(s) of genes coding for nonessential glycoproteins support the strategy to develop negative marker vaccines on this basis.

COOPERATIVE ACTIVITIES

Joint meetings were held in Tübingen (30. 06. 1992) and during the ELWW meeting on veterinary viral vaccines in Praeskilde (Denmark, 26. — 28. 11. 1992). All partners of the project participate in this ELWW and made contributions for the revision of the 'Blue Brochure'. Scientists from all laboratories attended the 17th International Herpesvirus Workshop in Edinburgh (01. — 08. 08. 1992) where also aspects of the common project were discussed.

Exchange of material: Nucleotide sequences from gG of BHV-1 strains Schönböken and LA and antisera raised against vaccinia- and bacteria- expressed gG were sent from G. M. Keil to J.van Oirschot who provided G.M.Keil with the corresponding sequence of the BHV-1 strain Lam and monoclonal antibodies directed against gE. J. van Oirschot obtained from P. P. Pastoret monoclonal antibodies and provided P. P. Pastoret with blood samples from cattle inoculated with various BHV-1 strains for determining cytotoxic T-cell responses against BHV-1. G. M. Keil received vaccinia recombinants from U. H. Koszinowski for the preparation of antisera. The resulting sera were sent to U. H. Koszinowski. P. P. Pastoret gave data on the glycoproteins involved in the cellular immune response to G. M. Keil from whom he got a recombination plasmid for deletion of glycoprotein C.

Exchange of staff: Scientists from Liege repeatedly stayed in Lelystad for the collection of blood samples of infected cattle.

EUROPEAN DIMENSION

Transnational collaborations within Europe on the field of viral veterinary vaccines enable to concentrate research experience and technical 'know how' from laboratories of different countries for the development of new vaccines for use in the common market and worldwide.

JOINT PUBLICATIONS/PATENTS

Denis, M., Slaoui, M., Keil, G. M., Babiuk, L.A., Ernst, E., Pastoret, P.-P. and Thiry, E.(1993). Identification of different target glycoproteins for bovine herpesvirus type1-specific cytotoxic T lymphocytes depending on the method of in vitro stimulation. *Immunology* **78**: 7-13.

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AREA D:

PRENORMATIVE RESEARCH

- *IN VITRO* **EVALUATION OF THE TOXICITY AND PHARMACOLOGICAL ACTIVITY OF MOLECULES**
  (from page 303 to page 356)

- **BIOSAFETY**
  (from page 357 top page 426)

Development of human endothelial cell lines with preserved morphological and functional characteristics for *in vitro* toxicological and pharmacological testing (BIOT CT-900195)

COORDINATOR:
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PARTICIPANTS:
02 J. GORDON, British Biotechnol. Ltd, Oxford, UK
03 C. de CASTELLARNAU, Biomed. Res. Inst., Barcelona, E

OBJECTIVES

The objectives of the second period of work were:
(a) immortalize endothelial cells with new viral vectors;
(b) build vector set to manipulate oncogene dose;
(c) select oncogenes for further studies;
(d) characterize endothelial cell lines (retroviral).

RESULTS

Participant 01 is involved in immortalizing endothelial cells with retroviral vectors containing various genes and in establishing standard functional assays for these cells. During this period the following points have been achieved:
(1) two human endothelial cell lines with expanded life span;
(2) development of a new human endothelial line infected with polyoma middle T;
(3) development of murine endothelial cell lines infected with a new retroviral vector containing polyoma middle T;
(4) characterization of the endothelial cell lines for some biological parameters.

Part of the work was directed to characterize the cells that have been developed during the first year. In particular, two human cell lines infected with shc oncogene (Lshc SN1 and Lshc SN2) and two lines infected with ras oncogene (Lras SN1 and Lras SN2) have been expanded up to 54-70 population doublings and maintained in culture for more than one year. These cells retained cobblestone morphology and expression of specific endothelial cell markers such as von Willebrand factor, cadherin-5 and CD31/PECAM.

Northern blot analysis showed a good expression of cell mRNA for the infected oncogenes even at the highest cell replication number. The cell lines could not be activated by cytokines such as interleukin-1 and tumor necrosis factor to express specific adhesive molecules such as E-selectin and VCAM. Studies are underway to see whether this lack of reactivity is due to the loss of the specific receptors for these cytokines.

A new line of human endothelial cells has been developed by infecting the cells with the same retroviral vector used above in which has been inserted the polyoma middle T oncogene. These cells have been expanded up to 40 population doublings. They are positive to endothelial specific markers and retain cobblestone morphology. These cells, up to 40 doublings, express high levels of polyoma middle T mRNA by Northern blot analysis.

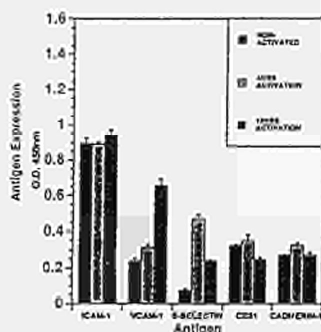
Additional studies have been done to develop immortalized mouse endothelial cells. These lines would be very useful to compare experimental data obtained in *in vitro* systems to *in vivo* models. Also in this case a retroviral vector containing the middle T oncogene has been used (N-TKmT, it expresses the selectable neomycin resistance gene from the Moloney murine leukemia virus long terminal repeat and the middle T oncogene from an internal thymidine kinase gene promoter). Endothelial cell lines have been obtained from heart, brain (B10V) and whole embryo (H5V). These cells present a normal endothelial cell morphology and express von Willebrand factor. In addition they can be activated by inflammatory cytokines such as interleukin-1 to express adhesive molecules (VCAM, ICAM and E-selectin), procoagulant activity, platelet activating factor, interleukin-6 and interleukin-1. When H5V cells were injected ino immunodeficient nude mice, but not in syngeneic recipients, they developed vascular tumors. Histologically the tumors consisted of large vascular lacunae, lined by endothelial cells and a prominent mononuclear cell infiltrate. Southern blot analysis of the established lesions revealed that only 5% of the cells in the neoplasm were trasplanted H5V cells.Thus H5V cells cause vascular lesions sustained to a large extent by recruitment of host cells and manifest full malignant potential only in immunocompromised hosts.These lesions have features similar to some vascular neoplasms in man such as Kaposi sarcoma.

Participant 02 has concentrated his work on immortalization strategies with oncogenic plasmids. Two oncogenes encoding SV40 Large-T-Antigen (SV40-LT-Ag) and c-myc have been selected.These oncogenes have been expressed under the control of the CMV and RSV promoters in order to observe differences in immortalization characteristics as determined by the level of gene product expressed.

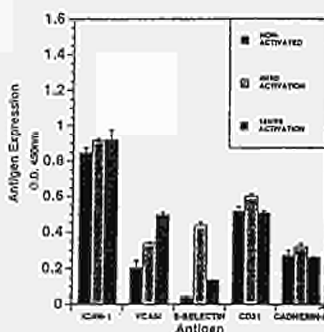
The SV40-LT-Ag can promote indefinite cell proliferation due to its ability to complex with the retinoblastoma protein and the p53 protein.These two proteins are known to block cell cycling in ageing cells and cause the cells to enter a crisis state leading senescence. The second approach is the expression of a de-regulated c-myc gene the product of which prevents cell cycle arrest into the G0-quiescent state and maintains cells in cycle. Another aspect of maintaining the cells in a continuous cell cycle is that the cells may die by apoptosis (programmed cell death). The oncogene bcl 2 is thought to co-operate with T-antigen or c-myc in promoting cell proliferations opposed to apoptosis. Co-transfection with a plasmid containing the bcl 2 oncogene may provide an extracomponent towards immortalizaton. Transfection of uman umbilical vein endothelial cells has been assessed using firefly luciferase as a reporter gene comparing calcium phosphate precipitation, DEAE-dextran transfection, electroporation and lipofection. The luciferase gene product converts luciferin into oxyluciferin and light is emitted. The amount of light produced indicates the efficiency of the transfection technique. Among the different techniques used elctroporation seemed the best for efficiency and cell survival. Cells transfected with the plasmid pDG24.4 (where the large T is under the control of the citomegalovirus promoter) and the plasmid pRSV-LT (where the large T is under the control of the Rous sarcoma virus promoter) by eloctroporation gave rise to two cell lines DG-CMV-LT-4 and DG-RSV-LT-2 respectively.

These cells express endothelial specific markers such as CD31 and cadherin-5 and can be activated by a cocktail of cytokines (interleukin-1, tumor necrosis factor and endotoxin) to express adhesive molecules such as ICAM-1, VCAM-1 and E-selectin up to passage 20-22.

CHARACTERISATION OF DG-RSV-LT-4 CELLS AT PASSAGE 5 BY ELISA

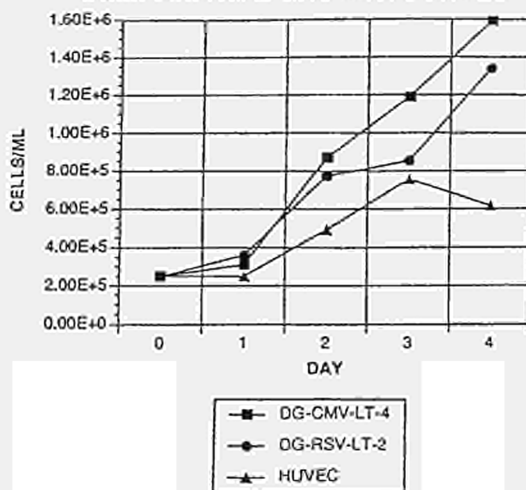


CHARACTERISATION OF DG-RSV-LT-4 CELLS AT PASSAGE 20 BY ELISA



The growth of DG-CMV-LT-4 and DG-RSV-LT-2 was compared to that of normal human umbilical vein endothelial cells.Both 'immortalized' cell lines exhibit an accelerated growth rate compared to normal cells.

COMPARATIVE GROWTH CURVES



Participant 03 started to apply the techniques set up during the previous period. In particular, this group analyzed the arachidonic acid (AA) metabolism of murine endothelial cell lines of different origin (skin, s-End; brain, b-End; thymic,t-End and embryonal, e-End). To establish the AA metabolic profile, the cells were

incubated with different concentrations of ^{14}C-AA and then the culture medium was analyzed by high performance liquid chromatography. The cell lines synthesized mainly prostaglandin (PG) E_2 followed by HHT, $PGF_{2\alpha}$, PGD_2 and HETE. Prostacyclin (PGI_2) the main eicosanoid formed by human endothelial cells of the umbilical vein was undetectable. The total eicosanoids synthesized through the cycloxygenase pathway were higher in t-End and b-End than in s-End and e-End. PGE_2 was the main metabolite and no differences in its production were found betwen the lines when thrombin-induced PGE_2 synthesis was performed. Exposure of the lines to inflammatory cytokines induced the production of PGE_2 and increased the converson of ^{14}C-AA to all eicosanoids only in t-End and b-End. No differences were found between IL-1α or IL-1β . These data indicate that in these lines PGE_2 is the main prostanoid produced in response to different stimuli. Heterogenity in response to cytokines among the lines was found with a maximal response in t-End and b-End.

HIGHLIGHTS / MILESTONES

(1) Three human endothelial cell lines developed by infection with retroviral constructs containing three distinct oncogenes (shc, ras and polyoma middle T) that retain endothelial specific characteristics up to 40-70 population doublings.

(2) Three murine endothelial cell lines infected by retroviral constructs containing polyoma middle T.

(3) *In vivo* assays for evaluation of endothelial cell line tumorigenicity.

(4) Optimization of human endothelial cell transfection using luciferase as a reporter gene.

(5) Construction of a panel of oncogenic plasmids

(6) Two human endothelial cell lines produced by transfection with two plasmids containing SV40 large T under the control of two different promoters.

(7) Characterization of these two lines for few specific markers and functional parameters.

(8) Characterization of arachidonic acid metabolism of four murine endothelial cell lines.

WIDER CONSIDERATIONS

The endothelium covers the internal lining of the cardiovascular system. Endothelial cell disfunctions are associated to the development of cardiovascular diseases such as myocardial infarction or atherosclerosis. The study of endothelial cells has been limited by their relative inaccessibility and short life span in culture. We are working on the possibility to develop immortalized endothelial cell lines to be used for pharmacological and toxicological tests in vitro. The general strategy is to insert into the cells new genes known for their ability to prolong the cellular life span. We are using different approaches (retroviral or plasmid vectors; different oncogenes) in order to obtain endothelial cell lines with expanded life but able to retain differentiated functional and morphological characteristics. These cells could constitute model systems for screening the pharmacological and toxicological potential of compounds and for identifying new therapeutic agents to combat aspects of endothelial cell disfunction.

COOPERATIVE ACTIVITIES

Meeting among participants: Madrid, 26-31 July 1992; London, 11-13 September 1992; Dublin, 8-10 December 1993.
Three months stage (from 1 March to 30 June, 1992) of C.De Castellarnau in E. Dejana Lab. Endothelial cell lines and materials have been continuously exchanged.

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Development of *in-vitro* neural systems for the identification of agents with toxicological and pharmacological potential (BIOT CT-900183)

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OBJECTIVES

Development of cultures and cell lines of neurons and glia in order to study basic pharmaco-toxicological mechanisms involving mostly glutamatergic and GABAergic neurotransmission to provide a basis for developing *in vitro* screening methods as defined in contract.

RESULTS

USTA has established a cell culture facility for primary neurons and astrocytes with help from RDSP. This facility has been used to further develop studies for development of screening methods for cytotoxic neuroactive agents and for development of screening methods involving end-points such as neurotransmitter release and reuptake, and receptor interaction and function.

The cytotoxicity screening studies involving cytoplasmic LDH release shows that N-methyl-D-aspartate (NMDA) and non-NMDA ionotropic receptors are involved in mediating cytotoxic actions of the neuroactive sulphur-containing amino acids (SAAs). NMDA and non-NMDA receptor antagonists can alleviate the SAA-induced cytotoxicity; moreover, a variety of signal transduction mechanisms involving cGMP and intracellular Ca^{2+} ($[Ca^{2+}]_i$) appear to be involved.

The normal and pathological action of endogenous excitatory amino acids (EAAs) such as glutamate (Glu) and the SAAs may also be mediated by the so-called metabotropic Glu receptors (mGluRs) which are coupled by G-proteins to a variety of cellular effectors. One such effector is the Ca^{2+}-dependent phospholipase-C which generates two intracellular messengers when hydrolyzing phosphatidylinositol. As part of the work for Year 2 of the contract, USTA have now completed a detailed study showing that SAAs exhibit contrasting mechanisms in stimulating phosphoinositide hydrolysis in primary cultures of cerebellar granule cells. Of much significance is the finding that one of the SAAs, L-homocysteine sulphinate (HCSA), acts as a selective agonist at the mGluR-1 subtype. HCSA could therefore be of much importance as a pharmacological probe at this receptor. These results also point to a contributory mechanism by which SAAs may induce neurotoxicity. USTA have also been involved in re-evaluating the proposed selectivity of commercially-available pharmacological agents. The application of cultured cells as *in vitro* models has been invaluable in neurochemically dissecting and characterising the precise mode of action of these agents.

Joint research with BIBRA on studies towards the development of screening methods for excitotoxic agents involving proto-oncogene expression end-points has been initiated. RDSP has further developed primary cultures of neurons and astrocytes in order to study neuro-differentiative and degenerative agents in such cultures to provide information of potential end-points for screening methods. The *in vitro* cell culture technology has recently been extended to include co-cultures of GABAergic neocortical neurons and cortical astrocytes. It has been demonstrated that this culture system can be used to study dynamic biochemical and physiological interaction between neurons and astrocytes with regard to GABAergic neurotransmission. This system appears to be useful for *in vitro* characterisation of drugs which interfere with GABA transporters and which therefore are potential anti-convulsants.

A neuronally secreted protein which induces astrocytic GABA transporters has been purified and partially characterised. Antisera have been raised using the N-terminal peptide sequence. This result is the first demonstration of neuronal regulatory control of an astrocytic function which directly influences neuronal activity, *viz.* neurotransmitter release. This complex regulatory situation stresses the importance of subtle end-points for screening methods.

As an extension of previous screening studies in which cultures of neurons and astrocytes were used to identify GABA transport inhibitors, it has been shown using a seizure prone mouse strain (FRINGE) that N-DPB-THPO (*N*-4,4-diphenyl-3-butenyl-4,5,6,7-tetrahydroisoxazolo[4 ,5-*c*] pyridin-3-ol) acts as an anticonvulsant *in vivo*. This is an example of the predictive value of in vitro cell culture model systems in development of anticonvulsant drugs.

Studies of the action of $GABA_A$-receptor agonists on development and differentiation of neurons in culture have been continued. It has been shown that treatment of neurons with THIP (4,5,6,7-tetrahydroisoxazolo[4,5-c] pyridin-3-ol) not only enhances the expression of GABAA-receptors but also leads to expression of voltage gated Ca^{++}-channels. Using immunogold labelling it has been shown that in neurons exposed to THIP, GABAA-receptors and Ca^{++}-channels are tightly spatially coupled, i.e. located in neuronal plasma membrane within 40 nm of each other. The functional significance of this finding is currently under investigation. In an attempt to further characterize the mechanism for the neurotrophic/differentiative effect of GABA agonists it has been demonstrated that polyamines, which are involved in regulation of cell growth, arc necessary for this action of GABA. If the normal metabolism of these compounds is arrested by treatment of neurons with an inhibitor of the rate limiting enzyme for the biosynthesis of polyamines, GABA agonists are unable to promote the morphological differentiation of neurons.

Neurotoxic actions of excitatory amino acids.

The mechanism of the neurotoxic action of excitatory amino acids (EAAs) has been further investigated particularly with regard to the role of intracellular Ca^{++}-homeostasis. Using cultured cerebral cortical neurons it has been demonstrated that different excitatory amino acid receptor subtype selective agonists induce neuronal damage by interference with Ca^{++}-homeostasis in different ways. Thus, N-methyl-D-aspartate appears to preferentially activate release of Ca^{++} from internal stores whereas AMPA ((RS)-2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionate) and kainate preferentially act by stimulating Ca^{++}

influx which, in turn , has little or no effect on intracellular Ca^{++} stores. A detailed study of the action of sulphur containing EAAs on Ca^{++}-homeostasis will be performed during early 1993 in order to further characterize their neurotoxic actions established during the period covered by this report. These findings will be important to defining neuroprotective strategies for excitotoxicity.

BIBRA has continued to study the actions of differentiative agents on neurons with the aim of developing screening methods for differentiative agents based on oncogene expression. The PC12 cell line is typically characterised as responsive to nerve growth factor (NGF) by arresting cell division and differentiating to a neuronal phenotype exhibiting many features typical of a sympathetic neuron. An early consequence of culturing PC12 cells with NGF is a significant increase in the expression of certain proto-oncogenes e.g. *c-fos, c-jun*. We therefore have two areas of interest when studying this particular cell line:

1) as a model of the molecular events associated with differentiation to a neuronal phenotype, and
2) as an established neuronal cell culture for studying the modulatory effects of drugs and chemicals.

Following treatment of PC12 cells with NGF (300 ng/ml of a 7.5s preparation) there was a transient expression of *c-fos* mRNA detectable as early as 15 min after treatment and declining to background levels by 120 min. The level of detection of the assay (dot-blotting) was about 5×10^5 cells. In collaborative experiments with Paul Rumsby's group at BIBRA we have developed a novel RT-PCR technique, employing an intron-containing competing fragment to increase sensitivity of the assay 100 fold; this allows us to analyse proto-oncogene expression in neural cell cultures established by our BRIDGE partners.

Expression of a related proto-oncogene, *c-myc*, was shown to be cell cycle related in PC12 cells. Following serum deprivation, a peak of *c-myc* expression occurred after 6 hours which was slightly increased in NGF treated cells. No significant modulation could be detected in expression of the *c-Ha-ras* proto-oncogene.

Both nicotine (10-100 µm) and muscarine (10-100 µm) appeared to induce a transient expression of *c-fos* mRNA in PC12 cells which had been cultured in the presence of NGF for 5 days or more. The level of *c-fos* mRNA expression was substantially less (about 5%) of that induced by NGF. The activity of acetylcholinesterase increased dramatically between 5 and 7 days after NGF treatment and stabilised at a level about 4-fold above baseline.

In collaborative experiments with TCDublin, PC12 cells were exposed to various benzodiazepine compounds in the presence or absence of NGF. Both diazepam (100 µm) and RO 5-4864 (100 µm) were able to induce *c-fos* mRNA expression in PC12 cells in the absence of NGF. When added simultaneously with NGF, diazepam, clonazepam, RO 5-4864 and PK 11195 could all super-induce *c-fos* mRNA expression in a dose-dependent manner.

In collaborative experiments with TCDublin, PC12 cells were exposed to the cytokines IL-1 and IL-6 in the presence or absence of NGF. PC12 cells were shown to respond to IL-6 (B-cell stimulatory factor) with a transient expression of *c-fos* mRNA; this effect was dependent upon serum deprivation and suggests that either IL-6 acts only at defined points in the cell cycle or that a natural inhibitor exists in serum. PC12 cells also responded to a high dose of IL-1β (100 ng/ml) with an increase in *c-fos* mRNA expression after 60 minutes exposure. No other effects were seen with this cytokine. However we were able to show that PC12 cells could express small amounts of IL-1α following 3-4 days treatment with NGF. Further

experiments in progress will assess the relevance of this apparent interaction between the immune and the nervous system.

CSIC has continued to develop and use brain slices and cultured neurons to study the toxicology of convulsant and non-convulsant polychlorocycloalkane pesticides at the level of synaptic function.

K^+-evoked [^{3}H]-Noradrenaline (NA) release from rat hippocampal slices was stimulated by relevant concentrations of γ-HCH (lindane, reduced by δ-HCH and diazepam) and was unaffected by α- and β-HCH, picrotoxinin, bicuculline and pentylene tetrazol. l- and n-type Ca^{2+} channels were not involved in γ-HCH stimulation of NA release. Voltage-dependent Cl- channels were involved in this stimulation as demonstrated by inhibition by 4,4'-di-isothiocyanato-stilbene-2,2-disulphonic acid. The present results demonstrate that neurotransmitter release studied in brain slices may be a useful assay method for testing the mode of action of convulsant and anti-convulsant agents on synaptic events.

Primary cultures of cortical and cerebellar neurons have been used to study the interaction of PCCAs with $GABA_A$ receptor function using a functional assay, $^{36}Cl^-$ uptake, and a binding assay for Cl^- channels, t-[^{35}S]-butylbicyclophosphorothionate (TBPS) binding. The organochlorine convulsant pesticide and lindane, aldrin, dieldrin and a-endosulphan completely inhibited GABA evoked $^{36}Cl^-$ uptake into cortical neurons in the concentration range (1-50 μM) with endosulphan being the most potent (EC_{50} 300 nm). The non-convulsant, tremor inducing organochlorine pesticide DTT was not inhibitory.

All organochlorine convulsant pesticides tested inhibited [^{35}S] TBPS binding to the $GABA_A$-receptor activated Cl^- channels with good correlation between their affinity for the Cl^- channel and their toxicity *in vitro* (LD_{50}). Stereoselectivity was also demonstrated for the HCCH isomers.

These results demonstrate the usefulness of primary cultures of GABAergic neurons in screening for interactions of convulsants and anti-convulsants with GABAergic synaptic transmission.

Studies of *in vitro* cytotoxicity of PSSAs (HCH isomers and cyclodienes) in cerebellar granule cells are in progress. Toxic effects are quantified by leakage of the enzyme lactate dehydrogenase from neural cells. Preliminary studies with g-HCH showed that cytotoxicity could be prevented in cortical cultures by adding GABA and benzodiazepines to the cell culture.

The interaction of HCCH isomers with secondary messenger system involving inositides has been studied using cerebral cortical and hippocampal slices, from developing (8-day) and adult rats in order to establish interference of these compounds with CNS maturation and plasticity. Both δ- and γ-HCCH isomers increased inositol phosphate (InsP) formation with *d*-HCCH being more potent, and with larger stimulations in the developing brain. This stimulation was receptor-mediated with effects on both glutamate and muscarinic systems. Subtle, but distinct, differences were observed for developing and adult brain slices and for different brain regions in interactions of HCCHs and neurotransmitter activation of InsP release. Further studies are in progress to study these effects in primary cultures of cerebellar granule cells.

HIGHLIGHTS / MILESTONES

(i) Re-evaluation of selectivity of action of commercially-available pharmacological agents of neurobiological significance.

(ii) Demonstration of the pharmacological potential of HCSA (an endogenous sulphur amino acid) as a selective agonist of the mGluR in primary cultures of granule cells.

(iii) The chloride transport blocker DIDS partially antagonized the γ-HCH facilitation of K^+-evoked release of NA in hippocampal slices.

(iv) Convulsant PCCAs (g-HCH or lindane, aldrin, dieldrin and a-endosulfan) inhibited GABA-induced Cl- uptake in cortical neuronal cultures.

(v) δ-HCH reduced InsP formation stimulated by glutamate and carbachol in regional brain slices of developing animals. Interaction of γ-HCH and δ-HCH with the InsP system depended on the maturation stage of the brain.

(vi) Development of competitive RT-PCR for the quantitative analysis of low-level *c-fos* mRNA expression.

WIDER CONSIDERATIONS

A number of pharmacological agents of neurological interest are commercially available. Since many of these compounds are used by researchers of multidisciplinary expertise to study the brain in healthy and disease states, it is important that the mode of action of such compounds be thoroughly investigated. Primary cultures of brain cells in combination with appropriately devised assays are proving invaluable as in vitro model systems in evaluating the mechanistic aspects of agents with pharmacological and cytotoxic potential.

COOPERATIVE ACTIVITIES

May 1992 — 2 workers from USTA went to RDSP for 14 days to undertake joint experiments on the use of fluorescent probes in monitoring intracellular Ca^{2+} levels in primary cultures of cerebral cortex neurons. October 1992 — 1 worker from RDSP went to USTA for 7 days — mutual exchange of techniques involving establishment of primary cultures of cerebral cortex neurons and second messenger assays. March 1993 — 2 workers from BIBRA went to USTA for 4 days to undertake joint experiments on screening for sulphur amino acids-induced expression of proto-oncogenes.
R. Rosa performed a short stay at RDSP labs to learn the methodology of Ca^{2+} determination by fluo-3 in neural cultures. Studies on HCH isomers were initiated.

Meetings:
All participants — Dublin — December 1992

Visits:
BIBRA — St. Andrews — March 1993 (3 day experiment — 2 people)

Material exchange:
Dublin — BIBRA — cytokines, benzodiazepines
BIBRA — St. Andrews — solutions for mRNA extractions, SAAs

Technology exchange:
BIBRA — St. Andrews — mRNA analysis of *c-fos*

Future collaborative experiments:
BIBRA — St. Andrews — SAAs and *c-fos* expression
BIBRA — Dublin — benzodiazepines, cytokines

EUROPEAN DIMENSION

The benefits of transnational collaborations outweigh any difficulties that have been encountered. The advantages are most obviously seen in

(i) the cost-effectiveness and efficacy of undertaking work requiring new technologies and/or a multidisciplinary input in the pursuit of information of mutual interest to the parties involved;
(ii) the additional incentives and responsibilities generated by working with peer groups;
(iii) the increased awareness of ongoing research activities — in the broadest sense — in partner countries.

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Definition and scientific validation of an *in vitro* test for the screening of tumor promoters and protective chemicals, based on gap junctional intercellular communication assays in human and animal cells (BIOT CT-910261)

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OBJECTIVES

Work on cytotoxicity evaluation for compounds being tested with the dye transfer assay; Exploration of gap junctional intercellular communication (GJIC) mechanisms through study of biochemical and molecular parameters: use of different cell types and study of the connexin gene expression; transfection of cells with specific connexin (Cx).

RESULTS

INRA laboratory studied the mechanisms of the stimulation of cell-cell communication. The stimulating effects of retinoids and flavonoids on communicating capacities of rat liver epithelial cells were studied at the molecular level. In all cases, the expression of connexin 43 was enhanced (followed by western-blot analysis for quantification and immunofluorescence for localisation). Depending on the molecule, this stimulation was related or not to an increase or stabilization of corresponding mRNA (northern blot analysis).

To assess the cytotoxicity of substances added to the culture media, the neutral red assay and proliferation assay were compared.

New cell systems have been developped: rat liver epithelial cells transfected with c-fos and c-jun proto-oncogenes; c-fos transfected cells have been characterized for their sensitivity to TGF-β. They will be used as model systems to study the mechanisms of action of cell-cell communication modulators.

Two aspects related to the possible role of gap junctional intercellular communication (GJIC) have been investigated in the **Unit of Multistage Carcinogenesis of IARC:** a possible tumor-suppressor function of GJIC by the use of connexin-transfected human cancer cells (HeLa), and a dye transfer assay for detecting putative tumor-promoting (or anti-tumor-promoting) agents both in the *in vivo* and *in vitro* situations.

The transfection of connexin genes into non-communicating cells may help to understand the role played by GJIC in the regulation of cellular physiology. As most of the human tumor cell lines tested so far do not communicate through gap

junctions, it is possible by this transfection strategy to see whether GJIC can 'normalize' those cells.

The human cervical carcinoma HeLa cell line transfected with different connexin (Cx) genes were used (in collaboration with **Bonn laboratory**) to see whether the recovery of GJIC was correlated with any change of their growth ability in both *in vitro* and *in vivo* (tumorigenicity) situations. Cx 40, 43 and 26 were used and, while all the transfected cells communicated extensively (tested by dye transfer), it seems that only the expression of Cx26 was able to significantly reduce the growth rate of the cells. The growth of Cx 26-transfected cells was markedly reduced (80% inhibition) in soft agar assay, but the most spectacular result came from the total inhibition of tumorigenicity, whatever the cell number injected into the nude mice (10^6 and 10^5 cells). This result is particularly interesting as Cx 26 was isolated as a putative tumor-suppressor gene in human breast cancer. Such experiments will be repeated with other Cx 26-transfected clones in order to confirm this result.

The *in vivo* dye transfer assay in rat liver, established in this laboratory, might also be suitable for testing the effect of potent rat liver tumor-promoting agents. Treatment with phenobarbital (PB), DDT, etc..., showed a marked inhibition of the *in vivo* dye transfer. Interestingly, among the chemicals tested, some flavonoids (collaboration with **INRA** and **L'Oréal**), suspected to be anti-tumor-promoting agents, induced a significant increase of GJIC in this assay.

At the **Karolinska Institute**, the effect of the skin tumor promoter, TPA, and the putative liver tumor promoter, endosulfan, (which are both potent inhibitors of GJIC), on the expression of Cx 43 has been investigated in the rat liver epithelial cell line, IAR20. Both compounds increased the phosphorylation of Cx 43 and reduced GJIC during the first hour of treatment, and in the TPA-treated cells, a slight reduction of the amount of Cx 43-protein was also observed after one hour treatment. However, after 4 hours treatment with TPA, a normal distribution of Cx 43 was observed which coincide with a normal GJIC. Endosulfan treatment did not affect the amount of Cx 43 but after 4 and 24 hours of treatment, when the communication was still totally inhibited, a reduced phosphorylation grade of the protein was evident. These results indicate that the two tumor promoters inhibit cell communication by different mechanisms.

The expression of Cx 32 in livers from rats treated with some PCB-congeners in an initiation/promotion assay has also been investigated with immunoblotting as well as immunostaining of liver sections. The planar 3,4,5,3',4'-PeCB induced a potent reduction of the amount of membrane bound fraction of Cx 32, whereas the non planar congener 2,3,4,3',4'-PeCB caused only a small decrease of the protein. In collaboration with **IARC**, it was demonstrated that Cx 32-mRNA was unaffected by treatment with either PCB-congener, which indicates an altered protein expression or function. However, it was observed that the Cx 26-mRNA level was slightly increased by 3,4,5,3',4'-PeCB treatment.

Brescia-Milan University works on the evaluation of chemical cytotoxicity on the IAR20 cell-system by comparative use of the neutral red and proliferation assays. Ten substances were analysed (lindane, trypan blue, rhodamine, caffeine, saccharin, dieldrin, 4-α-PDD, B-a-P, B-e-P and CCl4). As a general and constant observation, the neutral red assay, in spite of being technically simpler and quicker, demonstrated to be less precise and sensitive than the proliferation assay. While both methods gave the same ID 100 values for all tested compounds, the ID 80, 50, 20, and 0 values revealed a significative difference according to the assay used.

The results obtained with the neutral red assay demonstrated also higher degree of variability (within the same experiment) and lower reproducibility (among independent experiments) in comparison to the results obtained with the proliferation assay. In conclusion, the proliferation assay seems more suitable for the estimation of cytotoxic effect of chemicals prior to testing their possible time- and dose-dependent influence on intercellular communication.

The effect of protein kinase C (PKC) specific modulators on cultured bovine endothelial cells (EC) was studied to clarify the relation between PKC activity and junctional permeability (PKC activation has been proposed as a key event in down-regulating cell-coupling). The classical PKC activator, TPA, was found to inhibit in a dose and time dependent manner the EC-GJIC, this inhibition being continuous and irreversible. Neither the down-regulation nor the H7-induced specific suppression of PKC activity modified TPA effect. On the other hand, diC8 and bFGF, in spite of their PKC-activating capacity, failed to reproduce the TPA-induced inhibition of GJIC. Moreover, the growth factor caused an increase of the extent of EC-coupling. This observation suggests that PKC is not involved in EC-junctional gating regulation.

Work in the **Institute of Genetics at the Bonn University** is focused on the functional expression of homomeric mouse connexin channels in human HeLa transfectants. Most, if not all, mammalian cell types express more than one Cx protein. Probably these proteins form different gap junction channels between the same primary cells. In order to dissect the contribution of these channels to total GJIC, two approaches were followed:

(1) Antibodies to Cx 40, 37, 31 and 45 have been raised in addition to the previously characterized antibodies to Cx 32, 26 and 43.

(2) The mouse Cx genes for Cx 26, 31, 37, 40, 43 or 45 have been transfected into human HeLa cells which are defective in GJIC. The resulting transfectants express the expected Cx transcripts as well as protein, and are restored in dye coupling as well as electrical coupling. In HeLa Cx transfectants, the permeability of homogeneous Cx channels to fluorescent tracer molecules of different molecular size is compared. In addition, a collaboration with **Lyon, Brescia-Milan**, and **Stockholm** has been initiated to assay the effects of certain tumor promoters on GJIC using the HeLa Cx 43, 40 and 26 transfectants. Furthermore, the tumorigenicity in nude mice and other transformation parameters of HeLa Cx transfectants are measured in comparison with parental HeLa cells in collaboration with **IARC**. These studies should answer the question of whether restored GJIC correlates in HeLa transfectants with reduced tumorigenicity.

L'Oréal laboratory was mainly involved in cytotoxicity evaluation. The neutral red assay (FRAME In VitTox Protocol, number 3a, based on the uptake of a supra vital dye -neutral red- that accumulates in the lysosomes of viable uninjured cells) was selected to evaluate the toxicity of compounds before being tested in the dye transfer assay using IAR20 cells. In parallel, the MTT assay will be performed for the same purpose in **FRAME** laboratory. The objective was to harmonize toxicity evaluation in the different laboratories (INRA, Brescia-Milan University, IARC) and to examine if the neutral red protocol is more adequate than the other protocols which are already in use. For example, the cytotoxicity is determined by the cloning efficiency in the metabolic cooperation assay in V79 cells. However, different compounds were tested with the neutral red assay: 4-α-PDD, TPA, PB, DDT, BHA (compounds used for the standardisation of the dye transfer assay by

microinjection), as well as flavonoids. In general, the results with neutral red were more pronounced than those obtained with the cloning efficiency in V79 cells or the cell counting with IAR20. As Brescia group suggested in their concluding remarks, the proliferation assay (cell counting assay) seems to be more adequate than the neutral red assay in screening compounds for GJIC.

In collaboration with the **INRA** and **IARC** groups, additional experiments were performed to test modulating (antipromoting) effects of flavonoids on GJIC using metabolic cooperation assay in V79, scrape-loading and microinjection assays in IAR20 cells.

HIGHLIGHTS/MILESTONES

Bcsidc thc cvaluation of chcmical cytotoxicity for the dye transfer assay, studies were performed on the effect of potent tumor-promoting agents on GJIC mediated by specific connexins. New cell line systems were initiated with the use of HeLa transfectants; next studies shoud answer the question of whether restored GJIC correlates in those cells with reduced tumorigenicity.

WIDER CONSIDERATIONS

Cx-transfected HeLa cells can be ideal tools for studying the effect of tumor-promoting agents on specific connexins. This is an important question as tumor-promoting agents act in a tissue specific way. This could be correlated to the tissue specificity of expresssion of Cx. Such 'transfectant' system could be used as an assay for detecting putative tumor-promoting agents which may be tissue- (and therefore Cx-) specific.

COOPERATIVE ACTIVITIES

Meetings:

— BAMMIF, London, GB, 21-24/04/92: First European Workshop on Biotechnology Applications of Microinjection, Microscopic Imaging and Fluorescence; participation of INRA, Brescia-Milan University and L'Oréal.
— Collège de France, Paris, FR, 16/10/92: First meeting of french laboratories working on GJIC; participation of INRA, IARC and L'Oréal (presentation of the ELWW on Gap Junctions).
— EC-BRIDGE sectoral meeting, Dublin, IR, 8-10/12/92: In Vitro Evaluation of Toxicity and Pharmacological Activity of Molecules; participation of INRA, IARC, Brescia-Milan Univ., Karolinska Inst., Bonn Univ., FRAME and L'Oréal. Our group meeting took place there and contacts with some ELWW participants were also made during this meeting.

Exchanges and collaborations (1992-1993):

— **L'Oréal-INRA:** one day meeting every month to discuss results on flavonoids, exchanges of cells (V79 to INRA, IAR20 to L'Oréal).
— **L'Oréal-FRAME:** mailing of IAR20 cells and chemicals (4-α-PDD, TPA, PB, DDT, BHA) to Nottingham where they will perform cytotoxicity tests and the dye transfer assay (March,1993).
— **INRA-L'Oréal-IARC:** collaborative study of the effects of retinoids and flavonoids on GJIC.
— **INRA-IARC-Bonn University:** exchanges of molecular tools and protocols to study modulator induced changes of Cx regulation.
— **IARC-Karolinska Institute:** Improvement of immunostaining technique (April 92).

- **IARC — Brescia & Milan University:** Work on immunostaining techniques and Cx gene transfection.
- **Karolinska Institute-IARC:** 1 month stay in order to improve the measurement of immunostained tissues and cells by using a new image analyser in Stockholm (Dec. 92).
- **Karolinska Institute-L'Oréal:** 1 day meeting in Stockholm to discuss cytotoxicity, image analyser performances and structure-activity relationships (Nov. 92).
- **Bonn University-IARC-Karolinska Institute:** exchanges of Cx gene transfected HeLa cells.

Interactions with other BRIDGE groups:
- London (April 92): BAMMIF (organized by P. BACH, BIOT- CT91-0266); abstracts of the workshop will be published in Plenum Press (1993);
- Dublin (Dec.92): EC-BRIDGE sectoral meeting, organized by Cl. WILLIAMS, (BIOT- CT90-0183) with the BRIDGE groups of the pre-normative research area; highlights of the meeting will be published in ATLA (1993).
- Paris, meetings between the coordinators of 2 other BRIDGE groups (A. BARBIER, BIOT- CT90-0186; and B. COULOMB, CT90-0193) and ours to: — form a new ELWW (6/07, 27/07 -92) and prepare the 'blue brochure' in the field of 'In vitro integrated approach to skin pharmaco-toxicology'; — to organise (19/03/93) the first ELWW meeting on 'Cellular Interactions' for Nov.93.

EUROPEAN DIMENSION

After the publication of the 1992 Progress Report and our ELWW brochure 'In the field of: GJIC and screening of tumor promoters', new european laboratories contacted us to exchange information and to evaluate the possibility to join the ELWW and to collaborate with some of our partners. We will soon be able to set up a real european network in the areas of gap junctions and skin pharmacology.

JOINT PUBLICATIONS/PATENTS WITH TRANS-NATIONAL AUTHORSHIP

BEX V., HONIKMAN-LEBAN E., MAZET F., FLECHON B., MERCIER T., SHAHIN M. M. and MARTEL P., In vitro modulation of gap junctional intercellular communication by retinoic acid: functional, molecular and structural studies, VI International Congress of Toxicology, Rome, June 28-July 3, 1992.

MERCIER T., HONIKMAN-LEBAN E., CHAUMONTET C., MARTEL P. and SHAHIN M. M. (1993) Studies on modulating effects of retinoic acid and retinol acetate using dye transfer and metabolic cooperation assays, Fundam. Appl. Toxicol. (submitted to publication).

MESNIL M., PICCOLI C., WILLECKE K. and YAMASAKI H., Involvement of gap junctional intercellular communication aberration in human multistage carcinogenesis: its use as a carcinogen detection assay, EC-BRIDGE sectoral meeting, Dublin, Dec. 8-10, 1992.

OTHER PUBLICATIONS/PATENTS

CHAUMONTET C., MARTEL P., SANCHEZ I., SEILLAN C. and SUSCHETET M., Effects of flavonoids on gap junctional intercellular communication of rat liver cells, Food and Cancer Prevention, Norwich (UK), Sept. 13-16, 1992.

FRANSSON-STEEN R., FLODSTROM S. and WARNGARD L. (1992) The insecticide endosulfan and its two stereoisomers promote the growth of altered hepatic foci in rats, Carcinogenesis, 13, 2299-2303.

FRANSSON-STEEN R.and WARNGARD L. (1992) Inhibitory effects of endosulfan on gap junctional intercellular communication in WB-F344 rat liver cells and primary rat hepatocytes, *Carcinogenesis*, **13**, 657-662.

HEMMING H., FLODSTROM S., FRANSSON-STEEN R., WARNGARD L. and AHLBORG U. G. (1992) Inhibition of intercellular communication in cells in culture by polychlorinated compounds, *Chemosphere,* **25**, 939-945.
HENNEMANN H., DAHL E., WHITE J. B., SCHWARZ H. J., CHANG S., LALLEY P. A., NICHOLSON B. J. and WILLECKE K. (1992) Two gap junction genes, connexin31.1 and 30.3, are closely linked on mouse chromosome 4 and preferentially expressed in skin, J. Biol. Chem., **267**, 17225-17233.
HENNEMANN H., SUCHYNA T., JUNGBLUTH S., DAHL E., SCHWARZ H. J., NICHOLSON B. J. and WILLECKE K. (1992) Molecular cloning and functional expression of mouse connexin40, a second gap junction gene preferentially expressed in lung, *J. Cell Biol.,* **117**, 1299-1310.
HONIKMAN-LEBAN E., CATROUX P., METEZEAU P., KIEFER H., BAUMANN I., COTTIN M., ROUGIER A., DOSSOU G. K. and SHAHIN M. M. (1992) Possible application of image laser cytofluorometry in cellular dye transfer and in determination of free calcium, *Biol. of the Cell,* **76(2)**, 250.
HONIKMAN-LEBAN E. and SHAHIN M. M., Gap junctional intercellular communication and screening of potential tumor promoters, World Congress on Cell and Tissue Culture, Washington D. C., June 20-25, 1992.
LAGARRIGUE S., SEILLAN-HEBERDEN C., MARTEL P.and GAILLARD-SANCHEZ I. (1992) Altered response to growth factors in rat epithelial liver cells overexpressing human c-fos protein, *FEBS Letters,* **314(3)**, 399-403.

FIRST EUROPEAN WORKSHOP ON BAMMIF, London, April 21-24, 1992
Abstracts of the workshop will be published in Plenum, (in press)

CHAUMONTET C., ZANETTI C., FLECHON J. E. and MARTEL P., Application of microinjection and immunostaining techniques to the study of the stimulation of gap junctional intercellular communication.
HONIKMAN-LEBAN E. and SHAHIN M. M., Comparison of fluorescent dye transfer in intercellular communication studies using scrape-loading and microinjection techniques.
MAZZOLENI G., CAMPLANI A., TELO P., TANGANELLI S. and RAGNOTTI G., A highly sensitive video recording system connected to a microinjector helps the quantification of results and the time-laps monitoring of cellular events.

EC-BRIDGE sectoral meeting, Dublin, December 8-10, 1992:
Highlights of the meeting will be published in ATLA (1993)

CHAUMONTET C., BEX V., MERCIER T., LAGARRIGUE S., SANCHEZ I., SEILLAN C. and MARTEL P., Modulation of gap junctional intercellular communication in rat liver epithelial cells.
COOPER A., FENTEM J. and BALLS M., Inhibition of intercellular communication: an *in vitro* assay for detecting potential tumor promoters.
HONIKMAN-LEBAN E. and SHAHIN M. M., Gap junctional intercellular communication: an assay for screening of tumor promoters and 'antipromoters'.
MAZZOLENI G., CAMPLANI A., TELO P., TANGANELLI S. and RAGNOTTI G., Modulation of GJIC in cultured endothelial cells.
MESNIL M., PICCOLI C., WILLECKE K. and YAMASAKI H., Involvement of gap junctional intercellular communication aberration in human multistage carcinogenesis: its use as a carcinogen detection assay.
WARNGARD L., FRANSSON-STEEN R. and HEMMING H., Inhibition of dye transfer induced by environmental pollutants and its relevance for tumor promotion.
WILLECKE K., ELFGANG C., LICHTENBERG-FRATE H., BUTTERWECK A. and TRAUB O., Functional expression of homomeric mouse connexin channels in human HeLa transfectants.

Development of a predictive *in vitro* test for detection of sensitizing compounds (BIOT CT-900186)

COORDINATOR:
1) A. BARBIER, Sanofi Recherche, Montpellier, F
PARTICIPANTS:
2) J. BOS, Univ. of Amsterdam, Amsterdam, NL
3) J. KNOP, Universität Mainz, Mainz, D
4) J.P. LEPOITTEVIN, ULP, Strasbourg, F

OBJECTIVES

Participants 1, 2 and 3: : Determination of optimal conditions for hapten-processing in vitro using respectively guinea-pig, human, and murine cells.

Using epidermal cells prepared according to the optimized conditions previously determined, the search for the best conditions of hapten processing in vitro have to be performed.
Validation of this haptenization has to be done with the several sensitizers or irritants prepared by participant 4 by measuring cytokine production and/or morphological analysis of the cell surface markers.

Participant 4: Synthesis of 5-Me-PDC, 6-Me-PDC and of soluble derivatives of PDC.

The contribution of the Dermatochemistry laboratory is to provide the other teams with reference, standardized haptens. As the major difficulty for in vitro assays is the testing of insoluble compounds, the main work of this laboratory for the reporting period is also to prepare soluble derivatives of sensitizers and non-sensitizers.

MAJOR PROBLEMS ENCOUNTERED

The major problem encountered by participant 1 was the difficulty to find specific markers or antibodies for guinea-pig cytokines or other proteins. This difficulty lead to limit the evaluation to cytokines for which biological test is applicable.

Moreover, J.L. Stampf, the researcher in charge of this project for personal reason stopped definitively his work on October. He has been replaced by Dr H. Rizova, only on February the 15th.

For participant 2, the main difficulty was the obtention of isolated human Langerhans cells (LC) in a reproductible manner and in sufficient quantity. This difficulty lead the team to replace these cells by human peripheral blood monocytes since these cells are like LC, MHC class II expressing, bone-marrow derived antigen presenting cells (APC).

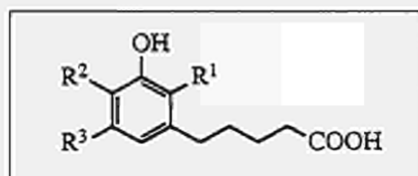
The participant 3 encountered his major difficulty in testing water-insoluble haptens like PDC and oxazolone, in epidermal LC internalization assay. This will lead to test new hydrophilic derivatives or micellation of chemicals by methods to be defined.

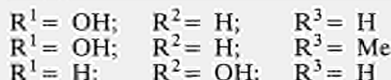
RESULTS

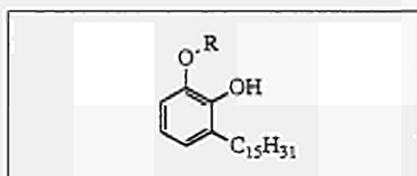
Participant 4: Synthesis and evaluation of water-soluble analogs of lipophilic haptens.

The major problem with organic xenobiotics is their low solubility in culture medium. Some of them (DNCB, DNFB, Oxazolone..) are slightly soluble in water but others as PDC (the main allergen from poison ivy) are not. We have developed two approaches to this problem. The first one is to introduce water soluble functional groups far from the reactive sites of the molecule, the second one is to synthesize pro-drugs by introducing water soluble groups able to be removed by cell metabolism, change in pH....., in order to release the hapten in situ.

These two approaches have been applied to PDC and analogs, leading to water soluble compounds. These compounds are under in vivo evaluation to check the conservation of allergenic property. These compounds will also be tested in vitro by coparticipants.



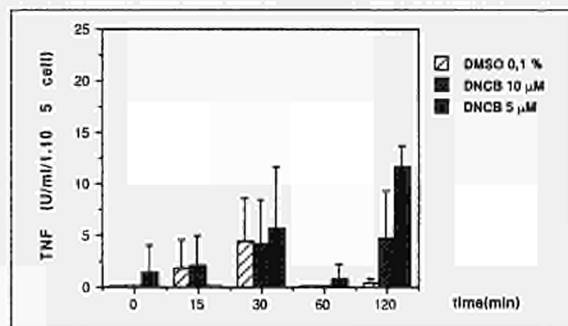

R^1 = OH; R^2 = H; R^3 = H
R^1 = OH; R^2 = H; R^3 = Me
R^1 = H; R^2 = OH; R^3 = H



R = β-Glucose R = α-Mannose
R = β-Galactose R = β-Xylose

Participant 1: Determination of optimal conditions for hapten-processing using guinea-pig cells.

As results obtained in the first year showed some difficulties with cell suspensions (high background production of cytokines linked to the cell preparation), the second year-program was carried on with the determination of the optimal conditions as regards the haptenization of epidermal cells in culture: simplified Green medium with feeder layer, hapten solubilized either in medium or in DMSO 0.1%, length of pulse 30 minutes (to avoid non-specific response, for example SLS induced after 1hour of contact the secretion of IL-6), washing, sampling of the medium at various times for cytokine measurements.

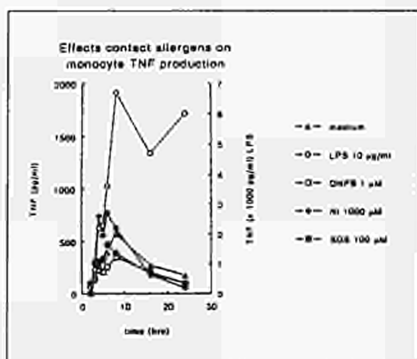


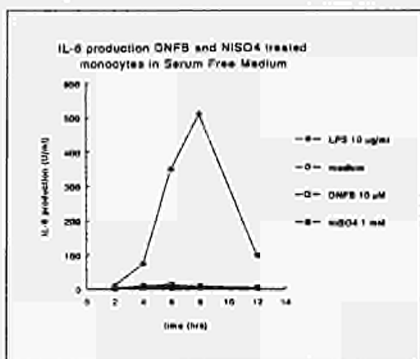
As specific markers or antibodies for guinea-pig are not available, measurements were limited to two cytokines, for which bioassays are possible: IL-6 (B9 cell line assay) and TNFα (L929 cytolytic assay), and to PGE2 for which a radioimmunoassay is available.
(Figure 2)

In these conditions, results showed that IL-6 and PGE2 production was not specifically enhanced by sensitizers versus irritants, and TNFα production was always small and extremely variable (see Figure 2 on the previous page).

Participant 2: Effects of contactallergens and irritants on human peripheral blood monocytes

One major problem of the monocyte system is the high background production of IL-6 and TNFα, and probably other monocyte produced cytokines like IL-1 and IL-8. In order to lower cytokine background production we decided to culture monocytes for 24 or 48 hours in so-called serum-free macrophage medium (SFM). This was done because of activation of monocytes either during adherence to the plates or by the presence of endotoxins in sera fractions in the culture media. After isolation, peripheral blood monocytes were cultured in 24-well plates in SFM. After 24 or 48 hours adherent monocytes were treated with LPS, $NiSO_4$ and DNFB for 30 min. at 37°C, 5% CO_2. After pulsing cells were washed (in plate) three times and cultured further in SFM. At different time points supernatants were taken for studying cytokine contents. Results of two experiments show that background production of monocyte IL-6 production is drastically reduced to 1-5 U/ml in each time point. However, the same reduction in IL-6 production appeared in LPS control (See figure below). This is about 20-40% of LPS responses in the experiments in normal serum-containing medium. IL-6 production of monocytes after incubation with DNFB and NiSO4 were at the level of the background: no significant changes in IL-6 production.





In the same experiments MHC class II (i.e. HLA-DR) expression of monocytes was investigated after 4 and 24 hours following treatment with compounds: no major changes in HLA-DR expression could be found.

Activation of Antigen Presenting Cells (APC) might be dependent on binding of stimulatory agents to proteins present. Since in SFM there are no protein present, this might partially explain the results in IL-6 production after treatment with sensitizing agents. In the future the experiments described above will be done with the exception of adding protein sources to the monocyte cultures during treatment with contactallergens and irritants. This protein source can be endotoxin free Bovine Serum Albumin.

Participant 3: Cytokine induction and mRNA analysis on cultured keratinocytes and modulation of receptor-mediated endocytosis after contact with haptens

The production of TNFα and IL-8 specific mRNA by long term-cultured human keratinocytes and HaCaT cell line was studied in vitro by Northern blot analysis. A strong upregulation was seen for IL-8 specific mRNA during the first hours after stimulation with haptens like PDC and DNFB as well as the tolerogen 5-Met-PDC and the non-immunogenic derivative PDV. Comparable data were obtained for SLS. Therefore no-hapten specific effect on the regulation of relevant keratinocyte-derived cytokines was detectable.

Further progress was made in developing a suitable test system for endocytotic activation of murine epidermal Langerhans cells (LC). According to methods described in the last annual report, internalization experiments using fluorochrome labelled second-step antibodies were performed. Beside a semi-quantitative assessment of activation by monitoring the intracellular distribution of internalized material and calculating a stimulatory-index these methods can be used for flow cytometric analysis. The pulse-width of intracellular structures containing internalized fluorochrome-coupled antibodies reflected the state of activation during treatment with different sensitizers, irritants and non-immunogenic compounds. A good correlation was found for this parameter and the stimulatory-index calculated from direct analysis of cells by fluorescence microscopy. This method will allow a more rapid and reliable performance of in vitro testing. A promising attempt to transfer these data to the human system was made. Dentritic cells (DC) from human blood were found to exhibit a comparable pattern after stimulation with haptens as seen for murine epidermal LC. DC could be distinguished from other MHC class II-bearing cell types by their lack for certain cell-lineage-specific markers. An optimized method using human DC for testing of activating properties of haptens in the human system is in development.

HIGHLIGHTS / MILESTONES

When irritant-contact effects are compared, no specific cytokine production by cells following hapten contact has yet been found, either using guinea pig and murine epidermal cells or human monocytes.

By contrast promising results were obtained when considering the specific endocytosis of class II molecules in LC following contact with sensitizers.

In view of these differential results, the teams of this BRIDGE program will focus, for the next year on the confirmation and the validation of the class II molecules endocytosis in LC as a possible in vitro test for detection of sensitizers.

WIDER CONSIDERATIONS

Allergic Contact Dermatitis, a distressful disease is induced by substances present in our environment (household products, cosmetics, plants, jewellery....). New molecules, especially those which may enter in contact with skin, have to be tested to assess their non-sensitizing effect. Up to now, only in vivo methods are used for this safety assessment and are recognized in the guidelines. The scientific community has become aware of the necessity of reducing when possible laboratory animal testing by alternative in vitro studies. It is the purpose of our investigations. Results obtained after 2 years of this program, show that some of the expected possible in vitro model (as production of cytokines by epidermal cells) would not seem able to discriminate sensitizers to irritants, whereas other tests (as

endocytosis of class II molecules by Langerhans cells) are very promising and are now under confirmation and validation.

COOPERATIVE ACTIVITIES

Two work meetings were organized with all participants in this second year of the Bridge project The first meeting was held in Amsterdam, on the occasion of the 11th meeting of the European Research Group on Experimental Contact Dermatitis, October 13, 1992. The second meeting took place at Mainz University, March 9, 1993. An additional informal meeting between the four teams of the project was held on the occasion of the Dublin CEC meeting (8-10 december, 1992). The Dublin meeting was also the opportunity to meet all the other teams involved in the ELWW on 'In vitro integrated approach to skin pharmaco-toxicology' and to decide the organization of the first official meeting of this ELWW.

The booklet of this ELWW has also been finalized.

Cooperative activity was expanded to intergroup technician formation: a technician from Montpellier spent one week in Mainz to take in hand some technics developed in Pr Knop's laboratory.

EUROPEAN DIMENSION

This BRIDGE program is the occasion to fruitful development of contact between several teams working in the same area. This cooperation was not easy to put in place: one year has been necessary to get mutual confidence. Now, this cooperation is fully effective with a lot of basic or technical exchanges. The second year of this program is also the beginning of expanded exchange between ELWW teams. We hope that the forthcoming meeting of this ELWW will be the occasion of additional international collaborations.

JOINT PUBLICATIONS / COMMUNICATIONS:

Barbier A., Stampf J.L., Lacheretz F., Bos J., Kapsenberg M., Pistoor F., Knop J., Becker D., Kolde G., Lepoittevin J.P., Mabic S. Development of a predictive in vitro test for detection of sensitizing compounds. Communication at the C.E.C. Sectoral Meeting on In Vitro Evaluation of the Toxicity and Pharmacological Activity of Molecules, Dublin 8-10 December, 1992.
European Laboratory Without Walls: In the field of In Vitro integrated Approach to Skin Pharmaco-Toxicology, DGXII, C.E.C. 1993

OTHER PUBLICATIONS / COMMUNICATIONS

Communications at the C.E.C. Sectoral Meeting on In Vitro Evaluation of the Toxicity and Pharmacological Activity of Molecules, Dublin 8-10 December, 1992:
Barbier A., Stampf J.L., Pelegrin M., Lacheretz F. In vitro effects of sensitizing compounds. The Guinea-pig cell culture model: Preliminary results.
Knop J., Becker B., Mohamadzadeh M. In vitro induction of epidermal cytokines and endocytic uptake of MHC class II molecules by contact sensitizers.
Mabic S., Lepoittevin J.P. Synthesis and evaluation of water-soluble analogs of lipophilic haptens.
Pistoor F., Bos J., Kapsenberg M. Contactallergens induce cytokine production in human peripheral blood monocytes in vitro.

OTHERS

Barbier A. Mise au point d'une méthode In Vitro pour la détection de substances sensibilisantes. Communication au stage de formation permanente: Les méthodes alternatives en toxicologie expérimentale, I.U.T. Montpellier, 11 september, 1992

Barbier A. Développement in vitro de tests prédictifs pour la détection d'agents sensibilisants. Communication aux 4èmes Journées de Pharmacologie Cutanée, D.E.A. de Biologie Cutanée et Cosmétologie, Paris, 26 March, 1993.
Mabic S., Lepoittevin J.P., Stampf J.L., Pare M., Benezra C. Induction of tolerance to poison ivy allergens in mice using water soluble derivatives of catechols. Eleventh Meeting of the European Research Group on Experimental Contact Dermatitis, Amsterdam 12-13 October, 1992
Mabic S., Lepoittevin J.P. Glycosylation of catechols. Journées Janssen-Chimie Strasbourg, Strasbourg 17 March, 1993

Pharmacology and toxicology of differentiated cell types, their cell-cell and cell-matrix interactions in an *in vitro* reconstructed human skin model (BIOT CT-900193)

COORDINATOR:
B. COULOMB, L. DUBERTRET, Hôp. H. Mondor, Créteil, F
PARTICIPATES:
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2) Ch. LAPIERE, Univ. Liège, Sart-Tilman, B
3) Th. KRIEG, Univ. Köln, Köln, D
4) N. FUSENIG, DKFZ, Heidelberg, D
5) P. GIACOMONI, L'OREAL, Clichy, F

OBJECTIVES

The objective of this program is to reproduce physiological or pathophysiological cellular regulations using in vitro reconstructed human living skins to develop predictive pharmaco-toxicological models.

The living human skin equivalent models were completed with different cell types (normal or pathological) or by adding other components of the extracellular matrix and new parameters for analysis have been defined.

During the second year of the program, among the multiple interactions that the skin models allow to modulate, several cell-cell and cell matrix interactions have been shown to be determinant in the pharmacological or toxicological cell response. The better understanding of the mechanisms involved already permits to study wound healing promoters, antipsoriatic drugs and pigmentogenic with these skin models. Again, exchange of staff, tranfer of methodology, joined experiments were performed in accordance with specific questions of each laboratory.

RESULTS

Participant No. 1:

1. Normal fibroblasts: During wound healing some fibroblasts are resting while other are activated and participate to the tissue reconstruction (they proliferate and remodel the matrix). We are now studying interactions between fibroblast populations of different growth potentiality. Prelimiary results tend to show that interactions between originally growing and resting fibroblasts differs in monolayer and in a collagen matrix.

2. Protein Kinase C (PKC): We could demonstrate that a PKC inhibitor (GF109203X) inhibited fibroblast growth while it stimulated keratinocyte growth. In addition the presence of EGF reinforced these effects. These results suggest that PKC controls in a different manner the growth, and the response to growth factors, of these two cell types. We are now working to defined the specicity of this inhibitor for PKC in fibroblasts and keratinocytes, in order to be able to use it as a relevant tool for determination of PKC involvement.

3. Keratinocyte Growth Factor (KGF): The ability of fibroblasts to stimulate keratinocyte growth is correlated with the expression of KGF mRNA in fibroblasts of the dermal equivalents.

4. Sclerodermal fibroblasts: When compared to normal fibroblasts, sclerodermal fibroblasts (provided by No. 3) were shown to stimulate growth of normal keratinocytes. mRNA from both type of fibroblasts, grown in the presence or absence of keratinocytes, are now analysed.

5. Fibroblasts from keloids: The collagen matrix regulate the growth of normal fibroblasts. Fibroblasts from keloids seems to escape to this matrix regulation.

Participant No. 2:

1. Analysis of the mechanisms involved in the transfer of information from the in vitro reconstructed matrix to the resident fibroblasts (F): The extracellular matrix (ECM), recognized by a set of membrane receptors, the integrins, operates a pre-transcriptional regulation of the phenotype of F. A down-regulation is observed for instertitial collagens whereas their degradative enzyme, collagenase, is up regulated. The gingival (HGF) and periodontal ligament (HPDL) F are similarly regulated. In collaboration with the participant No. 3, the nature of the integrins involved in the recognition of type I collagen fibers is under investigation. By using specific inhibitors and release of IP3, the involvement of protein-tyrosine kinase and phospholipase C in the signaling pathway could be demonstrated. The regulation of the collagenase gene was shown to depend on activation of c-fos and c-jun and the presence, in nuclear extracts, of AP1 able to bind to an oligonucleotide containing the TRE sequence of the human collagenase promoter. The latter was shown to be rigorously conserved up to -319 while three dimorphic sites were detected in the 5' upstream region.

2. Differentiation features induced by in vitro reconstructed matrix: Normal human F in culture on plastic secrete collagen under the form of unprocessed procollagen molecules similarily to PCP-N-I deficient F in the inherited ED VII C disorder that we recently described. A close contact between normal F and collagen fibers results in an activation of the PCP-N-I. The formation of a network of microvessels induced by recovering human umbilical vein endothelial cells (HUVEC) with interstitial collagen matrix does not depend on a hyperproliferative phenotype, on a modified Il-1 alpha expression, on the synthesis of basement membrane components or the production of instertitial collagenase. It is paralleled by an inhibition of the beta-actin mRNA expression. The procedure for reconstructing a living skin equivalent with stratified epidermal structures presenting adequate differentiation features is standardized and the effect of retinoids on this differentiation programme is under investigation.

3. Measurement of mechanical forces as a pharmacological tool: Various types of human cells (skin F, HGF, HPDL, smooth muscle cells, myofibroblasts) have been shown to be able to generate mechanical forces on a resistant polymeric collagen support that can be modulated by various stimuli (electromagnetic fields, physiologic mediators, pharmacological agents).

4. Cell-cell exchange of information: The influence of keratinocytes on HUVEC differentiation is under investigation. In the skin equivalent, keratinocytes diminish type I collagen mRNA level in F and increase collagenase production. The various mechanisms involved in the remodeling of the ECM by F under the influence of neoplastic epithelial cells have been analyzed.

Participant No. 3:

The main topic is to study the interaction of mesenchymal cells with a surrounding three-dimensional collagen type I matrix in vitro. Culture of fibroblasts and of endothelial cells in this system results in profound changes in cellular metabolism. Both cell types are capable of contracting the matrix to a tissue-like structure, however, the contractile forces exerted by endothelial cells, derived from human umbilical cords, are lesser than those of fibroblasts. Previous studies had shown that in fibroblasts the contraction depends upon the expression of the collagen receptor a2β1 of the integrin family. Studies now in progress will show whether this also holds true for endothelial cells.

1. Extracellular matrix: In addition to collagen, cell function and metabolism can be modulated by proteoglycans, which are major structural constituents of extracellular matrix. Commonly, they consist of a protein core sequence to which are attached glycosaminoglycan side chains (GAGs) composed of different disaccaride units with varying sulfatation grades. Identical core proteins can be glycosylated with different GAG side chains, thereby introducing a high degree of variability in modulatory action.
Gel contraction by fibroblasts was not changed by addition of GAGs with varying sulfatation degrees, whereas different GAGs caused inhibition of contraction by endothelial cells. High sulfatation correlated with further inhibition of lattice contraction.
By Northern blot analysis, collagen, collagenase and IL-6 mRNA levels — as far as transcripts were detected — were also found to be differentially regulated in the two cell types, indicating cell-type specific modulatory action by proteoglycans.
Work now in progress relates to the identification and elucidation of possible differences in the expression of receptors for proteoglycans/GAGs.

2. Cell-cell interaction: In a joint experiment (No. 1 & No. 3) dermo-epidermal interactions were studied, employing collagen lattices onto which biopsies of epidermis were applied. In specific, differential dermal influence was studied by seeding fibroblasts derived from healthy donors into one set of lattices, whereas the other set was populated by fibroblasts from patients with systemic sclerosis. Interestingly, the latter stimulate epidermal proliferation. Studies now in progress aim at identifying which factor might be involved.

3. Signal transduction: Further studies have begun investigating the molecular basis of signals, elicited by the cell-matrix interaction, and their transduction pathways. Special emphasis has been placed on identifying differences in cells cultivated in different environments, i.e collagen-coated dishes which provide limited contact of fibroblasts with a nonfibrillar matrix, and collagen lattices, where three-dimensional contact with an organized, fibrillar collagen network is given.
Using Western blotting and immunodetection with anti-phosphotyrosine antibodies, enhanced tyrosine phosphorylation of a Mr = 120000 protein (pp120) and a Mr = 42000 (pp42) could be demonstrated. This effect was observed in fibroblasts in both culture types, suggesting that tyrosine phosphorylation is involved in signal transduction triggered by two- and three-dimensional collagen-fibroblast contact. It remains to be seen whether the phosphorylation of pp120 and pp42 plays a direct role in regulating the biosynthesis of extracellular matrix proteins in fibroblasts or rather is involved in promoting alterations of cell morphology and spreading.

Participant No. 4:

1. Keratinocyte and mesenchymal cell proliferation: Keratinocyte-mesenchymal cell (fibroblasts; FB) and capillary endothelial cells (EC) interaction has been further studied concerning its effect on cell proliferation and differentiation as well as the molecular mechanisms involved, particularly peptide growth factors. In organotypic coculture proliferation is mutually stimulated in both cell compartments, as determined by histone 3 expression and immunofluorescence labelling of cell incorporating BrdU (markers of S-phase), or by mesenchymal cell counting following collagen gel digestion with collagenase (method from No. 1). While fibroblast numbers increased steadily in non-contracting gels this was regulated by keratinocytes and beginning gel contraction after one week of culture. Altough DNA synthesis was further increased, mesenchymal cell number relatively decreased, probably due to increased cell death as consequence of gel contraction and/or regulating factors from the epidermis.
Keratinocyte growth was strongly stimulated in organotypic cocultures, though proliferating cells remained located predominantly in the basal layer. This is similarly seen with non proliferating (X-irradiated) mesenchymal cells, but reduction in fibroblasts number seemed to reduce their proliferative stimulus to keratinocytes to rather normal levels. Associated with this hyperproliferation keratinocytes aberrantly expressed the mesenchymal cytoskeletal protein vimentin in lowermost cell layers. Following transplantation of organotypic cocultures into an in vivo environment (nude mice) both proliferation and differentiation markers rapidly normalized. This indicates that the in vitro models simulating hyperstimulated, irritated or wounded skin can be further modulated by altering external conditions.

2. Factors regulating epidermal-dermal interaction: In monolayer cocultures with postmitotic (X-irradiated) mesenchymal cells (FB and EC feeder layer) keratinocytes are maximally stimulated to proliferate. By mRNA analysis, it could be demonstrated for the first time that keratinocytes induce gene activation in mesenchymal cells and, in addition to interstitial collagenase (typeI), expression of growth factors (KGF, IL6, GM-CSF). Keratinocytes expresses IL-1α and also induced expression of IL-1α receptor mRNA in fibroblasts. Preliminary experiments, however, revealed that addition of growth factors (KGF, IGF1) or conditionned medium of coculture could not substitute the cocultured mesenchymal cells in sustaining clonal keratinocyte growth on plastic.

3. Development of a defined medium: A serum free and defined medium has been developed sustaining growth and differentiation of a subclone of the immortalized HaCaT cell line on plastic and collagen gels. Starting from a commercially available medium (KDM, Clonetics), $Ca2+$ concentration have been adaptated to fibroblast requirements. The morphogenic effects of different retinoic acid concentration is now studied to establishe improved physiologic conditions and to analyse pharmacological effects of this retinoid. Interestingly, collagen gels contracted much less under serum-free conditions but this was inhanced by TGFβ1. Moreover in coculture with fibroblasts the growth inhibitory effect of TGFβ1 on HaCaT cells was abrogated and instead a proliferating epithelium with improved structural organization developped. Finally, the presence of fibroblasts in collagen gels seemed to stimulate malignant HaCaT-ras clones to infiltrate the DE. This unable further analysis on the effects of defined growth and differentiation factors on keratinocytes and mesenchymal cells without disturbing interference of undefined serum or organ extract components.

Participant No. 5:

During the first year, we have analysed the requirements for growing normal human melanocytes in culture, alone or in the presence of normal human keratinocytes, and we have set up methodologies for the quantification of intra-cellular melanins.
The second year was devoted to the establishment of a correlation between the pigmentation which can be obtained in vivo and the accumulation of melanin within cultured melanocytes.
Repeated ultraviolet irradiation, at suberythemal level in the so-called UV-B range (20-100 mJ/cm≤) as a function of the photo-type induce pigmentation in humans within a few days.
In twelve independent experiments, we have observed that repeated low doses of irradiations (2.4 mJ/cm≤ per day) at 285 nm usually promote the doubling of intra-cellular melanin in cultured melanocytes within four days after the first irradiation. In one instance however, we have observed that the level of melanin increased up to fourfold, and in two other instances the level of melanin did not increase beyond 130 % of the control. Wo do not know whether the content of melanin levels off after day 5.
The intra-cellular melanin level generally increases smoothly for two to three days after the first irradiation, then increases steeply on the fourth day. In the instance in which the final level was four times the control, the melanin content doubled during the day after the first irradiation.
The amount of intra cellular melanin depends on several parameters (donor, presence of serum, other culture conditions etc...) but is generally found tabe between 9 and 58 pg/cell.
We have also analysed the effect of xenobiòtics, like furocoumarins, on the accumulation of melanin within melanocytes in culture. It is know that psoralens and UV light, in the so called UV-A range, induce strong pigmentation in human skin.
We have observed that repeated daily irradiations (7 J/cm≤ at 365 nm for four days) do not increase the intra-cellular level of melanin irrespective of the presence of DMSO. When the irradiations are performed in the presence of 8 methoxy psoralen, (5-20 µg/ml) the level of melanin is multiplied by three within 2 days.
5-20 µg/ml Angelicin or Khellin or 5-methoxy psoralen when present during irradiation increase by about 50 % the level of intra-cellular melanin within two days after the first irradiation.
When the irradiation is performed in the presence of 8 methoxy psoralen, the melanocyte undergo morphological modifications, melanin is secreted and forms aggregates.
The third year will be devoted to the analysis of UV and/or xenobiotics on the behaviour of melanocytes in reconstructed skin.

HIGHLIGHTS / MILESTONES

Cell-cell and cell-matrix interactions: Cell-cell and cell-matrix interactions are regulated by induction of specific growth factors which have been identified and characterized at molecular level. These regulations are mutually induced by the different components when they are combined in a tissue specific manner.
Receptors responsible for mediating mechanical forces from the extracellular matrix to fibroblasts have been identified. The intercellular system transmitting the signal from the receptors to the intracellular environment results in a reprogramming of fibroblast metabolism.

Pharmacology: By modifying the arrangement of the different compartments it was possible to modulate the in vitro epidermis to mimic normal or diseased skin (wound healing or psoriasis).
A defined medium has been developed, enabling studies of drugs and growth factor action in the absence of influences of unknown serum factors. Immortalized keratinocyte cell lines, with maintained capacity for differentiation, can be used to replace primary epidermal cultures.
Melanogenesis can be induced by UV light and xenobiotic treatment.

WIDER CONSIDERATIONS

The present state of these in vitro human skin equivalent models represents a close approximation to human skin enabling further studies on regulatory mechanisms important for tissue homeostasis and diseased states. The established models allow analysis of the influence of external factors on a multicellular organ system promissing relevant data for the in vivo situation.
We combined our BRIDGE program with those of SANOFI-RECHERCHE 'Sentitizer and allergy' (A. Barbier) and L'OREAL 'Gap junctions and tumor promoters' (E. Honikman-Leban) to form a large ELWW '*In vitro* integrated approach to skin pharmaco-toxicology'.

COOPERATIVE ACTIVITIES

Meetings:
04-05/04/92: No. 2 and No. 3 in London
22/04/92: No. 1 and No. 2 in Créteil
16/05/92: No. 2 and No. 3 in Cologne
20/05/92: No. 3 and No. 4 in Cologne
04/07/92: No. 2 and No. 3 in Liège
27/08/92: No. 2 and No. 3 in Malmö
31/08-04/09/92: No. 1 and No. 4 in Paris
26-27/10/92: No. 1 and No. 4 in Heidelberg
05/11/92: No. 1 and No. 2 in Créteil
08-10/12/92: **Plenary meeting in Dublin**

Exchange of staff:
17/04/92: No.5 -> No. 1 (Joint experiment in Créteil)

Exchange of material / methodology:
No. 1 -> No. 5: Procedures for establishing dermal equivalent
No. 1 & No. 3: joint experiment (exchange of cells & RNA)
No. 2 & No. 3: joint experiment (exchange of RNA)
No. 3 -> No. 2: integrins c-DNA

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Establishment of immortal differentiated hepatocyte lines from transgenic mice and their use for studying viral chemical agents (BIOT CT-900189)

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OBJECTIVES

Analysis of hepatocytes cell cycle progression, determine the usefulness of SV-202 transgenic hepatocytes as a reporter cell line for identifying indirectly acting mutagens. Specifically, analysis of cell cycle characteristics of normal hepatocytes from rats and mice and control mechanisms that govern the progression through the cell cycle, mainly in the G1 phase & during G1/S transition.

MAJOR PBOLEMS ENCOUNTERED

- Although hepatocytes undergo division in culture under appropriate culture conditions, they divide only once. Culture conditions are being systematically tested to screen for improved conditions to stimulate several divisions of normal, non-immortalized hepatocytes in primary culture and in subcultures.
- Fetal hepatocyte line FMH-202 has been shown to be useful for assays to identify indirectly acting mutagens at low levels by means of their clastogenic activity in the absence of exogenous metabolic activating systems (e.g. S9 liver extracts). However, dimethylnitrosamine (DMN) is active at relatively elevated levels (10^{-3} M), and it remains to be seen, whether culture conditions can be improved to render the mutagenic activity of DMN higher in this system.

RESULTS

Abstract

There is great demand for stable hepatocyte culture systems in areas such as pharmacology, toxicology, virology, carcinogenesis and biotechnology. At present, hepatocytes in primary culture maintain their differentiated functions only for a few days, which limits their usefulness in long-term studies. Therefore, hepatocyte lines were derived from transgenic mice that bear an immortalizing transgene which is expressed in the liver prior to the appearance of tumor cells in the animal. Resulting hepatocyte lines are immortal, non-tumorigenic and stably maintain their differentiated functions for extended periods. A new hepatocyte line has been derived from an adult mouse and is in the process of being adapted to multiply in serum-free medium in response to EGF and insulin. To identify indirectly acting mutagens, transgenic mouse hepatocyte line FMH-202 has been shown to be useful as an *in vitro* assay system for routine determinations of potentially mutagenic compounds by employing cytogenetic methods (clastogenic activity and induction of sister chromatid exchanges). Furthermore, within the realm of growth control studies it was shown that p34cdc2 is expressed in the G2 and M phases of the cell cycle, suggesting that it plays a role in the control of G2/M transition. Furthermore, it was demonstrated that p34cdc2 or p33cdk2 are not expressed in S phase,

and that cyclins D1 and E are present during the all phases of the cell cycle, however with clear increases in the G1 phase.

1) Establishment of an adult hepatocyte line from transgenic Delta-SV-202 mice

Transgenic hepatocyte lines were established from an adult mouse (12 weeks old) bearing the Delta-SV-202 transgene, in which the human growth hormone (hGH) gene was partially deleted in order to avoid the possibility that the hGH gene in the original construct (SV-202) might interfere with the process of immortalization of the hepatocytes. As expected from previous results involving the FMH-202 line, the new Delta-SV-202 line is not clonal, since cells require neighbours, i.e. cell-cell contacts for growth and maintenance of the differentiated hepatic phenotype. The cells grow in MX-83 medium free of serum supplemented with EGF and insulin. About 6 passages after cultivation, the cultures were free of contaminating cells. Cells multiply once every 48 hours in response to the growth factors. Following the 8th passage, cells no longer require EGF and grow in response to insulin. The efficiency of transfer is low, but cells can routinely be maintained with passages occurring once every ten days. We have begun to analyze the differentiated status of the cells and their capacity to activate indirectly acting mutagens.

2) Characterization of hepatocyte line FMH-202 for short-term test systems *in vitro* to identify indirectly acting mutagens

The established transgenic hepatocyte line FMH-202 was employed as a test system for identifying genotoxic activity of the indirectely acting mutagen dimethylnitrosamine (DMN). Few if any known cell lines are endowed with the capacity to metabolically activate at least 4 classes of foreign compounds as shown by the FMH-202 line as reported in the previous Progress Report. Such metabolically activated potential mutagens were detected by cytogenetic means (chromosome aberrations and induction of sister chromatid exchanges [SCEs]). However, one compound used as an example of another family of indirectly acting mutagenic compounds (dimethylnitrosamine [DMN]), the cell line responded to this mutagen, however at concentrations of about 10^{-3} M. It is possible, that this rather low responsiveness of the cells is due to the medium composition (i.e. lack of nicotinamide, of other nutrients and cofactors).

3) Growth control of hepatocytes and hepatocyte lines: control mechanisms governing progression through the cell cycle

The control of the hepatocyte cell cycle is unique: indeed, although highly differentiated at the adult stage, these cells remain able to divide. In culture, they are capable of undergoing one round of division independently of growth factors. Little is known in this general field and no data on liver cells have are available as yet. We have demonstrated in normal hepatocytes the expression of the kinase proteins cdc2, cdK2 and cyclins and characterized their sequential activation of throughout phosphorylation/dephosphorylation processes.

a) Cell cycle protein

We have postulated that this specific control results from critical regulations of cell cycle proteins in G0/G1 and/or G1s transitions.

In order to study them we have employed *in vivo* and *in vitro* models in which the cells are well synchronized: regenerating hepatocytes following partial hepatectomy, and primary cultures of normal adult hepatocytes synchronized by exposure

to sodium butyrate, which is well-known to block the cells in the G1 phase. First, we showed that p34cdc2 is expressed in G2 and M phases, suggesting a role in the control of G2/M transition as in most cell types. Second, we demonstrated that neither p34cdc2 nor p33cdk2 are involved in the commitment pathway of cells to enter S phase, leading to the postulate that both proteins play a critical role in determining the entry of cells into S phase. Finally, we observed that cyclins D1 and E are present all along the phases of the cell cycle. However, these two proteins are mainly present in G1 and at higher levels, suggesting, that they are good candidates for being involved in the regulation of the progression through G1 and in the transition from G1 to S phase.

b) Effects of a growth factor (EGF) and cell-cell communication

For these studies we took advantage of an unusually extended G1 phase of the cells. Early G1 corresponded to the transient expression of c-fos and c-jun, mid G1 to the appearance of c-myc and junD, and late G1 corresponded to the expression of c-myc and of p53. Interestingly, we showed that rat hepatocytes spontaneously entered the G1 phase but continued requiring growth factor stimulation (EGF) to progress through S phase, the restriction point being located in late G1. Some differences appear between cells of one species to another, e.g. between rat and mouse hepatocytes.

A role of cell-cell communication on the cell cycle was strongly suggested by results in which several techniques were employed to disrupt liver tissue (e.g. by collagenase perfusion of the liver), and by re-establishing cell-cell contacts in culture. Tissue disruption and cell isolation and their cultivation resulted in the induction of hepatocytes to enter the G1 phase. It correlated with a drastic and transient increase of c-fos and c-jun, and 4 hrs later of c-myc. Addition of EGF allowed hepatocytes to enter S phase and to progress through mitosis. When placed in coculture, hepatocytes remained blocked in G1 and could not be induced by any growth factors to enter S phase.

c) The cell cycle of immortalized hepatocytes

One cell line of immortalized hepatocytes from transgenic mice bearing the transgene Delta-SV-202 has been studied and compared to normal primary hepatocytes and to transformed hepatoma cells. The first step in our studies was to select pure populations of cuboidal granula cells. Purity could be maintaind for a long time (more than 4 months in continuous culture), and we verified that selection did not alter the functional characteristics of the cells: high levels of albumin, transferrin and aldolase B expression were maintained.

As a second step, we succeeded in synchronizing these cells by using sodium butyrate. From these studies it appeared, that the length of the G1 phase was reduced from 2-3 days in normal primary mouse hepatocytes, to 24-30 hrs in immortalized cells and to 14-18 hrs in transformed hepatoma cells. A comparative study of the expression of cell cycle proteins and regulation by cell-cell communication and growth factors was made possible. However, final results are not yet available, however they will be presented at the end of the third period of the present project. Experiments are in progress to determine the role of cell-cell communication on the progression of cells through the cell cycle.

Another immortalized hepatocyte line bearing a construct with a HBV-DNA sequence integrated into c-myc as a transgene (M.A. Buendia, Paris) has been established in culture and synchronization assays have been performed. This cell

line will allow us to determine whether cell cycle alterations vary according to the transgene employed for immortalization.

HIGHLIGHTS / MILESTONES

Establishment of a novel adult transgenic hepatocyte line and ts initial characterization, as well as its use for cell cycle studies.

WIDER CONSIDERATIONS

This differentiated liver cell line will be useful for studies in biotechnology, virology and toxicology. From initial data, the cells appear to be useful for short-term test systems *in vitro.*

COOPERATIVE ACTIVITIES

Two extensive meetings were held in France and in the laboratory in Hannover during the second year of contract.

PUBLICATIONS

LOYER, P., GFLIASE, D., MEIJR, L. & GUGUEN-GUILLOUZO, C. Oncogenes and cell cycle in adult hepatocytes. In: 'Cellular and molecular aspects of cirrhosis' (Clement, B & Gillouzo, A. Eds. Les Editions INSERM, Prtis. John Libey Eurotext, London, 1992. Vol. 216, pp. 277-285.

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Development of immortalized human articular cartilage cell lines for use in physiological, pharmacological and toxicological *in vitro* investigations (BIOT CT-900196)

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OBJECTIVES

1. The characterization of approx.30 clones of immortalized chondrocytes obtained in the first year of this contract.
2. Further investigations of the use of isolated chondrocytes in in vitro models for the study of tissue breakdown and repair in inflammatory conditions.
3. Studies of the model of experimental arthritis in the rat and the isolation and characterization of cartilage cells from diseased connective tissues.
4. The characterization of chondrocyte autoantigens in rheumatoid arthritis and the preparation of a chondrocyte expression library from immortalized human chondrocytes.

RESULTS

1. THE CHARACTERIZATION OF APPROX. 30 CLONES OF IMMORTALIZED CHONDROCYTES OBTAINED DURING THE FIRST YEAR OF THIS CONTRACT

The plasmid which permitted to isolate immortalized clones of human chondrocytes carried a temperature-sensitive(ts)SV40 large T antigen. In this case, large T antigen expression is controlled by the temperature.

Cells transfected by such temperature-sensitive oncogenes have been reported to be conditionally transformed. They only express the immortalized phenotype at the permissive temperature of 33°C and reverse to a non-transformed phenotype (with restored differentiated functions) when cultured at the non-permissive temperature (39°C).

a) *Southern blot analysis was performed to check the integration of the plasmid DNA into the cell genome* of the different clones, and to evaluate the number of copies integrated and the number of integration sites. The number of copies integrated is very different from one clone to another (from one to more than 20 copies per genome) and generally more than two insertion sites are present.

b) *The study of cell growth was performed* during a two weeks' period. For some clones, growth rates at 33°C and 39°C were approximately the same. However, for most clones, growth rate was higher at non-permissive than at permissive temperature. Thus, the growth curves display a pattern which is the opposite of

what was expected. The fact that cells grow quicker at 39°C than at the permissive temperature indicates that the large T antigen is apparently not totally inactivated.

c) *Study of the extracellular matrix components. Synthesis rate and type of collagen.* Collagen was extracted and purified from non-transfected human chondrocytes and from immortalized human chondrocyte clones after 48 hours' incorporation of tritiated proline in confluent cultures. The temperature did not dramatically modify the rate of proline incorporation. Collagen type was studied by SDS-PAGE analysis of the intact chains of collagen. All the clones tested synthesized alpha 1 chains (which could indicate the presence of type I collagen trimer or type II collagen) and a variable amount of type III collagen (which is a sign of 'dedifferentiation' of the chondrocytes). The only clones synthesizing weak amounts of type III collagen were PAPY-10 and HUM-25. For some of the clones synthesis at 33°C and 39°C seemed to differ slightly. SDS-PAGE analysis of these peptides seems to confirm the presence of type II collagen for at least one clone.

d) *Study of the extracellular matrix components. Synthesis rate and type of aggregans.* Aggrecan synthesis rates and types of aggrecans synthesized were studied on monolayer cultured chondrocytes and on chondrocytes cultured in gelified agarose. Only the PAPY-clones were studied. Results are given in the table.

TABLE 1: SO4 INCORPORATION IN MONOLAYER CULTURED CHONDROCYTES AND IN CHONDROCYTES CULTURED IN GELIFIED AGAROSE (PAPY-CLONES)
clones ranked by synthesis rates; synthesis rates expressed as pG SO_4 in aggrecans/ 1.10E6 cells/hour

CL	mono 1	CL	mono 2	CL	agar	CL	%derm SO_4
6	6877	6	5889	4	855	1	27
4	6254	4	2968	10	704	4	30
10	4016	10	2377	6	701	6	34
7	3379	7	1834	9	654	10	34
9	2868	9	1820	8	458	8	36
11	2356	11	1654			9	37
15	1853	1	1338	7	377	2	38
8	1405	2	1219			13	39
2	1261	15	968	2	306	15	39
13	1197	8	968	3	240	7	40
3	1089	3	941			11	41
1	1027	13	751	1	171	3	44
12	951	12	703			12	45

The ^{35}S-incorporation experiments on monolayer cultured cells were done on two different batches of chondrocyte clones with a two months'interval (monol and 2).

The various clones considerably differed by their capacity to synthesize aggrecans. When cultured in agarose, the chondrocytes showed lower aggrecan synthesis rates. However, when ranked by their aggrecan synthesis levels, these cell lines followed approximately the same order.

A pilot investigation (HUM 1 clone) showed higher aggrecan synthesis rates at the non-permisive temperature of 39°C.

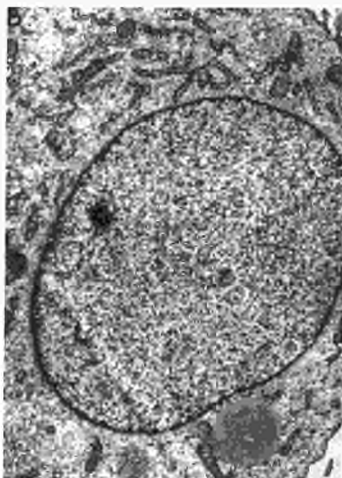
Degradation of the radiolabeled aggrecans with specific glycosaminoglycanases enabled us to characterize the different types of aggrecans synthesized.

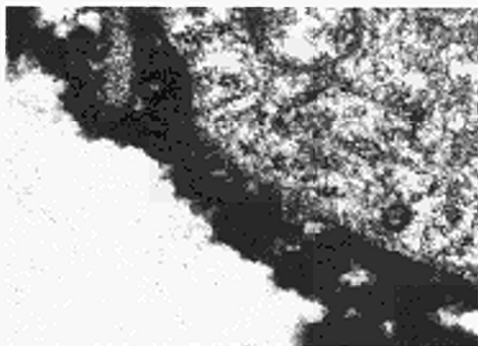
Aggrecans synthesized by phenotypically stable chondrocytes contain chondroitin-sulphate and keratansulphate. Dermatansulphate is not found in these cartilage-specific aggrecans. Fibroblasts synthesize aggrecans where dermatansulphate is the main (more than 50%) glycosaminoglycan. Chondrocytes that 'dedifferentiate' to fibroblasts in the monolayer condition in vitro produce increasing proportions of dermatansulphate-containing aggrecans.

After having been propagated in the monolayer condition the immortalized chondrocytes produced aggrecans with proportions of dermatansulphate ranging between 27 and 45% (table). The relative amounts of dermatansulphate found in the aggrecans is to be considered as a parameter reflecting the phenotypical stability of the chondrocyte in culture.

It was concluded that the immortalized cells had dedifferentiated to fibroblast-like cells after having been propagated in the monolayer condition.

e) *Microscopy of cells and the extracellular matrix.* As previously reported, immortalized chondrocytes cultured in gelified agarose,synthesize much less aggrecan (and collagen) than non-immortalized chondrocytes in the same culture condition. On light microscopy non-immortalized human chondrocytes appeared as single cells showing abundant metachromatically stained material around the cells. These pericellular deposits of aggrecans were present after three weeks of culture. They were less obvious around immortalized cartilage cells after three weeks of culture.





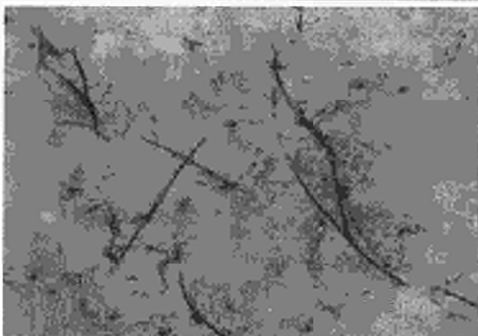


Fig 1: *Chondrocyte in agarose. Electron microscopy.*
A. normal non-immortalized chondrocyte in agarose.
B. electron-dense sheet of Alcian Blue-precipitated aggrecans around the cells.
C. pericellular collagen fibers.

Electron microscopic studies were performed on the chondrocytes and the pericellular material. Both immortalized and non-immortalized chondrocytes appeared vital and metabolically active, showing well developed rough endoplasmatic reticula (RER), many lysosomes and vacuoles (Fig. 1A, see previous page).

An electron-dense sheet of Alcian Blue-precipitated aggrecans was present around the non-immortalized cartilage cells after 3 weeks of culture in the agarose gel (Fig. 1B, see previous page). This electron-dense sheet appeared later around the immortalized cells.

Besides these electron-dense condensations indicating the presense of aggrecans, collagen fibers were clearly demonstrated in the artificial agarose matrix (Fig. 1C, see previous page).

Aggrecan aggregates were released from non-immortalized cartilage cell cultures after 3 weeks of culture and visualised after rotary shadowing.

Mature human articular chondrocytes predominantly synthesized small-size aggregates with 3 to 12 aggrecans per aggregate. However, a few large aggregates containing more than 100 aggrecans per aggregate were observed in each culture (Fig. 2A). Aggrecans synthesized by immortalized chondrocytes appeared later and seemed to be partially disintegrated (Fig. 2B).

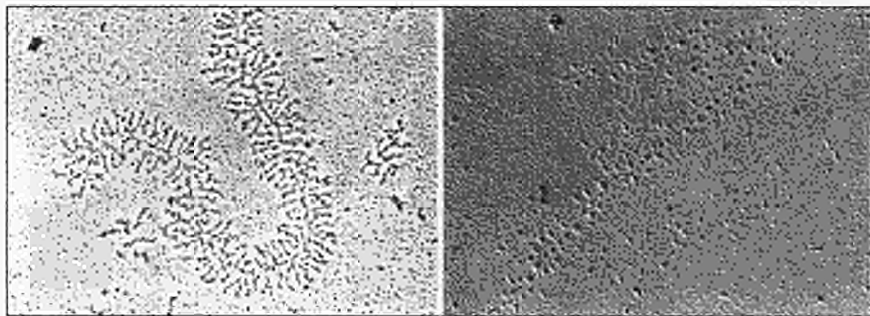


Fig 2: *Aggrecan aggregates synthesized by:*
A. non-immortalized chondrocytes in agarose.
B. immortalized chondrocytes cultured in gelified agarose.

f) Conclusions

Articular chondrocytes from adult donors have been immortalized after (ts)SV40 large T antigen transfection. The cells were then propagated in order to obtain large numbers. Hereafter, the chondrocytes showed low collagen and aggrecan synthesis rates which seriously hindered phenotype analysis. Not all immortalized cartilage cell lines were equallly (un)productive. When propagated in the monolayer condition, all immortalized chondrocytes have largely lost the potential to synthesize typical cartilage cell products. They have acquired the synthesis functions of fibroblasts.

2. FURTHER INVESTIGATIONS OF THE USE OF ISOLATED CHONDROCYTES IN *IN VITRO* MODELS FOR THE STUDY OF TISSUE BREAKDOWN AND REPAIR IN INFLAMMATORY CONDITIONS

Chondrocyte — macrophage polymorphonuclear cell joint co-culture as an in vitro model for the study of tissue breakdown and repair in immunologically mediated joint diseases.

For co-culture the chondrocytes are suspended in agarose and the agarose is allowed to gelify in micro 'insert' culture wells, the bottom of which consists of a porous filter membrane. These 'insert' culture wells are then placed in standard culture dishes in which the monocytes (MNC) are grown. This co-culture system is a model for inflammatory cell/connective tissue cell interactions. The chondrocytes show a normal PG-metabolism when they rest on non-activated MNC. When the same chondrocytes rest on LPS-activated MNC, which release abundant amounts of cytokines in the incubation medium, their PG-metabolism is greatly disturbed. They stop synthesizing PG and release neutral metalloproteinases, which destroy the formerly synthesized PG. This whole process is easily followed by analysis of the radiolabelled PG released in incubation media or retained in the gel.

An analogous co-culture system for chondrocytes and human polymorphonuclear neutrophils (PMN) has been prepared.

3. THE CHARACTERIZATION OF CHONDROCYTE AUTOANTIGENS IN RHEUMATOID ARTHRITIS (RA) AND THE PREPARATION OF A CONDROCYTE ESPRESSION LIBRARY FROM IMMORTALIZED HUMAN CHONDROCYTES

a) *characterization of autoantigens in (RA).* The majority of RA sera contain antibodies which detect a 60 KD antigen in the human chondrocyte expression library constructed as part of this contract. Sequencing should now proceed rapidly.

b) *preparation of a chondrocyte expression library from immortalized human chondrocytes.* Using the immortalized chondrocytes, prepared in Paris as part of this project, the London group has now constructed an additional expression library. The genes expressed in this library will be compared to those in the original one in order to arrive at an estimate of the degree of 'dedifferentiation' chondrocytes undergo in the process of immortalization and subsequent propagation.

c) *definition of the role of autoimmunity to chondrocyte antigens in TNF-alpha transgenic mice.* Because of the high degree of homology between human and rodent connective tissue components, the human chondrocyte expression library will be used to look for possible autoimmunity to chondrocytes antigens in the TNF-alpha transgenic mice. This will be done by screening the library with sera from the transgenic mice with/without arthritis.

CONCLUSIONS, WIDER CONSIDERATIONS AND MAJOR PROBLEMS ENCOUNTERED

1. Autoimmunity and the pathogenesis of theumatic joint disease

The definition of chondrocyte autoantigens in the pathogenesis of important human diseases such as rheumatoid arthritis, is a major development in our understanding of these diseases and could lead to disease-specific immunotherapeutic intervention.

2. The treatment of rheumatic disorders absorbs a substantial part of the health care budget. There are no *in vitro* systems that give sufficient information on the etiopathogenesis of cartilage destruction and the effects of pharmaceutical substances on this process. Well-defined connective tissue cell lines, i.e. cartilage cells, suited for studies in the field of degenerative and inflammatory joint diseases, are not available. Efforts are now being made to redifferentiate these chondrocytes so that their function in *in vitro* models is equal to their function *in vivo*.

Co-culture systems with these chondrocytes as target cells and cells that participate in inflammation have been prepared. These system could replace some experimental animal arthritis models.

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The risk potential of biotechnology-derived polypeptides causing nephrotoxicity based on assessment in human and animal renal cell lines using molecular and cellular biology techniques (BIOT CT-910266)

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OBJECTIVES

The overall objective is to validate different *in vitro* test systems (single cell and multicellular) to elucidate the primary target cells, the pathomechanism and the cascade of toxic events caused by biotechnology derived polypeptides and a range of other nephrotoxins. Current objectives include the optimization of culture systems for primary cells, the design of novel cell lines, characterization of these cell lines and development of sensitive methods to detect mechanisms of and means of ameliorating cellular damage by nephrotoxic compounds.

RESULTS

I. Techniques in In Vitro Nephropharmacotoxicology

A. Numerical modelling and structure activity models

The London group reported on the computer based prediction of pharmacotoxicological data based on numerical modelling derived from the physicochemical properties (electric charge, acid and base strength, conjugated system and solubility) of organic compounds and used these to predict the accumulation of a range of analogues in organelles. A structure activity relationship model has been developed as a prototype PC-based expert system and applied to help understand the adverse effects of a series of phenothiazine analogues or metabolites in 3T3-cells, which are uniquely sensitive to chlorpromazine (CPZ). The intracellular distribution of CPZ was predicted from the expert system and confirmed microscopically to accumulate in mitochondria. Thus the use of tetrazolium reduction to formazan was a rational method for assessing cytotoxicity based on mitochondrial function. The resulting structure activity data showed the importance of the N,N-dimethyl-propanamine moiety and suggested metabolic oxidation of the unoxidised 6,7,8,9-ring position, or 6,9-dihydroxy groups as of key importance in cytotoxicity.

B. Fluorescent probes

Fluorescent probes help assess some adverse biochemical effects and the functional assessment of cytotoxicity.

1. Spectrophotometric measurement of probes.

Peroxidative activation of CPZ (see numerical modelling above) is supported by the 3-5-fold oxidation of 2',7'-dichlorofluorescein to fluorescein by reactive intermediates in 3T3-cells compared to control conditions. The protective effects of alpha-tocopherol and L-ascorbic acid support a free radical mechanism.

2. Applications of flow cytometry

The Dublin group used flow cytometry (in collaboration with thc Innsbruck group) to sort and compare fused cells (see below) from unfused primary cells or cell lines. Similarly, cell viability and alterations in pH and free $[Ca^{2+}]_i$ following exposure to hypoxia/reoxygenation and gentamicin in LLC-PK_1 and MDCK cells are being used to establish the mechanistic basis of nephrotoxicity. The dose and time dependency of changes in cellular viability and intracellular calcium caused by cyclosporin A (CyA) in LLC-PK_1 and MDCK cells was assessed. Cell viability was also monitored by the neutral red cytotoxicity assay following treatment for 4, 6, 8, 12 and 24 hours with doses of CyA encompassing both 'chronic' and 'acute' dosing regimens.

For determination of $[Ca^{2+}]_i$ cells were treated with lethal and sub-lethal doses of CyA for 24 hours. A dose and time dependent loss of cellular viability was seen in both cell types for concentrations of CyA ranging from 500 ng/ml to 100 μg/ml. At the earlier time points of 4 and 6 hours the LLC-PK_1 cells exhibited a greater loss of viability compared to that in the MDCK cells. CyA for 24 hours caused dose dependent increases in $[Ca^{2+}]_i$ of 107 ± 13, 128 ± 10, 226 ± 67 nM relative to control values at concentrations of 5 ng/m, 50 ng/ml and 5 μg/ml CyA respectively. The ability of CyA to cause increase in $[Ca^{2+}]_i$ at sub-lethal doses indicates that alterations in $[Ca^{2+}]_i$ may play an important role in the mediation of it's toxicity. This indicates a possible site selective action of CyA along the nephron of the kidney.

Flow cytometry has been used to monitor cell viability and intracellular alterations in pH and free $[Ca^{2+}]$i following exposure to hypoxia/reoxygenation and gentamicin in LLC-PK_1 and MDCK cells. Dose response and time-duration of exposure relations have been established. The flow cytometer provides a novel approach to establish the mechanistic basis of nephrotoxicity in relevant cell populations from the kidney. The technique is also applicable to monitoring cellular responses in general to toxic compounds.

Primary rat proximal tubules (RPT) cells were cultured and were fused with the Normal Rat Kidney (NRK) cell line using the fusogen polyethylene glycol. Prior to fusion the RPT cells were incubated with 1 mg/ml 3-3'-diodadecyloxa-carbocyanine perchlorate (DiO) and the NRK cells with 1 mg/ml 1-1-dioctadecyl-3,3,3',3'-tetramylindocarbocyanine perchlorate (DiI). DiO and DiI are fluorescent probes which label the plasma membrane with high specificity. Their presence in the cell membrane are reported not to affect cell viability or cell surface properties. These characteristics together with the fact that the dyes are well retained in culture suggests that they may be useful for long term monitoring of cell fusion. Fused cells which contain both of the fluroescent dyes were analysed using a BD FACStar Plus Flow Cytometer gating out the cells containing only one of the dyes.

The fused cells were grown in glucose-free DMEM supplemented with pyruvate and 10% dialysed foetal calf serum for selection purposes. DNA analysis by flow cytometry using 100 µg/ml propidum iodide revealed a different profile for the fused cells when compared to the NRK and RPT cells. Additional evidence for fusion of the two nuclei resulting in a heterokaryon was obtained by light microscopy. These findings indicate that a successful fusion procedure was employed and that the technique of analysing and sorting fused cells using the long term tracing carbocyanine dyes in the flow cytometer may provide a useful procedure in fusion experiments. These fused cells may prove to be useful models in the screening of various compounds including those of environmental interest that may be toxic to the kidney.

3. Application of light microscopy

The London group have shown heterogeneity in cell culture and cell mapping in multicellular structures *in vitro* using fluorescent probes and conventional imaging. 'Monocultures' show that even well defined cells have a varying degree of biochemical heterogeneity, necessitating assessment of cells at an individual level in order to document classical discrete biochemical activities which may be key to better mechanistic understanding.

The 2- and 3-dimensional relationship between different cell types is well appreciated in terms of anatomy and pathology. This has been achieved by conventional histochemistry and cytochemistry, which facilitate the expression of heterogeneity in 2-D. Investigations that used serial 2-D sections and 3-D reconstructions were an essential part of the early developments in anatomy. This approach has also been applied to investigate the expression of a range of physiological functions in large multi-cellular structures, for example freshly isolated rat glomeruli, where the spatial architecture of cells is the same as that seen *in vivo*.

4. Application of real-time confocal microscopy to 3-D imaging

The London group used real-time confocal microscopy on two fluorescent channels in transmission and reflectance modes for 3-D reconstruction (Silicon Graphics). 2-D (Image-1) and 3-D (Voxel View) image analysis has been used to assess changes in freshly isolated whole Wistar rat glomeruli exposed to cyclosporin A (CyA). Confocal microscopy was used to reconstruct the volume of the glomeruli by optical sectioning, and the intracelluar structure of the glomerulus was assessed by selectively staining the cells with Nile Red. This selectively stains what appears to be epithelial cells in cultures and not mesangial cells. Thus it was possible to assess the volume of the whole intact glomerulus and the associated epithelial and non-epithelial cell volume. CyA caused a dose related decrease in the volume of the whole glomerulus. It is still to be established within which cell which cells this volume takes place.

5. Electrophysiological studies

Electrophysiological studies on cells grown on microporous filters have been used by the Dublin group to investigate the integrity of intercellular junctions. Gentamicin was shown to disrupt the integrity of the junctions in LLC-PK_1 and MDCK cells. Magnesium aspartate hydrochloride was shown to prevent the damage caused by gentamicin in both *in vitro* and *in vivo* experiments. This may provide a very useful approach to reduction of nephrotoxicity by aminoglycosides and possibly other nephrotoxic compounds.

II. Investigation of the effects of chemicals on cells

A. Glomeruli

1. Human mesangial cell (HMC) cultures

Human mesangial cells synthesize fibronectin (FN) which amounts to about 2% of total protein synthesis. The Giessen group have found that newly synthesized FN is secreted into the culture medium and incorporated into a fibrillar extracellular matrix (ECM). After incubation with ADR (2 µg/ml) an accumulation of FN in the ECM was revealed by immunofluorescence staining. Correspondingly, radioactively labelled, immunoprecipitable FN was significantly increased in the extracellular compartments by 23% compared to controls. Concomitant with the ADR-induced accumulation of newly synthesized FN in extracellular compartments, immunoprecipitable FN in the cells was reduced by 22%. In contrast to the quantitative changes of newly synthesized FN in the three separated culture compartments, the total amount of newly synthesized FN was not elevated in ADR-treated cultures compared to controls. Qualitative characterization of the FN pattern revealed a diminished number of degradation products in the culture medium of ADR-treated HMC. As demonstrated by Northern-hybridization the total stock FN-mRNA level is increased by ADR. However, the levels of translationally active FN-mRNA measured by *in vitro* translation were unchanged following administration of the drug.

These data suggest that ADR exerts two different effects on the FN metabolism of HMC *in vitro*:

i. an increased transcription of FN-mRNA and
ii. a decreased degradation of FN secreted by the cells. By interfering with the FN degradation ADR induces an extracellular FN accumulation. This is answered by the cells with a negative feedback control of FN expression on the level of translation.

2. Isolated glomeruli

The Hannover group have used the large isolated glomeruli of the Atlantic hagfish *Myxine glutinosaas a* **multicellular *in vitro* test system** to study the ADR-effects on overall protein turnover (synthesis/degradation) in glomerular cells during the development of ADR-glomerulopathy. The glomeruli of pretreated hagfish were incubated *in vitro* with radioactive precursors (^{3}H-amino acids, ^{3}H-uridine) to determine total glomerular amino acid uptake, *de novo* protein synthesis (as amino acid incorporation into TCA-precipitable proteins) and *de novo* RNA synthesis. Total glomerular protease activity was measured by the azocasein test. ADR decreased the total glomerular amino acid uptake - not due to a reduction of the glomerular inulin space. In contrast, the incorporation of amino acids into TCA-precipitable proteins was enhanced after ADR-treatment, which was also reflected by an enrichment of total protein in the glomerulus. The inhibition of amino acid uptake could be prevented by the sulfhydryl-donor, N-acetylcysteine (NAC). whereas NAC had no influence on the increase in protein synthesis. Total *de novo* RNA-synthesis and glomerular total RNA-content were reduced after ADR-treatment. The proteolytic activity was diminished in glomerular homogenates of ADR-treated animals.

The results obtained from the studies with isolated hagfish glomeruli indicate that ADR interacts in this multicellular *in vitro* model with overall cellular protein turnover via different pathomechanisms:

i. The reduced total glomerular amino acid uptake is possibly due to radical-mediated disturbances of membrane function.
ii. The increased protein synthesis is best explained by a net-effect of synthesis and degradation due to metabolic disturbances in protein synthesis and/or protein degradation. However, there was no concomitant increase in RNA-synthesis. It is suggested that inhibited transcription and translation are compensated by a decreased proteolytic degradation. This leads to an enrichment of protein in the glomerulus and might finally cause structural and functional changes of the glomerular filtration barrier for water and proteins (Fig. 1).

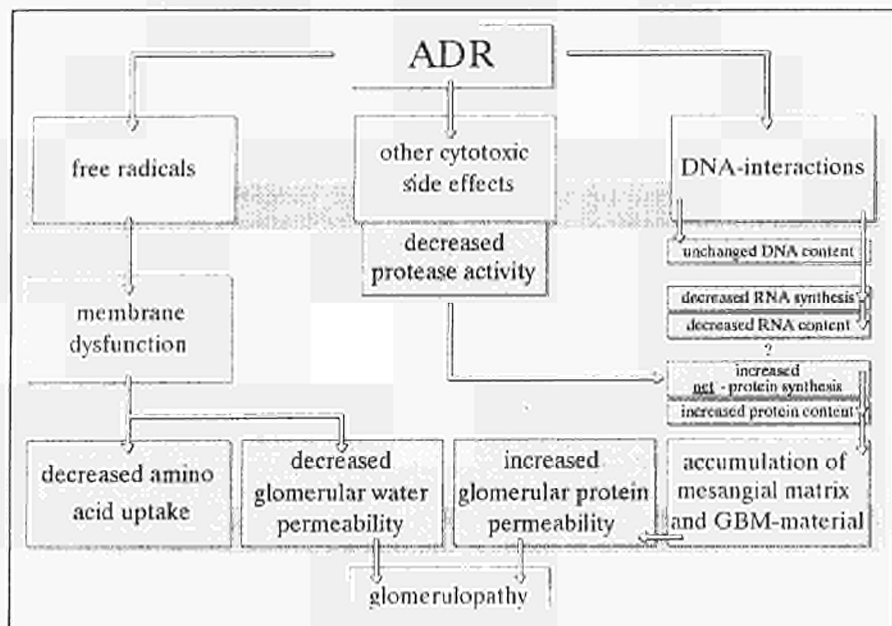

Fig. 1: *Cascade of pathological events contributing to the development of glomerulopathy*

In conclusion, in the two *in vitro* systems (isolated glomeruli, human mesangial cells) the primary target sites, the pathomechanism, and the cascade of pathologic events of nephrotoxins could be elucidated. As shown with the model compound adriamycin the nephrotoxic effects finally causing a glomerulopathy are due to several pathomechanisms summarised in Fig. 1 where the roles of free radicals, DNA-interactions and other cytotoxic side effects such as the altered activity of proteolytic enzymes are presented schematically. ADR disturbs the cellular balance of protein synthesis and degradation via these pathomechanisms in both test systems. In human mesangial cells, ADR interferes with proteases causing extracellular FN-accumulation and by a regulatory feedback-mechanism causing an inhibition of cellular FN-production, which results in an unchanged net *de novo* FN-synthesis. However, this regulatory mechanism is regarded insufficient to counteract during long-term ADR-treatment. This is confirmed by the studies on whole isolated glomeruli revealing an accumulation of proteins, due to an inhibited proteolytic degradation and an increased net-protein synthesis. When extrapolating these results on the *in vivo* situation, this could lead to an enrichment of

extracellular matrix proteins in the glomerulus thus finally causing structural and functional changes of the glomerular filtration barrier for water and proteins.

The *in vitro* findings from HMC and isolated glomeruli of a diminished proteolytic degradation confirm *in vivo* data on ADR-treated rats showing a loss of urinary FN-degradation products of renal origin. An altered urine pattern of FN-degradation products could be an early non-invasive marker for structural and functional changes.

Human mesangial cells in culture and isolated glomeruli therefore provide useful *in vitro* test systems to elucidate the primary target cells, the pathomechanism, and the cascade of toxic events of nephrotoxins.

B. Tubular Cells

The Lausanne group have studied the mechanisms of basolateral uptake of chelating agents in single S_2 segments of rabbit proximal tubule which had been incubated individually *in vitro* (without luminal perfusion) for investigating transport characteristics of 3 chelating agents diethylene triamine pentaacetic acid (DTPA), 2,3-dimercapto-1-propane sulphonic acid (DMPS), diethyldithio-carbamate (DDC). The steady-state tubular uptake of radioactively labelled p-aminohippurate (PAH), tetra-ethylammonium (TEA), or zidovudine (Zid) was measured in presence of various concentrations (0.01-1 mM) of the chelators in the incubation medium. It is known that PAH and TEA are taken up at the basolateral side of proximal tubules through the transport mechanisms of organic anions and cations, respectively, while Zid may be a substrate of both systems.

In control conditions, tubular uptake (tubule/medium ratio) approximated 150 for PAH, 180 for TEA, 90 for Zid. DMPS reduced PAH uptake only (by about 70% at 1 mM), DDC decreased TEA uptake only (by about 60 % at 1 mM), while both compounds reduced Zid accumulation (by 50 to 80 %). The inhibition of uptake was less at lower concentrations of chelators. DTPA was without effect on PAH, TEA or Zid uptake at all conditions tested.

The specificity of this interference was further assessed by studying the interactions between the chelators and the transported substrates in an exchange cotransport setup (trans-stimulation). Exit of PAH and TEA from preloaded isolated S_2 tubules was accelerated ($t_{1/2}$ reduced by 37-60 %) by DMPS or DDC, both of which increased the rate of efflux of Zid.

Uptake and *trans*-stimulation experiments suggest that 2 of the chelators studied show affinities for either the organic anion or organic cation transport system of the rabbit proximal tubule. These '*in vitro*' properties appear to correlate with the net secretion reported to occur in whole kidneys, at least for DMPS. These studies also point to the intermediary position of Zid regarding the renal transport systems involved in its transport, as suggested also by previous '*in vivo*' experiments.

The Austrian group have described the characteristics of a new renal proximal tubular cell line established by cell fusion. Untransformed continuous proximal tubular cell lines of human origin are not available. Established renal cell lines possess an indefinite life span, but lose a number of properties *in vivo et situ* ancestor cells properties, such as gluconeogenesis, specific hormone receptors or specific transport systems. For this reason hypoxanthine-guanine phosphoribosyltransferase deficient mutants ($HGPRT^-$) of the pig proximal tubular cell line LLC-PK_1 were fused with primary cultures of human (HPT) or rat proximal tubular RPT cells using polyethyleneglycol. $HGPRT^-$ mutants were produced by mutagenization of LLC-PK_1 wildtype cultures with N-nitrosoguanidine and selection of the HGPRT

defect mutant cells with 8-azaguanine. The resulting hybridoma cells were selected on hypoxanthine-aminopterin-thymidine media and characterized by their karyotypes, their expression of proximal tubular marker enzymes and ultrastructure. The hybridoma cells differ from the parent cells with respect to dome formation and density of microvilli, which is markedly enhanced in both hybridoma types. Karyotype analysis after 6 passages (one passage: confluent monolayer culture split 1:7 and grown to confluency again) LLC-PK_1-$HGPRT^-$ and HPT hybridomas displayed cells with 61-65 chromosomes, in case of fusion of LLC-PK_1-$HGPRT^-$ with RPT most of the hybrid cells obtained contained 66-75 chromosomes after passage 26. Although these values are slightly less than the sum of the 2n sets of the fusion partners (LLC-PK_1 2n = 38, HPT 2n = 46, RPT 2n = 42) they indicate successful fusions and the establishment of stable hybrid cells, which can be considered new immortal cell lines. It is expected that these cell lines retained a number of properties lost in the LLC-PK_1 fusion partner. Furthermore, for the first time a permanent cell line with characteristics of the human proximal tubular cell is available. The functional features specific for the human proximal tubule have now to be characterized in detail. Both newly tailored cell lines appear to be promising new model systems for *in vitro* nephrotoxicity testing.

Gstraunthaler (Austria) compared two gluconeogenic substrains of continuous renal epithelial cell lines, designated LLC-PK_1-$FBPase^+$ and OK-GNG^+'. which differ markedly in their metabolism of L-lactate (LAC) and pyruvate (PYR). OK-GNG^+ cells consumed LAC at high rates in contrast to LLC-PK_1-$FBPase^+$ cultures. PYR consumption rates by LLC-PK_1-$FBPase^+$ cells were linear over time, without considerable LAC production, whereas PYR consumption by OK-GNG^+ cells was almost completed after 24 hour, and resulted in an equimolar production and accumulation of LAC in the culture medium, which was consumed by the cells thereafter. In addition, L-alanine (ALA) was found as an end product of LAC and PYR metabolism of both cell strains which was reutilized by the cell strains at different rates.

To further delineate these differences in LAC and PYR metabolism, specific metabolic inhibitors were applied: 3-mercaptopicolinic acid (3-MPA) as inhibitor of phosphoenolpyruvate carboxykinase (PEPCK), and aminooxy acetate (AOA) as inhibitor of transamination reactions. 3-MPA dramatically decreased rates of LAC consumption of OK-GNG^+ cells, whereas accumulation of ALA in tissue culture media was increased, especially OK-GNG^+ cultures. AOA exhibited remarkable effects on LAC and PYR metabolism of the gluconeogenic substrains. In OK-GNG^+ cells LAC consumption was inhibited by AOA, whereas PYR consumption by LLC-PK_1-$FBPase^+$ cells was slightly stimulated.

These metabolic differences between LLC-PK_1-$FBPase^+$ and $OGNG^+$ cells might be due to differences in the regulation of gluconeogenesis by the intracellular NADH-NAD ratio, and/or differences in the rate of mitochondrial substrate fluxes and shuttle activities, possibly as a consequence of differences in the subcellular (cytosolic *versus* mitochondrial) localization of PEPCK.

Besides these differences in PEPCK expression, which may allow the study the significance of mitochondrial PEPCK in renal gluconeogenesis from LAC, these cell strains may also provide suitable model systems to study *in vitro* the interrelationship between renal gluconeogenesis and ammoniagenesis, another prominent mitochondrial metabolic feature of proximal tubular epithelial cells. Both cell strains, OK-GNG^+ cells cultured on LAC and LLC-PK_1-$FBPase^+$ on PYR, clearly adapted to metabolic acidosis (pH 7.0) by an increase in ammonia production, which was accompanied by an increased ALA excretion of both cell strains.

However the ammoniagenic response was much more pronounced in $LLC\text{-}PK_1$-FBPase$^+$ than in OK-GNG$^+$ cultures.

Optimization of culture conditions in collaboration with the Rouen group indicate that a much improved culture of rat primary cells can be developed. The cells grown in collagen-coated disks in hormonally-defined and glucose-free medium have shown much improved maintenance of *in vivo* characteristics compared to cells grown in media containing serum and glucose. Under the new conditions cells demonstrated increased levels of the gluconeogenic enzymes glucose-6-phosphatase and fructose-1,6-biphosphatase. They also exhibited lower levels of the glycolytic enzymes hexokinase and lactate dehydrogenase. Electron microscopy revealed that cells grown under the new conditions had a much improved ultrastructure, with more intact mitochondria and an extensive brush border.

Cell fusion experiments by the Rouen group in collaboration with the Innsbruck group have been performed using rabbit primaries fused (using the fusogen polyethylene glycol) with either $LLC\text{-}PK_1$ wild type, $LLC\text{-}RK_1$ wild type or OK-ATCC wild type. The criteria for selection were the ability to grow in glucose free culture media and to be passaged for more than 10 times. None of the fused clones survived for more than 6 passages in the absence of glucose. Intermediary characterization of these lines at the 4th passage showed the absence of brushborder marker enzymes for the $LLC\text{-}RK_1$ fusion experiment and the very low expression of glutathione-S-transferase and fructose-1,6-bisphosphatase for the $LLC\text{-}PK_1$ fusion experiment. Growth rates of these fusion products were very low: at least one month was necessary between two passages. As a conclusion of these first attempts with cell fusion experiments we failed to isolate promising and stable fusion products owning both immortality and specific proximal tubule characteristics.

The Rouen group showed the impact of increasing oxygen availability has been assessed on cellular phenotype expression of rabbit proximal tubule cells in primary culture developed with variable glucose and/or insulin contents. To mitigate hypoxia at the cell/medium interface, cells were shaken for the whole culture duration and their expressed phenotype compared with those expressed by static cultures. Oxygen tension was kept constant in the incubator atmosphere. Glycolysis and gluconeogenesis pathways, detoxication system, and mitochondrial, apical and basolateral membrane marker enzyme activities were assessed. As measured by extracellular lactate content, glucose consumption and lactate dehydrogenase activity, less glycolytic metabolism was seen in shaken than in static cultures grown in the presence of insulin and glucose. pH stability of the culture medium was markedly improved with time in shaken cultures compared to static cultures which become rapidly acidic. ATP levels were higher in cultured cells than in freshly isolated proximal tubules. A slight decrease of intracellular ATP levels is observed when cells are grown in shaken conditions compared with still conditions. Deprivation in both insulin and glucose partially prevented the rise in glycolysis while no impact of shaking was evidenced on gluconeogenesis. In all culture media, shaken cells exhibited apical membrane markers, gluconeogenic and detoxication system activities similar to cells grown under static conditions. In high glucose medium, rocked cells exhibited lower levels of basolateral membrane marker Na^+/K^+-ATPase, and higher levels of the mitochondrial marker succinate dehydrogenase. In conclusion: the induction of glycolysis which appear in primary cultures of proximal tubule cells may be partially prevented by continuously shaking the cultures. Substrate oxidation seems to occur in a more efficient way in shaken cultures.

The Rouen group have assessed cephaloridine nephrotoxicity in rabbit primary cultures of proximal tubule cells for 72 hours at concentrations up to 5 mM. Culture medium was renewed daily. Intracellular levels of ATP were not altered for non-cytotoxic dosages of cephaloridine (< 2mM) alpha-methyl glucopyranoside transport and leucine incorporation rates into proteins decreased for concentrations > 0.6 mM. gamma-glutamyl transpeptidase activity increased for concentrations > 0.6 mM with a maximum reached for 2mM. Na^+/K^+-ATPase remained unaffected for non toxic dosages. Glutathione pathway activities were markedly altered by cephaloridine exposure: increased levels of GSH, GSH-reductase and GSH-S-transferases activities occurred after cephaloridine exposure in a dose dependent manner.

The Rouen group have characterized the OK clone provided by the Innsbruck group. Unlike the ATCC-OK clone, this clone was able to transport alpha-methyl glucopyranoside in a sodium dependent way, and expressed higher levels of succinate dehydrogenase. Similarly, with the ATCC-OK clone, gamma-glutamyl transpeptidase activity was at the limit of detection of the assay, no alkaline phosphatase activity has been detected and low levels of Na^+/K^+-ATPase and glutathione-S-transferase were observed.

HIGHLIGHTS/MILESTONES

— Development of computer-based numerical modelling utilizing expert systems to predict toxicity and the potential to start applying this to biotechnology products.
— Isolated glomeruli and human mesangial cell models have been developed to detect mechanisms of glomerular toxicity. Changes in fibronectin expression as detected in model systems may be used for both *in vitro* tests and non-invasive diagnosis in humans.
— Development of new culture conditions have allowed maintenance of differentiated function of rat and rabbit primary proximal tubular cells. Toxic responses to aminoglycosides and platinum derivatives in these cell culture systems reflect more closely *in vivo* toxicity profiles compared to tests with presently available cell lines.
— Cell fusion experiments with primary human cells from proximal tubules and established novel renal cell lines have yielded very promising cells for development of new permanent non-transformed and non-tumourgenic cells with human characteristics for toxicity testing.
— Electrophysiological techniques were developed using renal cell lines grown on microporous filters to detect short-term ion transport modifications and barrier function defects induced by nephrotoxic compounds.
— Flow cytometric methods have been established for cell viability testing, and the mechanistic basis of toxicity including pH and [Ca]i changes in response to toxic compounds and selecting fused cells in the process of developing new cell lines.

WIDER CONSIDERATIONS

III. Value added nature of the research programme.

A wider application of fluorescent probes and confocal microscopy is being used to assess sub-cellular biochemistry and ways from which the 2-D and 3-D spacial relationship between different cell types can be assessed.

A. Financial support

Funding for the research has been extended beyond the EC programme grant BIOT-CT90-083 C to include Austrian Science Foundation (Project P8074), NIEHS (P30 ESO3828/03-07), British Council and PCFC Research Initiative support for the Interdisciplinary Research Centre for Cell Modulation.

B. Collaboration beyond the Bridge consortium

The growing impact of the Bridge consortium is facilitating an additional number of collaborations. These include:
R. Horobin, Dept Biomedical Science, Sheffield University, U.K.
M. Moore, Plymouth Marine Laboratory, Plymouth, U.K.
Jean Cambar, Groupe d'Etude de Phisiologie et de Physiopathologie Renales, Faculte de Pharmacie, Bordeaux, France.

COOPERATIVE ACTIVITIES

Exchange visits and exchange of materials

All contractors met on 24/4/92 in London at the first European Workshop on Biotechnology Applications for Microinjection, Microscopic Imaging and Fluorescence. Progress reports from each laboratory were presented and plans for future work were agreed.

Ms. Edel Healy (Dublin) visited the Innsbruck laboratory from May 18-27, 1992. The technique of primary renal cell culture from rat and human was transferred from Dublin to Rouen. The technique of cell fusion of primary and established cell lines was transferred from Innsbruck to Dublin. Collaborative studies involving use of flow cytometry were initiated.

Dr. Jean-Paul Morin (Rouen) visited the Dublin laboratory from July 22-27, 1992. Exchange of methodologies for primary culture of rat and rabbit proximal renal cells was effected by experimentation and demonstration. Collaborative experiments were initiated.

Ms. Edel Healy (Dublin) visited the Rouen laboratory from April 1-9, 1993. Techniques of measuring gluconeogenic, glycolytic and drug metabolising enzymes were carried out in Rouen on rat primary cells cultured under different conditions in Dublin.

All the contractors met at the BRIDGE Sectoral meeting on *in vitro* Evaluation of the Toxicity and Pharmacological Activity of Molecules held in Dublin 8-10 of December 1992. Presentations were made by each group and future schedules for work were agreed.

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Analysis of gene transfer between microorganisms and plants (BIOT CT-910282)

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RESULTS

I. The establishment of an *in vitro* experimental system to investigate gene transfer from *Nicotiana tabacum* to *Agrobacterium* tumefaciens

Interkingdom gene transfer is a potential risk factor to be considered when genetically manipulated microorganisms and plants are to be released into the environment. Horizontal gene transfer from the bacterium *Agrobacterium tumefacien* to tobacco plants (*Nicotianum tobacum*) which leads to the formation of crown gall tumors, is well-characterized. Close cell-cell contact is a prerequisite for the transfer of the *A. tumefaciens* T-DNA to the plant cell during the tumor induction. The current investigation aims to determine whether the cellular contact within the tumor enables a horizontal gene transfer to occur in the opposite direction i.e. from *N. tobacum* to the bacterium.

In the previous BRIDGE Progress report, we described results of an experiment in which *A. tumifaciens* recovered from a tumor apparently had incorporated DNA originating from the plant genome (the *aacCl* gene-see below) thus implying horizontal gene transfer. Further investigation revealed that this signal was due to the non-specific hybridization of the probe to the *A. tumefaciens*' genome. Two factors were considered to have contributed to this false positive result: unreliable marker genes (the *A. tumefaciens* cells showed a high rate of spontaneous resistance to gentamicin which was used for the selection of transformants) and a lack of specificity in screening putative transformants by colony hybridization.

In order to circumvent these two experimental difficulties, the selection method was modified and the following marker genes were chosen:

a) *luc:* the luciferase gene of the north American firefly *Photinus pyralis*, modified to enable its expression and detection in bacterial and plant cells,

b) *nptII:* the aminoglycoside phosphotransferase gene which confers resistance to neomycin (Nm) and kanamycin (Km) and is selectable in bacterial and plant cells,

c) *aacCl:* the bacterial gentamicin acetyltransferase-3-I gene which confers gentamicin (Gm) resistance in bacteria.

These more specific genetic markers were cloned together to generate pIB19 which was used to transform *A. tumefaciens* strain LB4404. Leaf discs of the wild type *N. tobacum* SR1 were infected with *A. tumefaciens* LB4404 containing the plasmid pIB19 and cultured under conditions allowing regeneration of mature plants. The regenerated shoots and resulting transgenic tobacco plants were

propagated on medium containing Km and cefotaxime. The stems of the plants were infected with the wild type *A. tumefaciens* strain A281. After growth of the tumor, five were excised aseptically, macerated, and plated on medium supplemented with Km to select *A. tumefaciens* transformants to which the marker genes had been transferred. More than 1000 Km^r bacteria were isolated and found to be Gm^r and luc^+. Analysis of the plasmid profiles of these bacteria revealed that they were A. tumefaciens LB4404 (the strain originally used to transform the *N. tobacum* leaf discs). The plasmid pIB19 however, was not detectable in any of the strains tested, and some of the large plasmids indigenous to the strain had undergone rearrangements. Thus, the bacteria LB4404 persisted, despite the fact that the plants had been grown aseptically on selective medium containing cefotaxime and the long time interval (11 weeks) between the leaf disc transformation and the isolation of bactcria from the tumors.

In order to eliminate the possibility of bacteria persisting on the plants they transformed, transgenic plants of the type SRI+ (*aacCl, nptII, luc*) are presently being grown from sterilized seeds. The experiment will be conducted using the resulting transgenic plants as the starting material.

In many cases, it is impossible to precisely reproduce natural conditions in the laboratory and therefore other members of the consortium have carried out similar experiments investigating gene transfer from plants to bacteria under field conditions as described below.

II. Gene transfer from transgenic tobacco plants to bacteria in soil and on leaf surfaces under field conditions (RPA and UCB)

In order to address the question of whether genes incorporated into the plant genome are released and incorporated into bacteria of soil ecosystems, a PCR approach is being developed to isolate DNA from bacteria surrounding the roots as well as those on the leaf surface of transgenic tobacco. These bacteria will be isolated using procedures designed to:

a) avoid growth of the bacteria *in vitro* (i.e. growth of the soil bacteria *in vitro* is known to select for only a small subpopulation)
b) separate bacteria from free exogenous free DNA
c) avoid damage to the bacterial cell wall.

Bacterial DNA will then be extracted and analyzed by PCR for the presence of a specific marker of the tobacco genome as well as for the presence of *aacCl* marker gene present in the tobacco genome.

Tobacco plants 525-4 carrying one copy of the gentamicin resistance gene (*aacCl*) were used for a small scale field trial authorized by the French Commission du Génie Biomoléculaire during the spring of 1992. This assay was conducted both at the SEITA experimental farm in Bergerac and at the RPA's experimental research station in Chazay d'Azergues. These studies were devised to provide samples to detect possible transfer of *aacCl* to bacteria inhabiting the soil (SEITA) or plant leaves (RPA).

In order to establish the method we first focused on developing conditions for recovery of bacterial DNA from soil samples. An method modified from several published protocols was employed to obtain efficient recovery of soil bacteria. Using these conditions, 10^7 bacteria per gram of soil are routinely counted after labelling with acridine orange. Specific primers were selected from the *aacCl* gene and a *Nicotianum tobacum* RuBisCO gene. To avoid the possibility of recovering

a RuBisCO gene from photosynthetic bacteria, one of the primers was located within an intron found only in plant RuBisCO genes. These oligonucleotides allowed us to detect the expected DNA fragment after PCR amplification of 'total soil DNA'. Thus we were able to conclude that soil contains intact DNA and that chemical components found in the soil (humid acids, clays, etc.) do not preclude the use of PCR to recover specific fragments.

Indeed, in our attempts to isolate bacteria from soil, we learned that DNA may be protected in some physiochemical niches. Treatment of our bacteria with DNAseI at fairly high concentration (up to 20 mg/ml) followed by lysis, led to the amplification of fragments both with the *aacC1* and the RuBisCO primers. The most likely explanation for this result is that exogenous plant DNA was in some way protected from DNAseI digestion.

In conclusion, we have established an efficient method for the isolation of soil bacteria and demonstrated the application of the PCR reaction to detect DNA in soil samples. Further efforts will be made to determine the conditions which allow complete removal of exogenous DNA from bacteria. These techniques will be then extended to the analysis of bacteria on leaves before the end of this year.

In order to analyze the possibility of gene transfer under conditions that favour the transformed soil bacteria, we are constructing transgenic tobacco plants containing a native bacterial bromoxynil degrading gene. These plants will be then crossed with tobacco plants carrying this gene under the control of an eukaryotic promoter and hence fully resistant to the herbicide. After treatment with the herbicide and possible interkingdom transfer, the transformed bacteria will be able to use this compound as an supplementary nitrogen source i.e. providing them with a selective advantage. Tobacco plants for use in these studies will be available before the end of this year.

III. Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction (UCB).

Sensitive methods have to be developed for detecting specific microorganisms within soil microbial communities and for monitoring the fate of their DNA in natural ecosystems which are often complex and heterogeneous. We developed a new method based on the polymerase chain reaction for this purpose.

In order to improve thc scnsitivity of detection we have optimized each step of the protocol including bacterial isolation, cell lysis, DNA purification, and PCR amplification. Development of a protocol allowing efficient cell breakage was a critical step since many microorganisms cannot be lysed using classical techniques. DNA was extracted from soil microorganisms using successive cycles of sonication, micro-wave heating, and thermal shocks to increase the efficiency of the lysis. Purification of nucleic acids was achieved by passage through Elutip columns. Finally, PCR amplifications were optimized via biphasic protocols using booster conditions, lower denaturation temperatures and in the presence of formamide. This method has been applied to 100 mg of soil samples inoculated with *A. tumefaciens*. Specific primers chosen in the plasmid-borne *vir* genes have routinely permitted the detection of the *A. tumefaciens* strain when inocula ranged from 10^7 to 10^3 cells. Moreover, the strong correlation between the size of the inocula and the yields of the PCR reactions permitted assessment of the validity of the protocol in quantifying the number of microbial cells present in a soil sample.

Adhesion of microbes and macromolecules to soil components is known to be related to surface characteristics of both partners, such as charge, hydrophobicity, and microaggregate formation which are in turn influenced by soil pH and water content. These factors are also involved in the adhesion of nucleic acids released from plant, animal or microbial cells. Understanding the mechanisms involved in DNA adsorption onto soil particles could provide information for monitoring the fate of free DNA in the soil and its ability to transform soil microbes.

We investigated the interactions which could exist between clay montmorillonite with high surface areas and the DNA. The extent of DNA adsorption was affected by the concentration and valency of the cations used (Mg^{2+}, Ca^{2+}, Na^{+}), indicating a charge-dependent process. Calf thymus DNA was found to be highly adsorbed by smectite (up to 30 mg/g of dry clay). Adsorbed DNA was shown to be more resistant to degradation by DNase I than free DNA. Experimental data with plasmid and short linear DNA showed that protection against nucleases was only partial. Nevertheless, clay-adsorbed DNA was found to be biologically active, even after DNase I treatment, and could transform competent *E. coli* cells. These results show that persistence of DNA and gene transfer by genetic transformation may occur in soil.

IV. Effects of environmental conditions on conjugative gene transfer (UBA)

The efficiency of conjugation between different strains of *Rhizobium* and *E. coli* depends on the biological, chemical and physical characteristics of the soil. We have designed laboratory microcosms to simulate as nearly as possible a natural habitat and still allows careful control of defined physiochemical factors to determine their effects on the efficiency of conjugation. By using the microcosms/metal-pin sampler methodology, developed during the previous BAP project, of which this BRIDGE project is an extension and enlargement, we have now shown that environmental chemical and physical factors, such as water content, pH, and temperature, affect plasmid transfer efficiencies. In addition to affecting the dispersion rates of the cells in soil and thus potential establishment of mating pairs, such environmental factors specifically affect the conjugation system.

R. meliloti dispersion rates in soil were altered only at highly acidic or alkaline conditions ($9 > pH > 3$), whereas *E. coli* strains showed a stenotopic response under the experimental conditions tested. However, taking into a account exclusively gene transfer frequencies, both species showed a very similar response, and had a maximum at neutral pH.

Another effect of environmental conditions on gene transfer in the natural environmental may relate to the polyhydroxyalkanoate (PHA) content of the cells. PHA, an energy reserve in most bacteria, has proven to be an indicator of the physiological status of microbial communities in nature. Optimal environmental conditions for growth are associated with low PHA cellular content, whereas adverse environmental conditions for growth are associated with high PHA levels. In addition, there is some evidence that membrane-bounded PHA could play a role in DNA uptake.

Our first results show that PHA-rich *R. meliloti* cells have slightly higher gene transfer rates than PHA-poor cells when conjugating with other *R. meliloti* on agar plates. Membrane-bound PHA granules may play a role in facilitating plasmid uptake by the recipient cell. The accumulated polymer might also participate in a global cellular response to environmental stress conditions by providing additional energy to survive.

We have extended the study to the strains *R. meliloti* 24-1, *R. meliloti* 2011 and *R. meliloti* 130 in order to assess the importance of two factors: first, the culture conditions for polymer accumulation, and secondly, the possible influence of PHA accumulation on the intraspecific conjugation.

R. meliloti was grown in a modified yeast-mannitol medium where the carbon source was replaced by glucose, fructose, or acetate. Glucose (1% w:v) yielded the highest levels of PHA accumulation. PHA was analyzed by HPLC. Cells grown on 1% glucose accumulated up to 1.06 fg PHA $cell^{-1}$, whereas cells grown without glucose accumulated only 0.0065 fg PHA $cell^{-1}$. PGA-rich and PHA-poor cells are being used alternatively as donors or recipients to assess the possible influence of PHA accumulation on the frequency of conjugational gene transfer.

We have continued the study of plasmid interspecific transfer frequencies between strains of *E. coli* and *R. meliloti* in sterile non-amended soil Petri dish-microcosms. Cells were introduced in the microcosms at concentrations ranging from 10^7 to 10^8 cells per gram of dry soil. Microcosm water content was maintained at 40% (v:w). The strains *E. coli* 650-120, *E. coli* J53 and *R. meliloti* 2011, all of them carrying either the plasmid RP4 or a derivative, were used as donors. Recipient strains were *R. meliloti* 130 and *R. meliloti* Rm42-1. Using this system, genetically modified *E. coli* cells have been proven to have very low dispersing capacity, which presumably limits their ability to conjugate in soil ecosystems.

V. Evaluation of gentamicin acetyltransferase gene (*aacC1*) plasmid mediated transfer from genetically modified *Agrobacterium tumefacien* to *Rhizobium legominosarum* natural isolates and other bacteria

Experiments carried out under the BAP contract in collaboration with the Bielefeld group, allowed us to develop the appropriate methodology to assess possible transfer of the gentamicin resistance gene (*aacC1*) from genetically modified R. leguminosarum and A. tumefaciens into their wild type counterparts and Pseudomonas syringae. Under this BRIDGE contract, we are extending these studies in order to: (a) ask whether the *aacC1* gene can be transfered from genetically engineered *A. tumegaciens* (UBAPF2a,b) into *R. leguminosarum* natural isolates collected from soil samples in Greece and into other Gram-negative bacteria by conjugation, under laboratory and soil conditions; and (b) examine how stable the transfered *aacC1* gene is in the recipient microorganisms. As a first step towards these objectives, a number of R. leguminosarum natural isolates obtained from Greek soil samplcs (*R. lcguminosarum* GNI) were characterized with respect to their resistance to antibiotics, plasmid profile and growth properties. Several were selected as potential recipients for conjugation studies with *A. tumefaciens* (UBAPF2a-c). Conjugation was carried out on filters and potential transconjugants were obtained by counter selection and growth in medium containing ampicillin, gentamicin, and spectinomycin. In most conjugation experiments carried out with various *R. leguminosarum* GNIs no transconjugants were observed. However, when UBAPF2b was crossed with *R. leguminosarum* GNI3d or R. leguminosarum GNI1d, candidate transconjugants were obtained. Rigorous testing of these potential transconjugants for their ability to grow in fortimicin, to express the *aacC1* acetyltransferase enzyme activity, to promote nodulation in Vicia faba plant seedling and the presence of the plasmid with the *aacC1* gene revealed that these transconjugants were not authentic *R. leguminosarum* natural isolates bearing the *aacC1* gene. Despite repeated efforts we have not yet been able to verify transfer of *aacC1* gene from genetically modified *A. tumefaciens* UBAPF2a,b into *R. leguminosarum* natural isolates. Most recently we have also attempted to

investigate whether *aacC1* gene can be transferred from *A. tumefaciens* URAPF2 into other Gram-negative bacteria.

VI. Analysis of gene transfer apparently catalyzed by a single gene found in *Streptomyces* plasmid pIJ101 (IP)

Bacterial soil populations include a large number of Actinomycetes (primarily Streptomycetes) which could potentially play a major role in catalyzing gene flux in the environment. Bacterial plasmids have long been implicated in this process however, our understanding of their participation is based largely on characterization of one plasmid conjugation system operative in Gram-negative organisms. In the case of the best characterized *E. coli* plasmid, F, 20-30 genes are required for mating pair formation and DNA transfer. In contrast, our results show that very few genes are required for conjugal transfer of the best studied streptomycete plasmid, pIJ101 (8.9 kb). In *Streptomyces*, our genetic analyses of mating experiments seem to suggested that spontaneous mycelial fusions result in heterocaryon formation, however at least one plasmid-encoded gene, *tra*, is required for both plasmid transfer/establishment and genetic exchange (recombination) of chromosomal markers. A recent protein database search has indicated that the *tra* gene is similar to a regulatory protein needed for sporulation in *Bacillus subtilus*. This may suggest that the tra gene serves to activate a chromosomally encoded regulon required for both plasmid transfer and recombination.

Potential heterokaryon formation is now being analyzed using the the bacterial luciferase system. In this system the production of light requires both LuxA and LuxB, two subunits of the *Vibrio harveyi* luciferase enzyme complex. We have cloned genes encoding these two proteins using compatible vectors in the same host and have demonstrated light production by complementation in *E. coli* as well as *Streptomyces*. We have not yet been able to demonstrate heterokaryon formation in *Streptomyces* using this system.

We are also studying the participation of the *tra* gene in the conjugation event which allows plasmid transfer and genetic recombination using a construction we have made which places the *tra* gene under the control of a thiostrepton inducible promoter system developed in our laboratory (*ptipA*). Preliminary experiments indicate that transfer of this plasmid is inducible by thiostrepton. Its ability to promote chromosomal recombination in the presence of thiostrepton is now being assessed.

Previous studies (BAP) from our laboratory demonstrated that Gram-negative bacteria (*E. coli*) can transfer genetic material to Gram-positive bacteria (*Streptomyces* and *Mycobacteria*). More recently, electron microscopic studies have been undertaken in order to visualize how these genetic exchanges occur. Electron micrographs of mixtures of *S. lividans* and *E. coli* or *Mycobacterium smegmatis* and *E. coli* under conditions where conjugation is taking place have revealed bridges between the two organisms. The structure of these points of contact suggest a fusion-like event (perhaps similar to that observed between two *Streptomyces*) which may allow partial mixing of the two cytoplasms or even genetic recombination between the two organisms. Apparently, these fusion events, in conjunction with plasmid fertility genes, are the two events which are necessary for chromosomal recombination between a streptomycete chromosome and homologous or perhaps even heterologous DNA.

HIGHLIGHTS/MILESTONES

Successful application of the polymerase chain reaction (PCR) to recover DNA from soil samples.

WIDER CONSIDERATIONS

Our consortium is interested in understanding natural mechanisms of gene transfer between different bacteria and from plants to bacteria as it may occur in the ecosystem. This question, which has not attracted widespread interest in the past, has become of foremost importance today with the planned release of recombinant organisms in the near future.

COOPERATIVE ACTIVITIES

All members of this BRIDGE consortium were represented at group meetings held at the Instiut Pasteur (June, 1992) during the BRIDGE sectoral meeting on Biosafety in Wageningen (December, 1992). UBC and RPA are collaborating in field trials of recombinant plants.

EUROPEAN DIMENSION

Legislation designed to deal with the potential risks of interkingdom gene transfer in the environment is an issue important to the European/global ecosystems. The research carried our under this BRIDGE contract is designed to provide a rational framework to determine whether such EEC legislation should be considered.

PUBLICATIONS

PAGET, E., L. J. MONROZIER, and P. SIMONET. 1992. Adsorption of DNA on clay minerals: protection against DNaseI and influence on gene transfer. FEMS Microbiol. Letters **97:** 31-40.

PICARD, C., C. PONSONNET, E. PAGET, X. NESME, and P. SIMONET. 1992. Detection and enumeration of bacteria in soil by direct DNA extraction and polymerase chain reaction. Appl. and Environ. Microbiol. **58:** 2717-2722.

Fate of genetically engineered microorganisms (GEMs) and genetically engineered DNA sequences (GEDs) in some environmental hot spots (BIOT CT-910284)

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OBJECTIVES

a) Construction of bacterial strains ('GEMs') for microcosm studies.

b) Measurement of the effect of environmental factors on survival and gene transfer of GEMs.

c) Assessment of the capacity of the selected environmental spots to take up genes from introduced GEMs.

d) Monitoring the natural gene mobilizing potential of selected environmental spots.

e) Monitoring the interaction between introduced GEMs and the natural microbial community of the selected environmental spots.

f) Development of a soil DNA detection procedure

g) Development of biological containment systems

MAJOR PROBLEMS ENCOUNTERED

— The construction of the heavy metal resistance marker gene cassette took longer than anticipated. This was entirely due to the discovery that the full resistance phenotype required the presence and expression of previously unknown regulatory genes (*czc*RI, Diels, Dong, Baeyens and Mergeay, unpublished observations ; Nies, J. Bacteriol. 174: 8102, 1992). These have now been successfully cloned and confer suitable resistance levels.

— For testing gene transfer from GEMs introduced into environmental spots to the natural microbes present in that spot, we planned to make use of a Dap- *E. coli* donor strain (*E. coli* 1849, Robeson et al., J. Bacteriol. 153: 211, 1983). This strain is unable to grow in the absence of diaminopimelic acid and hence can easily be counterselected on conventional rich media allowing for straightforward isolation of indigenous microbes that have taken up donor marker genes. However, we found the Dap- strain to survive extremely poorly in mating experiments. Consequently, frequencies of gene transfer were low. Attempts to isolate more robust *E. coli* or *P. putida* Dap- strains were unsuccessful. As an alternative strategy to measure gene transfer into indigenous microorganisms, use was made of auxotrophic donor strains (selection on minimal agar plates) or marker genes that can not be expressed in the donor but can in certain indigenous bacteria (see further).

RESULTS

a) Construction of GEMs for gene transfer studies in microcosms of the selected environmental spots.

For microcosm studies bacterial strains (*E. coli, P. putida, A. eutrophus*) were needed with marker genes that occur unfrequently in environmental samples (hereafter called GEMs even if recombinant DNA techniques were not always used to obtain the strain). Genes encoding for resistance to heavy metals, to antibiotics and for the catabolism of certain xenobiotic organic molecules were used for this purpose. For gene transfer studies GEMs with marker genes on mobilizable and non-mobilizable plasmids and on the chromosome were required. These marker genes should be expressed in either a narrow or in a broad range of bacterial species depending on whether gene transfer is studied from GEMs to introduced strains or from GEMs to indigenous microbes, respectively. The genes encoding resistance to the heavy metals cobalt, cadmium and zinc (*czc* operon) are expressed in *A. eutrophus* but not in *E. coli* and *P. putida* and can be used to assay gene transfer from *E. coli* and *P. putida* into *A. eutrophus*. The genes encoding resistance to mercury as present in pQKH6 are expressed in α, β and γ Proteobacteria and are adequate for following its transfer into the indigenous microbial populations.

A DNA fragment containing the complete *czc* operon (*czc*RI*czc*CBAD) was cloned into pUC18, pRK415 (Tra-, Mob+ , IncP), pKT240 (Tra-, Mob+, IncQ), and into other plasmids designed to permit integration of the *czc* operon into the chromosome of *E. coli* (pOM40) and other Gram-negative bacteria (pUTminiTn5) [Mol]. P. putida KT2440 and A. eutrophus AE104 strains with chromosomal inserts of *czc*RI*czc*CBAD were obtained. Plasmid pQM901 was constructed from the epilithic plasmid pQKH6 (60 kb, Hg^R, Tra^+) by insertion of a Tn5-based transposon containing a kanamycin resistance and a catechol-2,3-dioxygenase gene (*tdnC*)[Cardiff].

b. Effect of environmental factors on gene transfer between GEMs.

Survival and gene transfer of GEMs in environmental spots may differ from those recorded in the laboratory due to the effect of environmental factors, e.g. the presence of nutrients from roots, temperature, indigenous organisms, soil compounds and toxic compounds. Progress was made in understanding the effect of some of these factors. Transfer of the epilithic plasmid pQM85 in homologous matings of *P. putida* cells (strain KT2440 to UWC3) on membrane filters was maximal at 15-20 °C. Another epilithic plasmid pQKH6 showed optimal transfer at 15-40 °C in the same assay system [Cardiff]. Optimal temperature for transfer of a F' plasmid in homologous matings of *E. coli* K12 cells (strains XL2 and RB85) was shown to be 37 °C both on plates and in silty clay soil microcosms, corresponding to the optimal growth temperature for *E. coli* [Oeiras]. In sterile soil the tested *E. coli* K12 strains survived poorly. However, when *Zea mays* plants were grown survival was much better. The *E. coli* K12 strains also seemed capable of colonizing the plant roots and of performing gene transfer in the rhizosphere, the preferential zone for bacterial growth and plasmid transfer being associated with the root hair zone. Bentonite was shown to reduce the frequency of transconjugant formation in this mating pair both on plates and in the soil microcosm. Bentonite slightly reduced the frequency of transfer of RP4 from *E. coli* K12 J62 to *P. putida* CTQm 106 or *P. fluorescens* CTQm103 and had no effect on the mobilization frequency of the *czc*CBAD and *czc*RI*czc*CBAD genes from *E. coli* to *A. eutrophus* in filter matings [Oeiras].

Previously, it was shown that in soil microcosms the *czc*CBAD gene fragment cloned in mobilizable plasmids (pRK290 and pKT210) and the *czc*RI*czc*CBAD gene fragment cloned in pBR325 can be transferred from *E. coli* to *A. eutrophus* [Gent]. Similar microcosm studies with two Portugese soils (silty-sand and silty-clay) showed very low or undetectable transfer of the *czc* genes from *E. coli* to A. eutrophus [Oeiras]. Experiments will be performed to understand the reason for this difference. The Gent group now reports that, in the soil microcosm system, retromobilization of IncQ plasmids with *czc*CBAD marker genes can occur from *P. putida* to *A. eutrophus* both in sterilized and unsterilized soil.

In a microcosm system simulating diseased plants (i.e. potato discs infected with *Erwinia carotovora*), RP4::Tn*bph* gene transfer between *E. coli* and *P. putida* was studied. It was found that *E. carotovora*-induced deterioration of the plant material did not affect the transfer rate [Gent].

c. Assessment of the capacity of the selected environmental spots to receive genes from introduced GEMs.

Transfer of the plasmid RP4::Tn*bph* (Tra+) from *E. coli* into the indigenous microbial population of sandy and sandy-loam soil polluted with biphenyl was observed. The *E. coli* donor strain is unable to grow on biphenyl as sole carbon source and is a methionine auxotroph allowing efficient selection for transconjugants and against donors on a mineral medium with biphenyl. Soil transconjugants carried plasmids indistinguishable in size from RP4::Tn*bph*. Similar gene transfer results were obtained using as donor a soil bacterium provided with the RP4::Tn*bph* plasmid. No transfer was detected in non-polluted soil [Gent].

Systems to study gene transfer from GEMs to the indigenous microflora were also used by Cardiff. A first system using Dap$^-$ *E. coli* donor strains was found not suitable for gene transfer studies (see above). A second system used a tryptophan auxotrophic *P. putida* PaW340 donor strain containing the engineered epilithic plasmid pQM901. This plasmid encodes genes for resistance to mercury and kanamycin and a gene responsible for conversion of catechol by catechol-2,3-dioxygenase to a yellow product. Mating of this strain with epilithic microbial populations resulted in the formation of mercury- and kanamycin-resistant indigenous transconjugants which all contained plasmids with a size similar to that of pQM901. Most transconjugants were found to express the catechol-2,3-dioxygenase gene. Among indigenous transconjugants were *P. putida* and *Alcaligenes xylosoxidans*. In a recirculating stream microcosm filled with River Taff water and natural River Taff stones and inoculated with the donor, epilithic bacteria could be recovered which could grow on minimal agar plates containing mercury and kanamycin (putative transconjugants). However, none of the isolated strains (all pseudomonads including *P. fluorescens*, *P. aureofaciens*, and *P. pickettii* species) were found to convert catechol into a yellow product. As similar levels of mercury- and kanamycin resistant prototrophs could be recovered from the microcosm before seeding with the donor strain, the isolated strains may be naturally resistant indigenous bacteria [Cardiff].

d. Natural gene mobilizing potential in selected environmental hot spots

A triparental mating method was used to isolate and to quantify conjugative plasmids in environmental samples. For this purpose an *E. coli* strain containing an IncQ plasmid carrying the *czc*CBAD genes, a plasmid-free *A. eutrophus* strain, and a microbial extract of an environmental sample are used as donor, recipient and helper, respectively. A control experiment showed a linear relationship between

the number of helper cells with IncP plasmids in the mating mixture and the number of transconjugants which eventually emerged [Gent]. Hence, triparental exogenous isolation may be used to quantify the number of IncQ-mobilizing functions in environmental samples. In addition, this method can be used to physically isolate mobilizing, broad-host range plasmids. Microbial extracts of soil and activated sludge samples were used as helper in the triparental exogenous isolation system. Transconjugants were isolated that contained IncQ-mobilizable functions (as shown by further mating experiments) and plasmids differing in size from the IncQ plasmid (as shown by gel electrophoresis). Of three soil and seven activated sludge plasmids, none hybridized with IncP, IncW and IncN probes [Gent].

e. Monitoring interaction between introduced GEMs and the natural microbial community

Experiments were performed to analyse whether introduction of GEMs into environmental hot spots could influence the composition and activity of the indigenous microbial flora. Inoculation of biphenyl-polluted soils with an *E. coli* strain that contains a RP4::Tn*bph* plasmid results in transfer of the biphenyl degradation genes into the indigenous microflora (see above). Upon prolonged incubation of these soils, oxygen consumption was found to be higher than in non-seeded soils. Similar experiments were performed with 3-chlorobenzoate-, naphthalene- and toluene-contaminated soils seeded with *P. putida* strains equipped with expressed degradation genes for 3-chlorobenzoate, naphtalene and toluene, respectively [Gent].

It has been suggested (Garland and Mills, Appl. Environm. Microbiol. 57: 2351, 1991) that the BIOLOG system, a bacterial identification system based on differential utilisation of carbon sources, can be used to classify heterotrophic microbial communities. Different dilutions of epilithic suspensions from Taff river stones were tested in the BIOLOG system. Optimal incubation times (24-48 h) and dilutions (1/100) were determined. Comparison of the populations of bacteria on the test stones before and after introduction of *P. putida* PaW340 containing the engineered epilithic plasmid pQM901 showed no differences in the output of the test that were greater than differences between duplicate stones [Cardiff].

f. Development of a soil DNA detection procedure

The soil DNA extraction procedure described in the first report was further improved (better removal of brownish compounds, inhibition of DNase activity, increased speed) and tested for PCR analysis of soil samples. The method allows simultaneous handling of large numbers of samples in a short time and without sample cross-contamination problems. Up to 30 mg of soil can be analysed in a single PCR reaction without inhibition by soil contaminants. Consequently, a theoretical detection limit of 33 DNA copies/g soil is obtained [Mol].

g. Development of biological containment systems

Earlier, we reported the isolation of an *E. coli* mutant strain that was less effective to act as a recipient for IncP plasmids compared to Wild Type *E. coli* strains. It was now found that this recipient-negative mutant strain contains (parts of) an IncP plasmid in its chromosome making the strain unsuitable for biological containment purposes [Gent, Mol]. No further efforts were performed to obtain IncP recipient-negative strains as recent data (e.g. see this report) indicate that IncP plasmids make up but a minor part of conjugative plasmids in natural environments.

HIGHLIGHTS/MILESTONES

- The introduction of a cassette of genes conferring resistance to the heavy metals cobalt, zinc and cadmium into a wide range of plasmids (mobilizable, non-mobilizable) and into the chromosome of *P. putida* and *A. eutrophus*.
- Observation that genes coding for degradation of xenobiotic organic molecules can be transferred from introduced GEMs to indigenous soil and epilithon bacteria.
- The exogenous isolation of broad-host range conjugative plasmids from soils that do not hybridize to IncP, IncN and IncW probes.
- Develoment of a soil DNA purification procedure suitable for routine analysis by PCR.

WIDER CONSIDERATIONS

Evidence is accumulating that conjugative gene transfer is a natural process that frequently occurs in the environment, in particular in nutrient-enriched environmental spots. We have shown that a transfer process occurs efficiently in certain types of soil [Gent] but not in others [Oeiras]. This indicates that frequencies of transfer of genes need to be established on a case-by-case basis at present, at least until we can determine the causal differences in the soils.

From a risk assessment point of view, the ubiquity of gene transfer between bacteria in nature may be interpreted as implying that man-made transfer of genes between unrelated bacteria does not pose high levels of hazard. However, it may also be inferred as indicating that introduction of a gene in one bacterium may eventually lead to its dissemination throughout the entire bacterial kingdom.

COOPERATIVE ACTIVITIES

Semestrial meetings in Oeiras (Portugal, 22.10.1992) and Cardiff (United Kingdom, 23.03.1993). Sectorial BRIDGE Biosafety meeting in Wageningen (The Netherlands, 06-09.12.1992). Exchange of strains, soil samples, methods and protocols. Occasional contacts (exchange of strains, methods and protocols) with partners of other BRIDGE projects, including BIOT CT-910293, BIOT CT-910288 and BIOT CT-910301.

EUROPEAN DIMENSION

The European collaborative work enriches a whole range of research activities in the partners' laboratories, especially as a result of exchange of different approaches. In particular it was felt that maximal benefits appeared after long-term collaborations (»3 years).

PUBLICATIONS

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Mergeay M., de Rore H., Top E., Springael D., Höfte M., van der Lelie D., Dijkmans R., and Verstraete W, Plasmid biology and risk assessment: Elements for a modelling approach of plasmid-mediated gene release in soil environments. Proceedings of the 1991 OECD workshop 'Mathematic modelling of gene exchange in soil' at King's College London, in press.

The effects of selection on gene stability and transfer in populations of bacteria in soil (BIOT CT-910285)

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OBJECTIVES

The primary aim for the third six monthly period was to study the effects of selection on introduced antibiotic resistance genes in soil populations of streptomycete inoculants by the addition of antibiotics to soil. Two antibiotics, neomycin, a water soluble antibiotic, and thiostrepton, a water insoluble antibiotic, were chosen for the study. The methods for antibiotic extraction and detection were further developed with the aim of achieving extremely sensitive assays to enable detection of production *in situ* and monitoring the rate of decay or immobilization of antibiotics added to soil. Evidence for antibiotic production in soil was also sought using streptomycetes producing neomycin and thiostrepton. The second objective was to continue to employ soil microcosms which allowed continuous rounds of germination and sporulation. The final objective of this period was to develop and test methods for direct monitoring of bacterial growth and differentiation in soil by differential DNA extraction and amplification of low levels of DNA by the polymerase chain reaction (PCR). This required techniques for quantitative PCR analysis of soil DNA.

Division of labour: Microcosms were developed by each participant but continuous enrichment systems were produced and used only by Athens. Extraction and detection of thiostrepton was developed by Warwick and methods for neomycin by Wageningen. Selection and gene transfer were investigated for plasmid-borne genes at Wageningen and Athens and for chromosomal genes at Warwick. Marked inoculants were used throughout and indigenous marked strains used in conjunction with type strains for experiments at Athens. Three soil types were identified as representative of the agricultural soil within the vicinity of each participating laboratory, the soils differed markedly in terms of clay and organic matter content.

MAJOR PROBLEMS ENCOUNTERED

Extraction and detection of low levels of neomycin in soil has proved difficult. After the addition of neomycin to soil a range of buffers were used to recover the antibiotic. Levels as high as 1000 $\mu g\ g^{-1}$ soil were not detected in the clay loam soil of Athens, while in the sandy loam soil of Wageningen detection of levels below 500 $\mu g\ g^{-1}$ were not possible. Since neomycin is a water-soluble antibiotic, this effect could be due to irreversible adsorption of the antibiotic to clay particles. To overcome this problem current work has focused on the use of supersensitive bioassay strains coupled with ion exchange resins, which, when added to soil, may exchange cations and reduce binding of the aminoglycoside.

RESULTS

1) ***Antibiotic selection on Streptomyces species:*** The effects of addition of thiostrepton and neomycin to soil were studied for a range of *Streptomyces* species.

a) The effects of thiostrepton: Various levels of thiostrepton were added to sterile batch microcosms (clay loam Warwick soil) containing the thiostrepton-sensitive *S. lividans* TK23. The antibiotic caused limited inhibition but appeared to enhance sporulation. This suggested that a response to premature death of mycelia produced from germ tubes, in this case, was increased spore formation. TK64(KT), a strain of *S. lividans* which contains a chromosomal amplification containing both thiostrepton, *tsr* and neomycin, *aph*II, resistance genes, was unaffected by the addition of thiostrepton. After soil turnover the population declined steadily at a rate of approximately 2 logs over 14 days. The reason for this decline is presently under study but may be due to the auxotrophic nature of the strain. The selective effect of thiostrepton on a mixed population of TK23 and TK64(KT) was investigated in sterile fed-batch soil microcosms (Fig. 1). TK23 was unaffected by the presence of TK64(KT), while the latter declined at a rate of 4 logs per 14 days showing that it was out competed by the prototrophic TK23 either in the presence or absence of thiostrepton. The initial decline in spore counts for TK23 was due to germination and evidence for killing was found at this time but the surviving mycelia appeared to sporulate more profusely and thereby aided the survival of TK23. Thiostrepton was extracted from soil and detected throughout the experiment using thiostrepton-specific ultra-sensitive bioassays and further confirmed by HPLC, levels declined from 50 $\mu g\ g^{-1}$ soil to 20 $\mu g\ g^{-1}$ and remained at this level.

The survival of thiostrepton-resistant TK24, lysogenized by KC301 containing *tsr*, was not enhanced by the addition of thiostrepton and this inoculant was out competed by TK23 in soil both with and without antibiotic additions. The results indicated that lysogens grew and/or sporulated less effectively and that although some lethal effects were monitored in the presence of thiostrepton, enhanced and more rapid sporulation in the sensitive, non-lysogenized strain counteracted this effect.

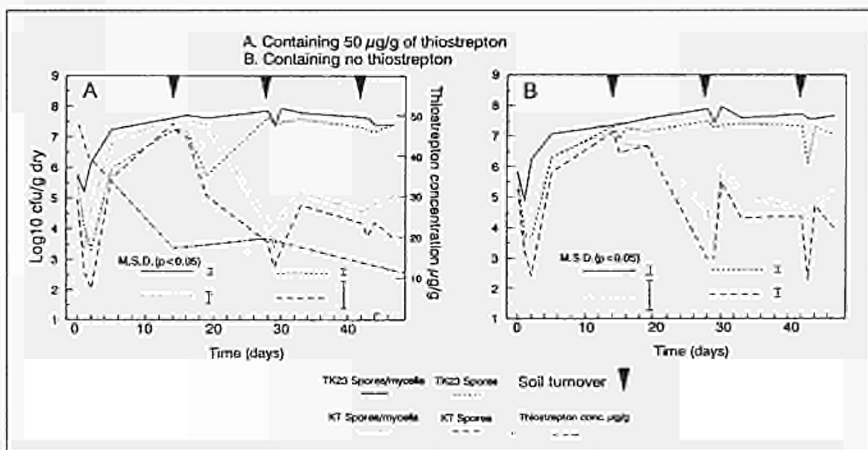

Fig. 1. *Effects of thiostrepton addition on S. lividans growing in clay loam soil.* **A.** *TK23: sensitive prototroph;* **B.** *TK64(KT): resistant auxotroph.*

Detection and in situ production of thiostrepton by *S. azureus* in soil: Initially the fate of thiostrepton (50 μg g^{-1}) in soil was investigated to determine detection limits for extraction and assay techniques. Thiostrepton was added to non-sterile soil and could be detected over a 70 day period however the level declined rapidly over the first 10-14 days, followed by a more gradual decline. The initial loss may be due to degradation or it may represent adsorbed antibiotic which could not then be extracted, the progressive loss over two weeks implicated biodegradation.

The growth of *S. azureus* in sterile amended (1% chitin) Warwick soil was assessed together with *in situ* production of thiostrepton. The antibiotic was detected at day 3 which coincided with a rise in spore numbers indicative of the first round of sporulation. Following 6 days incubation thiostrepton was detected at over 50 ng g^{-1} soil. This level was achieved with a viable count of approximately 10^8. Further studies are aimed at monitoring production in non-sterile soil and how this affects survival of sensitive and resistant strains of *S. lividans* following co-inoculation with wild type or thiostrepton-blocked mutants of *S. azureus*.

b) The effects of neomycin: The effects of neomycin were studied in fed-batch microcosms (clay loam soil) inoculated with *S. griseus* CAG17 (neomycin sensitive strain) and TK24(pIJ673) using Athens soil. The microcosms in this case were stirred each day to allow continuous growth of mycelia, and not as previous methods where sporulation was allowed to occur before amendment. Under these conditions the killing effect of neomycin should be maximised as fewer resistant spores were formed. However, no evidence was obtained for lethal effects of neomycin on *S. griseus* and this strain survived in higher numbers than *S. lividans* both with and without neomycin additions (0-1000 μg g^{-1}). No transconjugants were detected. The level of neomycin in the soil could not be monitored as the antibiotic was not extracted, even at levels of 1 mg g^{-1}. Irreversible adsorption was possible as previous studies have found this occurred with several aminoglycosides. Illite can bind over 40 g of neomycin per kg and this clay is present in the soil used. In addition stability will be poor for this water soluble antibiotic.

Results obtained with Wageningen soil were quite different to those reported above for the clay-loam soil. Various levels of neomycin, 0 to 1000μg g^{-1}, were added to sterile fed-batch microcosms (sandy loam soil) containing strains TK23 and TK24 (pIJ680, *aphV*) (Fig. 2). In the presence of neomycin the sensitive strain TK23 fell below detection limits ($<10^2$ c.f.u. g^{-1}) after only 30 days of incubation, while in neomycin-free soil the strain reached levels of up to 10^6 c.f.u. g^{-1}. This showed that neomycin was having a detrimental effect on the survival of TK23. TK24 (pIJ680) grew in the presence of neomycin even at 1000 μg g^{-1}. Growth of the resistant strain was greatest at the highest level of neomycin added, and this was confirmed by direct analysis of extracted soil DNA when probed for plasmid DNA. Exposure to high levels of neomycin may have affected copy number of the plasmid thus dramatically improving survival compared to the performance of the strain at lower levels of neomycin addition (Fig. 2, see next page), this phenomenon is still under investigation.

In contrast to the data obtained in both Warwick and Athens, antibiotic present in Wageningen soil appeared to exert a killing effect on a sensitive strain.

Thiostrepton additions to the Wageningen soil also resulted in killing of the sensitive strain *S. lividans* TK23. The resistant *S. lividans* TK24pIJ673 survived 500 μg g^{-1} thiostrepton. The inhibition of TK23 was most marked during the first week of incubation. When the two strains were co-inoculated transconjugants were

recovered although transfer frequencies in the presence of thiostrepton were reduced by a log to 10^4 due to killing of the sensitive recipient.

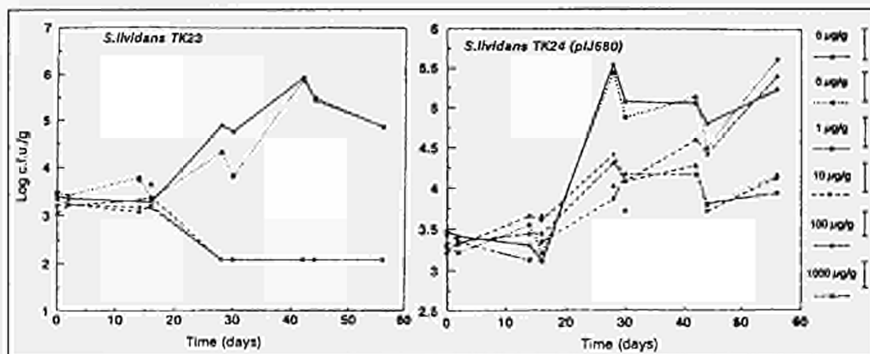

Fig. 2 *Selection for plasmid-borne neomycin resistance in sandy loam soil (neomycin levels are indicated TK23: sensitive pIJ680: resistance plasmid.*

2) ***Soil microcosms:*** Soil microcosms continued to be assessed and improved. Fed-batch microcosms currently used were of two types i) 10-20% of the soil was removed and replaced with fresh soil every 10-14 days, allowing re-germination of spores. ii) The soil was stirred daily in an attempt to obtain a continually growing mycelial population, although at present viable counts can not give an accurate estimate of the mycelial content so quantification by analysis of soil DNA is underway. Perfusion columns are being assessed to provide a system allowing continuous antibiotic enrichment of soil.

3) ***Monitoring DNA in soil:*** Collaborative work between Athens and Warwick using DNA extraction from soil has allowed comparisons to be made between streptomycete population levels in soils of different water contents. In addition to this, the use of differential DNA extraction has shown that a small increase in mycelial content of the soil can result in a significant difference in the presence of plasmid DNA. The extent of mycelium in soil is difficult to determine and large numbers of spores are often produced which can give a misleading impression of the population size in terms of biomass.

PCR primers have been designed for the *tsr*, *aphII* and *galK* genes and conditions for amplification optimised in each case. PCR is being used firstly to detect bacterial DNA in microcosms where numbers of bacteria are too low for detection by conventional plate counts, and secondly to assess the presence of both *tsr* and *aphII* genes in field samples. In the latter case field samples have been obtained and the DNA extracted.

4) ***Activity measurements:*** In addition to plate counts and DNA monitoring, methods of monitoring activity have also been developed. In Warwick the production of two enzymes, urease and chitinase, are being assessed. These enzymes are produced during mycelial growth and therefore give an indication as to the state of the streptomycete population. Another two enzymes, agarase and amylase have also been assessed for their usefulness in monitoring populations. In Athens the respiration rate of streptomycetes in soil, measured by monitoring carbon dioxide evolution, was useful for detecting differences in activity between amended and

unamended soil although the method was not sufficiently sensitive to detect the effects of stirring the soil during soil turnover.

HIGHLIGHTS/MILESTONES

Marked differences were detected between varying soil types concerning the effects of added antibiotics neomycin and thiostrepton on sensitive streptomycetes growing in soil, the presence of certain types of clay and the clay content was most important in determining the inhibitory effects of antibiotic additions to soil. In sandy loam selection for resistant strains was observed.

WIDER CONSIDERATIONS

The environmental implications of using antibiotic resistance genes for marking introduced organisms: the selective advantage of resistance genes in bacteria may be seen when high levels of antibiotic are present such as the levels of kanamycin in animal manure and slurries. The selective effects of such antibiotics will depend on the soil type as clay appeared to adsorb and inactivate the antibiotic neomycin.

COOPERATIVE ACTIVITIES

Over the last year there has been two plenary meetings, the first at Athens 23-25th May 1992, all participants were involved and results discussed. A further meeting was held at Wageningen 11th December 1992 involving all participants. A laboratory exchange was conducted in June/July 1992 between Athens and Warwick.

EUROPEAN DIMENSION

Exchange of skills and techniques, particularly the training of personnel in different laboratories. Opportunity to sample a wide range of soil types from differing geographical and climatic areas and use the soil in microcosm studies. Broadening our understanding of scientific approach and how this varies within Europe.

LIST OF PUBLICATIONS/PATENTS WITH TRANS-NATIONAL AUTHORSHIP

Karagouni A. D., A. P . Vionis, P. W. Baker and E. M. H. Wellington. The effect of soil moisture content on spore germination, mycelium development and survival of a seeded streptomycete in soil. Microbial Releases, **2**, 47-51.

OTHER PUBLICATIONS/PATENTS

Marsh P., I. K. Toth, M. Meijer, M. B. Schilhabel and E. M. H. Wellington. Survival of the temperate actinophage øC31 and *Streptomyces lividans* in soil and the effects of competition and selection on spread of lysogens. FEMS Microbiology Ecology, in press.

E.M.H., P. Marsh and I.K Toth. Molecular Ecology of Filamentous Actinomycetes in soil. Wellington, In: *Molecular Ecology of Rhizosphere Microrganisms*, ed. F. O'Gara, D.Dowling and B.Boesten. VCH, Weinheim.

Safety assessment of the deliberate release of two model transgenic crop plants, oilseed rape and sugar beet (BIOT CT-910298)

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OBJECTIVES

1. Collection and evaluation of hard data on pollen dispersal (oilseed rape and sugar beet).
2. Assess the feasability of outcrossing and viability of descendance (oilseed rape and sugar beet).
3. Assess the predictability of transferred gene activity (oilseed rape).
4. Assess the establishment of a new trait introduced into a population (oilseed rape and sugar beet).
5. Develop and test a computer model for objective 4.
6. Evaluate possible plant instability as a consequence of transformation (oilseed rape).

RESULTS

1. Introduction

This project finds its origin in BAP-project 371 on the 'Study of gene dispersal from plants produced by recombinant DNA'. Whereas in the BAP-project only small scale field trials were carried out, emphasis lies on large scale field trials in the present project. The two model crops are oilseed rape and sugar beet and will be discussed separately in this first progress report. It is the intention to compare the data obtained in both crops by the end of the project.

2. Oilseed Rape (OSR)

2.1. Distribution of introduced genes via pollen and seed

Field trials have been set up in Belgium and France with experimental surfaces exceeding 1 ha. Seeds harvested at various distances of a transgenic Basta resistant OSR plot (1990 trial) were sown and sprayed with Basta, several weeks after emergence. The results allow to estimate the dispersal of pollen as a function of distance to the pollen source.
Review of the results, taking into account additional parameters as reveiled by the computer modelling work, has led to a new design which will be applied in replicate field trials in France and Belgium.

2.2. *Effects of outcrossing to relatives*

2.2.1. *Performance of plants and populations*

Interspecific crosses after manual pollination and embryo rescue between OSR and *B. oleracea, B. nigra, B. adpressa, Sinapsis arvensis* and *Raphanus raphanistrum* show varying degrees of hybrid production depending on the species involved, on the sex of the parents and on the time of flowering.

Field experiments to measure the ability of spontaneous hybridization between OSR and *B. adpressa* or *S. arvensis* or *R. raphanistrum* is in progress. Preliminary results suggest that the genetic background of OSR may have a large effect on the success rate of hybridization. In insect free cages, transgenic OSR grown in a mixture with *S. arvensis, R. raphanistrum* yields no hybrids.

BC_1 progeny (after manual pollination and embryo rescue) after backcrossing with the diploid wild species yields very low numbers of plants. Similar experiments under field conditions are in progress as well as production of BC_2.

2.2.2. *Performance of the introduced gene*

Purpose of the work is to determine the influence of genetic background on gene expression. Most of the effort so far has been in creating transgenic OSR containing maize transposable elements in following combinations: Ac-streptomycin resistance excission marker; Ds-basta resistance. The tolerance to streptomycin of seedlings and cultured tissues has been determined using wild type plant genotypes.

2.3. *Stability of a GM plant*

The main objective is to accurately predict the level of transgene expression. SAR elements are hypothesized to decrease variability of gene expression. Chimeric gene constructs containing a SAR-element (A;TBS), GUS, the lhca*3 promoter and NPTII have been introduced in tobacco. The chicken SAR element (A) induces a 2-3 fold reduction in the variability of transgene expression.

2.4. *Computer modelling*

The results provide information on the mechanism and parameters that influence gene dispersal. Due to complex interactions that may exist, predictions will mostly be limited to those crops whose parameters of interest have been precisely evaluated. The experimental data from the field experiments are being included in the new models under development.

3. Sugar beet

3.1. *Dispersal of introduced genes via pollen*

80 Plants, transgenic for Roundup tolerance and GUS, were maintained in the field in an area of about 10 m^2. Pollen movement was monitored by the use of single row plots of male sterile *Beta vulgaris* at distances of 25.50 and 75 m. Mixtures of *B. maritima* genotypes were planted in 5 directions at the same distance as the male sterile *B. vulgaris*.

The analyses indicated that expression of GUS in the progeny of the segregating pollen donor was 57%. On CMS plants at distance zero, 48% of the germinating seeds were GUS positive. The comparable frequency of outcrossing to the CMS plants decreased with distance from the pollen source. The level of germination of

seeds harvested from *Beta maritima* was extremely low with 2 GUS positive on 15 000 seeds harvested.

3.2. Performance of plants and populations

A competition model to characterize the yield potential and plant weight at low densities, as well as a relative measure of competitiveness, were used in an experiment involving three Beta species: hybrids between transgenic ssp. vulgaris and 1) non-transformed ssp *maritima*, 2) non-transformed ssp. *vulgaris*, 3) non-transformed ssp *maritima.*

An assessment of stress tolerance towards limiting growth factors was carried out in the greenhouse for 1) transgenic *Beta vulgaris*, 2) non-transformed *B. vulgaris*, 3) non-transformed *B. maritima*, 5) *Hordeum vulgare* and 6) *Brassica napus*

WIDER CONSIDERATIONS

The range and scope of activities associated with the concept of 'plant breeding' have increased with time and with technological advances.
Initially, plant breeding involved only the selection of 'superior' plants and lines from available populations in cultivation, perhaps in land-race varieties, or in nature. Such selection would nowadays not usually be regarded as plant breeding. Conventional plant breeding which involves the cross-fertilization of selected parents, followed by the systematic evaluation of the resultant large and varied plant population, is an increasingly scientific process. In the past two decades this process has been improved by biotechnological techniques. Some of these may enhance and accelerate selection techniques, thereby shortening timescales in plant breeding programs (e.g. cell and tissue culture and RFLP probes). Others may promote the health status and rates of multiplication of improved varieties (e.g. micro-propagation). Finally, the biotechnological techniques may broaden the range of germplasm available to plant breeders by making accessible, or producing, new gene combinations containing desirable DNA sequences not available from other sources.

In understanding the impact of biotechnology on plant breeding it is essential to distinguish between analytical techniques, such as RFLP probes, which improve the efficiency of selection in breeding programmes, and 'engineering techniques' which exploit 'genetic modification' to produce transgenic plants.

Theoretically, it should be possible to incorporate any gene from any organism into any host plant. However, before recombinant DNA technology can become a generally useful technique for plant breeders, a number of steps have to be achieved. These include:

(i) gene identification
(ii) isolation of specific DNA sequences
(iii) DNA cloning
(iv) transfer of DNA sequences
(v) plant regeneration
(vi) appropriate gene expression in transformed plants
(vii) sexual transmission of the trait.

A number of genes controlling protein quality have been identified and isolated, and the genetic modification of seed quality genes is now becoming a reality.

The incorporation of resistance to pests, diseases and herbicides is also showing promise. However, it is quite clear that genetic modification technology is not a

substitute for conventional breeding but complementary to it. Once new genes have been introduced into a species, the plant breeder still has to carry out the painstaking and time consuming process of selection and multiplication.

In contrast to most new technologies, guidelines, regulations and in some cases legislation have been set up (e.g. by the EC, USA, Japan) to control the scientific and commercial development of biotechnological products. There exists public concern, and corresponding demands for biosafety assessments. In the EC, Council Directive 90/220/EEC on the deliberate release of genetically modified organisms describes the regulatory framework within which such releases can be authorized. The potential and limitations of the new technologies are as yet not fully appreciated and is therefore a demand for the precautionary approach reflected in such regulation.

The organization for Economic Cooperation and Development (OECD) has also provided an international forum for its 24 members (all the major developed countries) to discuss and formulate common approaches and principles.

In particular little is known about the relative performance of modified and non-modified plants in the field. Therefore, under the BAP and BRIDGE initiative, the Commission of the European Communities decided to support a number of independent projects for the development of appropriate monitoring methods which may generally be applicable to genetically modified plants. A range of relevant experimental data are being gathered. At all stages there is close consultation between the projects and national competent authorities.

Such an accumulation of data and information should enable future regulatory decisions on field work with genetically modified plants to be based on a detailed understanding of the parameters which are thought to have an effect on the survival and on the physical and genetic dispersal of plants and/or introduced genes in the environment.

COOPERATIVE ACTIVITIES

Activity	*Date*	*Place*	*Participants*
General project coordination meeting	March '92	PGS	All
Sugarbeet focus group meeting	June '92	Monsanto	Monsanto, Maribo, PGS
General project coordination meeting:	October '92	Paris Sud	All special topic: modelling
Oilseed rape focus group meeting:	December '92	PGS	Paris S, INRA, PGS special topic: dispersal studies
General project coordination meeting:	December '92	Wageningen	All BRIDGE sectorial meeting

EUROPEAN DIMENSION

Neither the risks, neither the necessary precautions associated with the deliberate release of genetically modified plants could be adressed properly on a local or member-state basis. This is clearly illustrated by the EEC directives on the deliberate release of genetically modified organisms, that give a blue print of a procedure and prerequisites to be implemented by each member state. As a consequence it is crucial to backup the implementation with safety assessment data at the same Community level. Especially since the kind of approach of the project involves implicit multisite evaluations and multinational interactions of competences.

In addition, since all field trials are subject of the local authorization procedures, this Community approach stimulates the interaction between the different regulatory committees and guarantees their involvement in the practical follow-up and conclusions.

Therefore both the Community aspect of the funding and the scientific/ regulatory involvement at national and Community level, certify an objective, unbiased evaluation of the data gathered by this consortium of specialist public and private organizations.

LIST OF JOINT PUBLICATIONS/PATENTS WITH TRANS-NATIONAL AUTHORSHIP

M.C. Kerlan, A.M. Chêvre, F. Eber, A. Baranger and M. Renard (1992) Risk assessment of outcrossing of transgenic rapeseed to related species: I. Interspecific hybrid production under optimal conditions with emphasis on pollination and fertilization. Euphytica 62, 145 - 153

Stability, genetic transfer and ecology of fungi used as biocontrol agents (BIOT CT-910290)

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OBJECTIVES

The aims of our original workplan have been got on time. The biological risks of the voluntary release of filamentous fungal strains in the environment have been estimated on:

- genetical point of view, studying genetic plasticity of fungal strains and genetic structure of fungal populations
- ecological point of view, by evaluating persistence and colonisation processes within variable environments.

RESULTS

Teams of the project have now an excellent methodological knowledge to study diverse processes of genetic plasticity of Fusarium and Beauveria under different environmental conditions. The project allows us to find new data which contribute to a better knowledge of fungal genetic plasticity mechanisms and risk assessment.

1. Typing

- Molecular phylogeny of Fusarium sp. have been established comparing sequences of the intron within nitrate reductase gene, sequences of the ITS, RFLP of the amplified IGS; RFLP using a dispersed repeated sequence. Applied to a population of non-pathogenic strains isolated from soil, those data revealed a large genetic diversity (team 2).
- In spite of its much more restrictive host spectrum, Beauveria brongniartii would be genetically more heterogenous than B. bassiana. For instance, ITS sequences revealed that strains of B. brongniartii isolated from a sugar cane pest (Hoplochelus marginalis) in Madagascar are belonging to a very original population within that fungal species (team 1).

2. Parasexual cycle

- intraspecific and then interspecific hybridization have been demonstrated in Beauveria by protoplast fusions under laboratory conditions (team 1). Molecular analyses by different probes revealed the aneuploid structure of those product of fusion. Some of them cumulate interesting properties of both parental strains; they are highly hypervirulent.
- Parasexual cycle of Fusarium has been researched under natural soil conditions by team 4. Mutant of antagonistic Fusarium oxysporum, carrying benomyl resistance or colour markers were obtained after exposure to UV light. A benomyl-sensitive antagonistic F. oxysporum was transformed to benomyl

resistance with a plamid carrying a tubuline gene cloned from Aspergillus nidulans. The benomyl-resistant phenotype of transformants was highly stable during in vitro culture in the absence of selection. A benomyl-resistant transformant of antagonisitic F. oxysporum has been co-cultured in vitro with a benomyl-sensitive F. oxysporum marked by orange pigmentation. Two sources of fungal biomass were tested in the experiments: conidial suspensions or mycelium. the developement of colonies on PDA amended or not with 10 g benomyl/ml was recorded after &, 2, 3, 7, 14, 21 and 28 days of liquid culture in the absence of selective pressure. Orange colonies were never observed on benomyl-amended PDA in this experiment, while the acquisition of benomyl-resistance marker by orange strains was previously accomplished after protoplast fusion.

3. Transposable elements

— Characterization of dispersed repetitive sequences led to the identification of a retrotransposon group in Fusarium; but we have no information on the activity of those repeated sequences. So the stable inactive repeated sequences are excellent markers to study the molecular genetic diversity and the genetic structure of fungal populations. Repetitive elements are widely distributed in Fusarium sp. and present in variable copy numbers (team 2).

— A strategy has been conceived to detect active mobile elements considering unstable mutants of a specific gene. So, the first bacterial type transposon has been reported in fungi, studying unstable mutants of the nitrate reductase of Fusarium. That transposon family, Fot, shares significant similarities with TC1. Fot elements are able to create mutation through insertions or imprecise excision (team 2).

— Those efficient strategies for transposons trapping are now utilized on Beauveria (team 1).

4. Transformation

Transformation with a heterologous gene

— A procedure has been conceived to increase rate of transformation in Fusarium (500 transformants/ùg ADN); 10 % of them are 'monocopy'; several others have copies on tandem; at least others have a too much complicated genetic organization. 'Monocopy' are generally more stable than others. With heterologous gene, most of insertions are ectopic while with homologous gene, 50 % of insertions go to the target gene and replace it (team 2).

— Foreign DNA may be inserted into entomopathogenic fungi by non-specific (illegitimate) integrate of plasmid DNA or by homologous recombination. The objective of this study is to determine how the stability of foreign DNA in entomopathogenic fungi is affected by the vector used to introduce it. In addition, at the last contractors' meeting, several of the participants expressed interest in the use of a reporter gene for tracking modified fungal strains in the environment. Team 3 is now attempting to provide this. We are also investigating the possibility that strains of the entomopathogenic fungal genera Beauveria and Metarhizium contain plasmids.

— A nia- mutant of Beauveria bassiana has been transformed with pMJT1, which carries the niaD gene of Aspergillus nidulans (team 3). Although transformation frequency was not as high as has been reported previously, it should be difficult to increase this. In order to examine the stability of the integrated plasmid,

revertants were obtained from the transformants by plating on media containing chlorate. Eleven strains capable of growth on chlorate were obtained from three of the transformants. Of these, one retained the capacity for growth on minimal media. At present these strains are being checked by Southern hybridisation to determine whether the transforming plasmid has been lost from the chromosome. It was observed that both the original strain and its transformants showed sectoring when grown on complete media. Purification of sectors has proved difficult, although cultures taken from within a sector do appear to sector at higher frequency. Sectors do not differ in growth rate, but may exhibit reduced sporulation. Sectors can appear from single spores at a frequency of approximately 0.5 %, but can revert to the original morphology. Sectoring does not appear to be linked to the stability of the transformants.
Transformation and co-transformation systems of the species Beauveria have been performed with Gus A, ben A and nia D (team 1). Stable transformants were obtained; molecular analysis suggested that they are 'multicopy'.

Homologous transformation

In order to develop a homologous transformation system for B. bassiana, Beauveria genes are being sought via heterologous probing. A 2.4 Kb fragment containing the A. nidulans SC gene was used as a probe against various digests of B. bassiana DNA (team 3). Team 1 is cloning nia A gene of Beauveria.

5. Presence of plasmids

Several strains of Beauveria bassiana and Metarhizium anisopliae have been screened for the possible presence of plasmids. Several small bands have been observed in two B. bassiana strains. However, these appear to be ssRNA as they are degraded by RNase. The slow rate of degradation suggests that they may be circular ssRNA.

6. Effects of environmental factors on genetic stability

Methodologies have been conceived to specifically follow the effect of relative humidity, thermic cycle and UV. Those parameters can also be integrated in order to simulate climatic conditions. Such microcosms are used to estimate persistence of fungal propagules as well as their genetic stability (team 1). More, the soil activity of conidia and mycelium can be separated by a specific methodology (team 3). Tracking of modified strains with lux AB from Vibrio harveyi is under process (team 3).

Team 4 did not observed any genetic exchanges between Fusarium strains introduced within soil on mycelial or conidial suspensions.

In steamed or natural soils, survival and development of Beauveria strains or interspecific protoplast fusion products have been evaluated by CFU counting and fluorescence microscopical examination (team 5). In steamed soils fungal propagules density is hundred times higher 10 days after inoculation; however such multiplication does not occur in non sterile soils. More, none differences have been observed between parental and aneuploïd strains.

HIGHLIGHTS / MILESTONES

Genomic plasticity of fungi is very important: retrotransposons, bacterian type transposons and parasexual cycle occured in both species studied. However, these phenomena are not frequent since they have not been got under natural conditions. Such studies are of good help for strain improvement by protoplast fusions.

WIDER CONSIDERATIONS

Since use of fungi will increase in industrial or agronomic applications it should be a priority for EEC to support researches on genomic plasticity in order to:

- evaluate frequences under natural conditions of DNA exchanges between strains
- use genetic exchanges for strain improvement.

COOPERATIVE ACTIVITIES

- The first meeting of the project participants occured on october 92 at La Minière. The second one will be held at Sheffield University; the last one at Turin University.
- Strains have been exchanged between teams 1 and 2; 1 and 3; 2 and 5; 1 and 4. Genes have been exchanged between teams 1 and 1; 2 and 3. Products of fusion have been exchanged between 1 and 4.

EUROPEAN DIMENSION

Such project facilitates drastically exchanges at the European level.

Genetic tools for constructing GEMs with high predictability in performance and behaviour in ecological microcosms, soils, rhizospheres and river sediments (BIOT CT-910293)

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OBJECTIVES

I. Vectors specialized in obtaining controlled gene expression under environmental conditions.
II. Barriers to gene transfer. Retrotransfer of rDNA.
III. Conditional suicide systems. Improvement of TOL regulatory circuits and metabolism of alkylaromatics in soils by contained bacteria.
IV. Construction of PCB-degraders.

RESULTS

To achieve the proposed goals, laboratory work was organized in three teams. The CSIC-CIB group at Madrid and group I at GBF were in charge of objectives I and II. The CSIC-EEZ at Granada and the group at Lyngby were in charge of objective III and participated in some of the experiments under objective II, while the University of Cork group and group II at GBF were in charge of objective IV. The CSIC-CIB group contributes to this objective as well. Microcosm test assays were performed by the CSIC-EEZ, U. of Cork and GBF-II group.

For objective I the following actions have been undertaken:

1.1. Engineering gene expression responsive to environmental signals

To develop new strategies for expression of heterologous genes under the control of environmental signals, a collection of transposon-based vectors to generate conditional phenotypes were constructed. Four recombinant mini-Tn5 transposons bearing outward-facing Pm, Pu or Psal promoters from the catabolic plasmids TOL and NAH7 of *Pseudomonas putida*, along with their cognate wild type or mutant regulatory genes (*xylS*, *xylR*, *nahR*) were constructed. Transcription from such promoters is activated when host bacteria encounters certain aromatic compounds, such as alkyl- and halobenzoates (XylS), alkyl- and halotoluenes (XylR) or salicylates (NahR). These transposons enable the generation of conditional mutations dependent on the presence of specific effectors, as well as the engineering of strains expressing heterologous genes that are regulated by aromatic inducers. The broad host range of the Tn5 transposition system and the stability of the inserted genes due to the loss of the transposase gene during delivery of the mobile element make these transposons particularly suitable for the construction of stable strains exhibiting conditional phenotypes.

1.2. Novel non-antibiotic selection markers

We have continued the characterization of selection markers alternative to resistances to antibiotics.

(a) Resistance to potassium tellurite. This resistance is originated from the activation of a cryptic Tc^R system in RP4 plasmid and can be excised as a 2.5 kb restriction fragment adaptable to our series of plasmid and transposon-vectors.

(b) Resistance to herbicide glyphosate: This is encoded by a modified *aroA* gene from *Salmonella*. Its utility as a selection marker is still unclear due to the very high rate of spontaneous resistance to the chemical. (c) Growth on lactose as the only carbon source. A *xyl*R'-'*lac*ZY cassette which expresses constitutively β-galactosidase and lactose permease activities was made. Strains which carry such genes can grow on lactose which allows their direct selection.

For objective II the following strategy has been used:

2.1. Retrotransference of chromosomal markers mediated by TOL

Bidirectional gene transfer involves the mobilization of a self-transmissible plasmid from a donor bacterium to a recipient one and its return to the donor bringing with it genetic information from the recipient microorganism. Retrotransconjugants are the donor bacteria that have received DNA from the recipient, and are discriminated from donor, recipient and direct transconjugants by appropriate markers. The *Pseudomonas putida* TOL plasmid pWW0 is able to retromobilize a kanamycine resistance marker integrated in the chromosome of other *P. putida* strains. The rate of retrotransfer is influenced by the location of the kanamycine marker on the chromosome and it occurs at a rate of 10^{-5} to below 10^{-9} retrotransconjugants per donor.

2.2. Barriers to gene transfer based on killing/immunity systems

Due to the high potential of lateral DNA transfer between microorganisms, recombinant gene containment circuits are being developed. These are based on two genes, the *col*E3 killer gene, encoding colicin E3 which kills microorganisms by inhibiting protein synthesis, and the immunity gene, *imm*E3 that codes for a repressor of the killing function and that binds to colicin E3 and prevents its action. The kill gene is placed adjacent to the rDNA, whereas the immunity gene is located separately from the rDNA so that co-transfer of both elements is unlikely. We have cloned and expressed separately *col*E3 and *imm*E3 genes. The plasmid pUTKm-*Imm*E3 contains the *imm*E3 gene within a mini-Tn5 and its expression is controlled from the β-lactamase and tac promoters. This construct allowed to integrate the constitutively expressed *imm*E3 gene in *E. coli* and *P.* putida. The *col*E3 gene has been cloned under the control of the Ptac promoter in the shuttle vector pVLT31, a plasmid able to replicate both in *E. coli* and *P. putida*, using *E. coli* CC118*Imm*E3 as a recipient strain. To test the killing system in *E. coli* we have transformed the non colicinogenic strain HB101 with pEDF5 (the plasmid that express *col*E3 under the control of Ptac) and pVLT31. We obtained about 10^6 transformants/µg pVLT31 while we found no transformant with pEDF5. In contrast, using *E. coli* CC118*Imm*E3 as a recipient strain the number of transformants with pEDF5 or pVLT31 was similar. To check if ColE3 functions in *P. putida* matings between E. coli CC118*Imm*E3 bearing pEDF5 and *P. putida* KT2442 or *P. putida* KT2442*Imm*E3 as the recipient were set up. Only when the recipient was *P. putida* KT2442*Imm*E3 was it possible to detect a substantial level of transconjugants.

The experimental work devoted to objective III has been aimed at the improvement of regulatory elements controlling catabolic pathways to be used in conditional killing systems. The model system under study is a modified catabolic pathway for the metabolism of nitrotoluenes by using a GEM containing TOL upper pathway for the metabolism of toluenes.

3.1. Isolation of mutant Pu* promoter with higher rate of transcription than the wild type promoter Pu

The *P. putida* TOL plasmid upper operon exhibits three regions critical for regulated transcription, namely, the -12/-24 site for RNA-polymerase/σ^{54}-binding, the -55/-67 region for IHF protein binding, and the -130(UAS2)/-170(UAS1) region, where two sites for XylR binding are located. The XylR recognition sequence UAS2 showed a perfect inverted repeat (5'-ATTTN$_4$AAAT-3') while UAS1 showed two mismatches (5'-CCTTN$_4$AAAT-3'). The two Cs that break the inverted repeat were changed as follows: C -172→T; C -173→A, CC -172, -173→AT. Transcription activation from mutant promoters was measured as β-galactosidase activity after fusion to *lac*Z; the better the palyndromic sequence at UAS1, the higher the transcription from Pu, with increases in activity of up to 50%.

3.2. Isolation of a mutant *Xyl*R regulator, *Xyl*R7 that recognizes nitroaromatics

The *xyl*R gene cloned in a plasmid was mutagenized *in vivo* with nitrosoguanidine. A XylR mutant able to stimulate transcription from Pu with nitrotoluenes was isolated and called XylR7. The mutant XylR recognizes xylenes but once activated by nitroaromatics retained the wild type capabilities regarding recognition of UASs sequences, and requirement for IHF and σ^{54} in promotion of transcription. Conditional suicide systems based on the use of the following couples, Pu/XylR, Pu/XylR7, Pu*/XylR and Pu*/XylR7 are under development.

3.3. Metabolism of p-methyl-^{14}C-benzoate in soils

We developed a model bacteria based on a modified TOL *meta*-cleavage pathway for metabolism of alkylaromatics. In complex environments, such as soils relatively rich in organic matter, metabolism of p-methyl-^{14}C-benzoate by *P. putida* (pWW0) takes place, which can be monitored as $^{14}CO_2$ evolution from the labelled alkylaromatic. Linear $^{14}CO_2$ evolution in soils takes place for at least a month, although efficient mineralization of the alkylaromatic requires appropriate mass transfer.

The third team of laboratories has directed their efforts to the isolation of new PCB degraders and competition studies between PCB degraders and wild type strains in different settings.

4.1. Isolation of microbes from polluted sites

A key issue in bioremediation is the ability of the microbes to survive and function in the target ecosystem. The working hypothesis was that microorganisms that are isolated from the target ecosystem should be the best candidates for reintroduction, since they have already proven to be well adapted to the system. Sediments at the river Elbe along a gradient of pollution were sampled. From the top layer of intact sediment cores different categories of microorganisms were isolated (Pseudomonads and biphenyl and chlorobenzoate degraders). Isolates were stored at —20°C on glycerol, and for subsequent experiments these frozen cultures, rather

than cultures propagated in the laboratory were used. In the first set of survival experiments, six Rif^r strains of Pseudomonas were chosen. Their survival curves in Spittelwasser sediments, which are heavily contaminated with more than 200 different compounds were compared to those of allochthonous wild type PCB degraders which came out of enrichments from all over the world. As expected, the Spittelwasser isolates survived better than allocthonous isolates.

4.2. Competition experiment in sugar beet rizospheres

Preliminary *in vitro* competition experiments in the presence of succinate and biphenyl (diauxic growth conditions) suggest that the recombinant *Pseudomonas* F113pcb was more competitive than wild type F113. To investigate the competitiveness of the GMO under selective and non-sclcctivc conditions in the rhizosphere sugarbeet seeds were co-innoculated with equal numbers of the F113pcb and F113 strains. Each strain could be isolated from root washings on the basis of different antibiotic resistant markers. Neither strain had a competitive advantage over the other in either amended of unamended soils. The ratio between the GMO and wild type remained the same after 25 days on plants.

The potential for lateral transfer of a chromosomally integrated *bph* locus from rhizosphere pseudomonad F113pcb to other microbes was investigated.

The following mechanisms for lateral transfer were evaluated:
1) Transposition of the element promoted by a trans-acting active transposon elsewhere in the genome.
2) Chromosomal mobilisation of the *bph* locus promoted by a CMA plasmid.
3) Retro-transfer into a plasmid bearing donor. To conclude, there was no evidence for transfer of the *bph* locus into homologous strains at detectable frequencies.

4.3. Non-disruptive monitoring of gene expression

As a model to validate in the field the expression systems developed in our laboratory, a series of approaches to study the expression *in situ* of the *bph*ABCD operon of *Pseudomonas strain* LB400, for degradation of PCBs is under way. Several mobile cassettes engineered within mini-Tn5 transposon vectors, carrying regulatory elements of the *bph*ABCD operon fused to *lac*Z and *lux*CDABE reporter genes were constructed. With the lacZ fusions activity is followed non-disruptively by exposing the cells (both in liquid suspension or associated to a solid support such as sepiolite) to the fluorescent substrate of β-galactosidase MUG (4-methylumbelliferyl β-D-galactopyranoside), whereas with the lux fusion, the reporter system can be directly monitored by emission of light without any external addition.

HIGHLIGHTS / MILESTONES

1. A number of transposon-based expression systems have been designed to obtain an efficient performance of recombinant phenotypes under environmental conditions.
2. ColE3/ImmE3 was shown to function efficiently in recombinant gene containment circuits.
3. The TOL plasmid pWW0 has been shown to be able to retromobilize chromosomal DNA.

4. The TOL upper pathway regulatory elements, the Pu promoter and the *xyl*R regulator, have been modified to increase the rate of transcription and the range of effectors, respectively.
5. Recombinant bacteria mineralize p-methyl-^{14}C-benzoate in complex microcosms.
6. Survival of bacteria isolated from polluted sites was shown to be higher than that of allochthonous wild type microbes when reintroduced in target microcosms.
7. No competitive advantage of the recombinant F113PCB strain bearing the *bph* trait over the wild type strain was found in rhizospheres.
8. No lateral transfer of the *bph* locus integrated in the chromosomes of F113pcb was found.

WIDER CONSIDERATIONS

Novel genetic information was introduced into *Pseudomonas* bacteria through a series of mini-transposons lacking transposases within the inserting element. These bacteria survived well in natural settings, eg, river sediments, soil microcosms and rhizospheres. Genes (including suicide killing functions) were expressed as expected and appeared to be stable. This proves that this strategy is an efficient approach to the engineering of strains for various environmental purposes.

COOPERATIVE ACTIVITIES

A plenary meeting was held in Granada. Lars B. Jensen from the Technical University of Denmark visited J.L. Ramos laboratory for three months to carry out joint work.
Dr. V. de Lorenzo and Dr. J.L. Ramos visited each others laboratory to further define collaborative links.
Dr. V. de Lorenzo visited Dr. S. Molin, Dr. K.N. Timmis and Dr. Dowling to further define collaborative links.
Dr. J.L. Ramos provided Dr. Dowling with suicide genes.
K.N. Timmis and J.L. Ramos exchanged strains.
A plenary meeting will be held in July in Segovia near Madrid.

LIST OF JOINT PUBLICATIONS

Michán, C., Zhou, L., Gallegos, M.T., Timmis, K.N. and Ramos, J.L. 1992. J. Biol. Chem. **267**, 22897-22901.
de Lorenzo, V., Eltis, L., Kessler, B. and Timmis, K.N. 1993. Gene **123**, 17-24.
Molin, S., Boe, L., Jensen, L.B., Kristensen, C.L., Gisskov, M., Ramos, J.L. and Bej, A.K. 1993. Ann. Rev. Microbiol. in press.
de Lorenzo, V. and Timmis, K.N. 1993. Methods in Enzymology. in press.
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OTHER PUBLICATIONS

Fenton, A.M., Stephens, P.M., Crowley, J., O'Callaghan, M. and O'Gara, F. 1992. Appl. Environm. Microbiol. **58**, 3873-3878.
Abril, M.A. and Ramos, J.L. 1993. Mol. Gen. Genet. in press.

Experimental and modelling studies on the fate in soil of introduced biologically contained bacteria (BIOT CT-910288)

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OBJECTIVES

Objectives for the reporting period were along the lines set in the last report to the EC, as well as in the workplan. Due to possibly insufficient functioning of containment genes in the soil pseudomonads to be used as carrier organisms for beneficial genes, alternate strategies of containment were envisaged. Thus, major aims have been to obtain further knowledge about the functioning of suicide principles in soil pseudomonads and the potential to use promoters/genes involved in starvation survival for biological containment. Testing of principles found in soil microcosm systems have been an integral part of the research efforts. Finally, a major aim has been the mathematical development of a model predictive of bacterial survival in soil, which takes localization of cells in the soil matrix into account.

MAJOR PROBLEMS ENCOUNTERED

The poor action of the *Escherichia coli gef* gene on the membrane of the soil pseudomonads used has made application of *gef* for biological containment of these carrier organisms in soil doubtful. An alternative biological control gene, a *S. marcescens* nuclease (*nuc*) gene, was found to function sufficiently under nutrient-rich conditions, however its function under starvation conditions was unclear. Therefore, it was decided to develop an alternate strategy based on passive containment using inactivation of genes of the starvation survival programme of soil pseudomonads. Tinkering with such genes could lead to organisms with adequate survival in the relatively nutrient-rich rhizosphere, whereas in the oligotrophic bulk soil and groundwater survival is impaired. In connection with this, contained organisms developed will be tested primarily in soil microcosms, since time constraints will preclude any field testing in the project period.

RESULTS

The project is composed of 4 areas of expertise, which are largely complementary and integrative. Thus, expertise in soil biotechnology of IB-DLO is combined with that in molecular microbiology of containment of the TUD, that in starvation and stress responses of the UG and that in mathematical modelling of biological systems of IMW-TNO.

The core of the project is formed by the IB-DLO efforts to develop contained bacteria carrying cryIVB genes for release into the rhizosphere of gramineous plants for biocontrol purposes, and to use mathematical modelling to describe bacterial fate in soil. *Pseudomonas* strains, i.e. *P. fluorescens* R2f and *P. cepacia* P2,

previously selected for their capacity to serve as efficient carriers, have been used for expression of *cry*IVB. Since a previous construct with *cry*IVB under the control of the *tac* promoter, C5t, had shown impaired survival in a sandy soil, it was decided to use expression specifically induced by root exudates as an alternate strategy. For containment of the modified organisms, carbon starvation induction was envisaged. Promoters triggered by exudates or by carbon starvation were identified and isolated using a promoter probe system based on a promoterless *lac*Z on a disarmed transposon.

Strategies for biological containment were both passive and active. Passive containment of soil pseudomonads based on impairment of genes essential in the response to carbon starvation survival or stress was feasible, since mutation of a regulatory gene involved in the response to carbon starvation drastically reduced the viability of *Vibrio* upon carbon starvation (UG). Active containment was based on host killing genes provided by TUD, i.e. the *gef* and *nuc*. The latter gene was modified such that the enzyme was expressed intracellulary.

Passive containment

Passively contained strains should show restricted survival under the starvation conditions prevailing at the non-target sites bulk soil or groundwater, via impairment of genes involved in the response to starvation stress. Knowledge on the starvation/stress response was obtained with *Vibrio* by the UG group. Eight starvation-induced genes, i.e. *csp* 5 and 6 encoding periplasmic proteins, 2 exoprotease encoding genes, 1 stress protein encoding gene (*dna*K), 1 responder (*csi*A) induced after the initial phase of starvation, 1 regulatory gene (*csr*I1) and *rel*A which encodes a ppGpp synthetase and regulates the stringent control regulon, have been cloned and partially sequenced. Starvation-specific mRNA has also been isolated to identify late starvation genes. Subtractive hybridization of labelled cDNA from mRNA made during late starvation, with mRNA made during growth could identify specific starvation transcripts.

Genes involved in global control were also looked for. Inactivation or deletion of *dna*K and *kat*F-like genes in *Vibrio* appeared unsuitable to obtain disabled strains, since *Vibrio dnaK*$^-$ mutants grew poorly and *katF* was suggested not to be a general responder to starvation conditions. *Usp*A (universal stress protein A) of *E. coli*, which was shown to be a general responder to growth arrest or perturbations in balanced growth but not to slow growth, constitutes a prime locus for constructing strains with reduced starvation survival. *Usp*A mutants grew at rates similar to the parent, were susceptible to killing and showed altered carbon assimilation and timing of expression of starvation proteins during prolonged carbon starvation. Immunological detection in *Vibrio* and *Pseudomonas* spp. suggested that UspA and its patterns of induction may be conserved among the gamma subgroups of purple bacteria. To analyse *usp*A regulation and obtain regulatory mutants, the *usp*A promoter and 1.5 kb of upstream sequence were transcriptionally fused with *lac*Z, permitting the search for upstream regulatory sequences using deletion and site-directed mutagenesis.

Since primary, single-transposon insertions may show insufficient weakening, such insertion mutants were used to introduce secondary transposons or point mutations to hit regulatory genes. Further, mutants in *rel*A and *csr*I1 showed wildtype behaviour during growth but poor survival and recoverability during starvation. These mutants down-regulated the synthesis of several C-starvation specific proteins. Proteins of the stringent control regulon and the carbon starvation stimulon, were identified allowing introduction of additional containment systems.

Hence, stably conditionally disabled bacteria may be obtained by inactivation/deletion of genes at different levels in the regulatory hierarchy within a stimulon as well as in different starvation/stress networks.

The TUD group has taken *P. putida* 2442 and *P. fluorescens* R2f as target strains to study physiological and molecular events during starvation survival with the aim to use knowledge obtained in the construction of contained organisms. First, both strains survived extremely well under carbon starvation conditions, with no loss of viability after over 30 days. Further, both organisms developed resistance to various stresses (heat, oxidative stress, osmotic stress, ethanol). Resistance developed fastest in *P putida*. Presently, mutants which show reduced survival under stress are being isolated, and several *P. putida* mutants with such phenotypes are available. In parallel, specific mutations were inserted in known regulatory genes (*rpoS, relA*) in the strains in order to test the significance of these for stress survival. Strains with impaired survival will become available for microcosm testing in the next months. A detailed physiological analysis of the stressed pseudomonads, including measurements of cell size, DNA content and ribosome content was undertaken by TUD, which will provide a unique platform for designing optimal strains for short-term competitive survival and long-term elimination in the environment. A characterization of the stress responses of both pseudomonads may be expected in the final report covering survival data, protein synthesis (total and specific as monitored on 2-D gels), cross-protection against other stresses, DNA replication, ribosome concentrations, and effects of various regulatory mutations.

IB-DLO has focused on *in vitro* and soil microcosm testing of available carbon starvation mutants of carriers, strains R2f and P2. The mutants used in these experiments, R2f mutant RA92 and P2 mutant PC3, were inoculated into growth medium or soil with their respective parent strains in a 1:1 ratio. This ratio did not shift upon growth in medium with glucose after more than 30 generations. Mixed inoculation of mutant and parent strains into carbon-free medium resulted in a shift to 1:4 (R2f, 14 days) and 1:50 (P2, 2 months). See Fig 1. Thus, both mutants were specifically impaired in survival in the stationary phase.

Soil/rhizosphere experiments in a model rhizosphere chamber were done with the same experimental set-up. When directly under the influence of plant roots, the RA92/R2f mixture didn't show large shifts in the 1:1 ratio, not even 1 month after introduction. However, the ratio shifted to about 1:10 both in planted soil 2-8 mm from the membrane and in unplanted soil. In contrast, the PC3/P2 mixture didn't show a major shift from the 1:1 ratio in planted or unplanted soil.

The difference in ecological behaviour of both mutants compared to their parent might reflect either a difference in behaviour of the parent strains in soil or a difference in the affected function. Strain P2 may be a better scavenger of scarce nutrients, since it shows optimal growth in relatively nutrient-poor media. R2f might represent a more copiotrophic organism, which shows a decline of viable counts after introduction into bulk soil. The mutant derivatives, RA92 and PC3, reacted differently to stress factors like N-, P-starvation, UV and heat. Overall, RA92 appeared to be a good candidate for a passively contained carrier strain of the *cry*IVB construct, although it is not completely killed shortly after escape from the vicinity of roots.

Active containment

The 2 host killing genes, i.e. the *gef* and *nuc* genes were tested in *P. putida* KT2442 and in *P. fluorescens* R2f. The *gef* gene only showed substantial activity against *P. putida*. The nuclease, however, was active against both strains. As shown by Contreras et al, a system based on the fusion of the Pm promoter from the TOL pathway and the *lac*I gene combined with a *lac*P-*gef* fusion in *P. putida* was shown to survive only in the presence of benzoates. The contained *P. putida* strain disappeared in soil without benzoates. Similar results were obtained with *P. putida* containing *nuc* instead of *gef*. This *nuc* based system is now being transferred to *P. fluorescens* R2f, which expresses the nuclease efficiently, after which it will be tested in soil microcosms at IB-DLO.

Containment via *nuc* controlled by a root exudate-induced promoter may give a clear switch between induced and uninduced conditions in the rhizosphere and bulk soil, respectively. To achieve such differential expression, a negative loop based on *lac*I will be used. The IB-DLO is currently cloning the root exudate controlled promoter identified in the R2f insertion mutant RIWE8. This mutant is specifically induced by proline, a minor component of wheat root exudate.

Mathematical modelling of bacterial fate

The model predictive of survival and spread of introduced bacteria in soil was based on the concept of bacteria living in soil pores of varying size classes. Bacterial mobility between pores could only occur with water flow, which also moved dissolved nutrients. Only one invading bacterial strain could survive per pore, whereas invasion of already occupied pores was abortive. Bacteria in larger pores were subjected to predation. A first version of the model considered survival and predation to depend only on pore size, whereas in a second version the pore water content also determined survival and predation and bacteria could become resistant and non-growing due to starvation. Such cells were assumed to be revivable at high nutrient levels.

Modelling of the dynamics of introduced cells versus a resident organism with similar fitness parameters showed the mere presence of a resident often excluded invading organisms. The first model indicated that favourable environmental conditions, i.e. high nutrient levels or low predation rates, led to less free pore space for the invaders and to lower densities. The second model suggested that the more easily bacteria became resistant, the more difficult it became for an invader to survive in the system.

Invading bacteria have a higher probability to enter larger pores than smaller ones. Further, they are assumed to enter only pores which are not completely water-filled. Raising the mortality rate in these larger pores enhances the probability that the invader becomes extinct, leading to unoccupied pores which are available for intruding bacteria. However, starvation resistance and cell death both lead to a reduction of the number of dividing cells in a pore. Changes in the rate of either process only had a considerable effect when that rate was the highest of the 2. Further, the rate at which the bacteria revived had little effect on bacterial invasibility. Revival of just 1 cell per pore resulted in a bacterial colony in that pore and activation of more cells did not change the ultimate number of cells. Reactivation probability under favourable conditions was only important in pores with a low number of starvation resistent cells. Besides, revival only impacted population dynamics when the number of pores with newly-activated cells was substantial compared to the number of pores already containing active cells.

The effect of the predation rate in wet pores was tempered by the fact that the pores, which have a large enough pore neck for predators to enter, are very often dry.

It appeared that if the number of pores occupied by the invading bacteria dropped too low, the invader population was doomed to go extinct. Depending on parameter values, however, it took a considerable time before extinction was reached.

HIGHLIGHTS/MILESTONES

Principles of use in the construction of passively contained bacteria for release are being unraveled at high speed. Several genes involved in the carbon starvation induced regulon have been singled out for insertional inactivation.

The feasibility of the use of carbon starvation mutants to obtain impaired survival in bulk soil, but not in the rhizosphere was shown, although the efficiency of killing may still be insufficient to use such mutants as carriers in a field experiment.

In addition, environmentally (root exudate) triggered promoters have been identified for the selective triggering of beneficial or containment genes. Using such promoters aspects of differential regulation of heterologous genes in carrier organisms for release can be developed.

WIDER CONSIDERATIONS

The current project develops fundamental knowledge in order to develop inherently safe (biologically contained) biological control agents for release into agricultural soils. Principles underlying environmentally triggered passive and active containment are being unraveled and applied to the biocontrol agents under development. In addition, a mathematical model predictive of field behaviour of released organisms, which takes soil aspects into account, is being developed and will be validated using results obtained in contained soil microcosms.

The project aims for the development and application of contained organisms expressing both their biocontrol and containment genes triggered by environmental signals, in order to satisfy requirements of both enhanced efficacy and reduced risk of spread or persistence.

COOPERATIVE ACTIVITIES

In the past project year, meetings between the different partners in the project have taken place on a regular basis. A group meeting was organized at IB-DLO in march 1992, after which a second meeting took place in september 1992, during the ISME meeting. IB-DLO staff have further travelled to Copenhagen in november 1992 to address specific progress. A last group meeting was then organized during the BRIDGE sectorial meeting in Wageningen in december 1992.

Several more informal meetings and contacts have taken place between different partners. Materials (strains, genes) have further been freely exchanged between various partners.

EUROPEAN DIMENSION

The international cooperation in this project has brought together a group of scientist with complementary expertise, who previously did not have formal cooperative links. Clearly, this has greatly stimulated both the development of

fundamental science in the different laboratories involved, as well as the applied aspects of successfully producing contained biocontrol agents. Due to the short time span of the programme (2 years), major breakthroughs and achievements are only expected in the coming research period.

PUBLICATIONS

Several manuscripts have been submitted by the different groups or are in press. A full account of these will be provided in the final report. No patents have been filed.

An experimental approach to investigate horizontal gene transfer between organisms (BIOT CT-910287)

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OBJECTIVES

The ultimate objective of this research is to measure experimentally the frequency of horizontal gene transfer between eukaryotic organisms. The chosen model involves the plant pathogen *Cladosporium fulvum* and its host species, tomato (*Lycopersicon esculentum*). The elements that are studied are the plant Tnt-1 and the fungal *Cf*T-1 retrotransposons. The objectives set in laboratory 02 (INRA, Versaille, France) for the reporting period were (1) the assessment of the expression of the tobacco Tnt-1 element in the tomato genome, especially in response to fungal factors, and (2) the construction of marked copies of the Tnt-1 element. The objectives set in laboratory 01 (UEA, Norwich, England) for the reporting period were (1) the construction of marked copies of the *Cf*T-1 element (2) Transform *C. fulvum* with these marked copies of these constructs.

RESULTS

Lab 01 Oliver's research group. Marked copies of the retrotransposon *Cf*T-1 have been produced using chimeric marker genes constructed in each of the collaborating laboratories. These marker genes have been inserted into a non-coding region of the retrotransposon. These chimeric genes provide hygromycin resistance under the transcriptional control of either the *gpd*A promoter of *Aspergillus nidulans* or the 35S promoter of CaMV. The former has been shown to be functional in *C. fulvum* (Oliver et al., 1987), while the latter, being, functional in plants, has not been previously used in any fungus. These marked copies of *Cf*T-1 have been sent to laboratory 02 in order to be cloned into *Agrobacterium* Ti vectors and used to transform tomato. These constructs have been used to transform *Cladosporium fulvum* and both constructs confer resistance to Hygromycin B. Preliminary evidence suggests that the 35S promoter of CaMV may be active in *C. fulvum*. If additional experiments confirm this finding, the 35S promoter will be used for the expression of the marker gene within the retrotransposon sequence since this promoter will remain active in both recipient organisms.

We have also been surveying ecologically and taxonomically related fungal species for *Cf*T-1 or related retroviruses. A related retrotransposon, *Cc*T-1, has been identified in *Cladosporium cladosporioides* by Southern hybridisation using a *Cf*T-1 probe. A highly conserved region of the reverse transcriptase gene has been isolated from *Cc*T-1 by PCR, and its sequence compared with that of the reverse transcriptase gene of *Cf*T-1. Phylogenetic analysis of these and other sequences from the same gene in other retrotransposons has revealed a close evolutionary relationship between *Cc*T-1 and *Cf*T-1. In parallel, the rDNA sequences of these and the other fungi used in this study have been compared and preliminary results suggest that *C. fulvum* is in fact a *Cladosporium* as previously reported by Cooke (1883) and not a *Mycovellosiella* as reported by von Arx (1983) and that it need not be placed in its own genus *Fulvia* as reported by Ciferri (1952). The close rela-

tionship between the retroelements identified in both *C. fulvum* and *C cladosporioides* may suggest that the retrotransposon was acquired prior to species divergence. This observation does not, however, rule out the possibility of horizontal gene transfer between the *Cladosporium* or other genus.

Lab 02 Grandbastein's research group. The expression of the Tnt-1 element was studied in tomato, after introduction, via *Agrobacterium* mediated transformation, of an LTR-GUS construct, composed of the Tnt-1 LTR fused to the β-glucoronidase reporter gene. Preliminary results obtained in tobacco plants transformed with the same construct indicated that the Tnt-1 expression in tobacco is not constitutive, and can be specifically activated by extracts from various fungi, or by proteinaceous elicitors from fungal or bacterial origin. Our results show the activity of the LTR-GUS construct is similarly regulated in the tomato genome, and is induced by extracts of the fungus *Trichoderma viridis*, and by crypogein, a proteinaceous elicitor produced by the fungus *Phytophtora cryptogea*. We are now testing *C. fulvum* extracts as well as intracellular fluids from *C. fulvum* infected tomato leaves, sent from laboratory 01. These results will indicate whether modifications to the promoter of Tnt-1 are necessary for the expression of retrotransposon genes in the infected tomato leaves. The expression of these gene will be a prerequisite for the horizontal transfer of this element to *C. fulvum*.

In parallel construction of marked copies of Tnt-1 is well under way. An artificial unique cloning site (*Mlu*I) at the end of the Open Reading Frame has been introduced since such a site was not naturally present in the element. Two constructs with this unique engineered site have been produced either under the transcriptional control of the original LTR, or under the control of a modified LTR which insures constitutive expression of the element in the leaves. Marker genes for antibiotic resistance are presently being introduced to the unique *Mlu*I site of these constructs. Marked copies of these Tnt-1 elements as well as marked copies of the *Cf*T-1 fungal retrotransposon which have already been sent from laboratory 01 will be cloned into Ti vectors of *Agrobacterium* and transfered to the tomato host.

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HIGHLIGHTS / MILESTONES

Our results show that the Tnt-1 tobacco element is expressed in an heterologous species such as tomato, and that the regulation observed in the tobacco genome is also conserved in tomato. This confirms that Tnt-1 is indeed a suitable tool for the project, since its transcription in tomato was required. These results also confirm that Tnt-1 expression is specifically activated by factors of microbial origin and represent thus one of the first direct indications of the response of a retrotransposable element to natural challenges such as plant-pathogen interactions.

WIDER CONSIDERATIONS

Activation of retrotransposable elements has often been correlated to stresses. If a retroelement is regarded purely as a selfish gene then in its bid to survive it may

be activated and, as a consequence, create fortuitous adaptive response in its host genome to environmental change. This work is the first direct report of the effect of a natural environmental change on the activation of a transposable element, and it is important to determine if this activation influences the plant response to the pathogen. Such studies may aid the understanding of the evolutionary role of these ubiquitous mobile sequences. It may be that under these conditions of stress the activated element may transfer horizontally to the invading host pathogen in the ultimate selfish act. An assessment of the risks involved in the release of genetically engineered organisms must take into account the frequency of this horizontal gene transfer. The assessment of the frequency of horizontal gene transfer places an upper limit on the safety of the deliberate release of engineered organisms and will indicate the likelihood of gene-escape to unrelated species in the environment.

COOPERATIVE ACTIVITIES

Chimeric marker genes under the transcriptional control of *gpd*A fungal promoter, or the CaMV 35S promoter have been exchanged together with tomato seeds. Marked copies of the *Cf*T-1 retrotransposon, as well as LTR(*Cf*T-1)-GUS constructs have been sent from Norwich to Versaille where they will be cloned into *Agrobacterium* vectors and transfered to tomato. Freeze dried fungal exudates and intracellular fluids from *C. fulvum* infected tomatoes have been sent from Norwich for testing in Versaille. The participating members met in May 1993 at UEA, Norwich.

PUBLICATIONS

Ponteau, S., Grandbastein, M.A., and Boccara, M. Microbial elicitors of plant defense responses activate transcription of a retrotransposon. Submitted for publication.

Safety of genetically engineered retroviruses used for gene transfer (BIOT CT-910286)

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OBJECTIVES

Task 2 (i), Milestone B: Description of long-term stability of expression of selected model proviruses
Task 2 (ii), Milestone D: Pilot results, frequency of recombination between exogenous and endogenous proviruses
Task 3 (ii), Milestone H: Expression activitiy at the pre-integration sites of selected model proviruses
Task 3 (iii), Milestone J: Identification of tissue-specific regulatory elements
Milestone K: Construction of tissue-specific retroviral vector systems
Task 3 (iv), Milestone M: Construction of locus-targeted vectors
Task 4 (i),(ii), Milestone O: Screening of infected animals for germ-line transmission
Task 5 (ii), Milestone S: Large-scale purification of recombinant virus

RESULTS

UA: Work is progressing with respect to the transfer, expression and stability of murine leukaemia virus derived vectors in cultured murine cells. The stability of expression of 18 proviruses, available as single integrants in cell clones, has been monitored over a five months cultivation period (Milestone D). Although all proviruses are physically stable, major differences in the long-term stability of expression levels are observed. Based upon this result and upon our earlier work we conclude that at least two *in cis* acting mechanisms may contribute to provirus expression. To further study these effects of the integration site we have developed a simple single side polymerase chain reaction method that allows the selective amplification and sequence analysis of the integration site of a vector provirus. Using this new methodology we have determined the integration site sequences for a number of proviruses and have begun an analysis of the transcriptional activity of the pre-integration sites (Milestone H).
To study rare recombination events between exogenous and endogenous proviruses we have developed transfer systems that are impaired in the normal integration machinery. Helper-free packaging cell lines yielding virus particles defective in the integrase have been produced and found to have the expected characteristics except that they produce only very low levels of virus protein. The available packaging cell lines are therefore not well suited to study rare recombination events (Milestone D) and we have gone one step back to obtain high-producer packaging lines.
A novel biological containment system for retroviral vectors is under development. This system is based upon the use of vectors requiring an artificial primer for reverse transcriptase. Only vectors produced in specifically engineered cells carry-

ing a non-natural tRNA-like RNA that matches the altered primer binding site on the vectors will be able to complete a replication cycle. We have demonstrated that this strategy works in principle and are currently refining the tools for this transfer system.

GSF: The Munich group has been investigating the promoter regions of genes that are expressed specifically in the mammary gland (Milestone J). One promoter of interest is that of the whey acidic protein (WAP). Two negative regulatory elements (NRE) have been identified in the WAP promoter. The most distal of these has been extensively defined and the repressor protein that binds to this NRE partially characterized. Since the distal NRE is a candidate determinant of mammary specificity, it is presently being introduced into a murine leukaemia virus (MLV) based retroviral vector (RV) upstream of the viral promoter to try to target virus expression to mammary cells (Milestone K). In a second approach directed towards the construction of tissue specific RVs, we have continued our efforts to produce RVs based upon mouse mammary tumour virus (MMTV). This has involved the characterization of two virally encoded regulatory factors, Naf and Sag, both of which are products of the enigmatic open reading frame located in the MMTV long terminal repeat. The Naf product was identified as a repressor of transcription from MMTV based constructs. In further analyses data have been obtained to suggest that Naf can affect transcription from viral promoters other than MMTV. In contrast, expression of the Sag (superantigen) product results in the deletion of whole classes of T-lymphocytes that are expressing a particular Vß chain as part of the T-cell receptor. Although both Naf and Sag are encoded by the MMTV LTR, we have shown that they are independent products of this region. Further, a novel MMTV promoter has been identified upstream of the previously described promoter that can be shown to generate transcripts encoding Sag. These factors may prove useful as a means to manipulate or target expression of RVs (Milestones J and K).

A number of RVs designed to show locus targeting (Milestone M) have been constructed by the Munich group. All of these vectors are based on MLV and carry varying lengths of segments of the herpes simplex virus thymidine kinase (HSV-tk) gene inserted at different locations within the RV genome. The RVs have been packaged and used to infect a cell line carrying a single copy of the HSV-tk gene and cell clones have been isolated after negative selection for the loss of HSV-tk activity. Possibly, this loss has been caused by integration of an RV genome, due to homologous recombination, into the HSV-tk gene. This is currently being analysed by Southern blotting and PCR analysis.

Finally, MLV-based RVs have been introduced into mice to determine the risk of germ line infection (Milestone O). The infected animals have been mated and the offspring analysed for the presence of RV DNA. Preliminary results indicate that some of these progeny mice may have acquired RV sequences, but that they are probably mosaics, since not all cells of the analysed mice carry RV.

IIGB: Work has progressed according to the contract with respect to the tasks concerning targeting and genetic stability of retroviral vectors and risks of germline transmission after somatic infection. The potential of addressing retroviral vector expression by introduction of tissue specific promoters has been studied in detail (Milestones J and K). The regulatory elements were those derived from the *HLA-DQA1* gene, a member of the MHC class II multigene family, whose products are fundamental for antigen presentation and regulation of the immune response. The MHC class II genes are expressed in certain lineages of the lympho-erythropoietic system and thus constitute an ideal tool for targeting expression to different cell

types of the lymphoid and the erythroid cell compartment. This study has included i) the dissection of the elements participating in the control of the *HLA-DQA1* gene in different cell lines and mutants and ii) the introduction of the controlling elements thus identified into retroviral vectors and comparison with the level of expression attained by conventional expression vectors. Reporter genes such as the CAT or *hph* were used for monitoring promoter activity. As previously reported the retroviral sequence context generates interference with the regulated expression of the cloned *HLA-DQA1* promoter in most cell types analyzed. We have developed a mutational analysis to isolate retroviral vectors which would overcome such negative effect and allow re-expression of the *hph* reporter gene fused to the *HLA-DQA1* elements inserted into a retroviral construct. This analysis has indicated i) that the phenotypes of the vectors are stable and apparently independent of the integration sites; ii) that it is possible to isolate by positive selection rare mutants which allow re-expression of the reporter gene and iii) that some of these alterations are inside the retroviral vector genome. We have rescued some of these mutations and we have cloned one of the sequences responsible for the phenotype. A molecular analysis is now in progress to correlate the phenotype to a structural change in any of the viral or cellular functions present in the vector.

In collaboration with the group at GSF we have developed a system to test the risk of infection by retroviral particles of spermatogonia in mice; this methodology involves transplantation of retrovirus producing cells into the testis of the animals (Milestone O). Analysis of the progeny of treated males mated to uninfected females has shown that the tracer retroviral vector genome can be detected in the offspring. Further analysis is required to assess the mode of transmission.

UCB: The UCB participant has mainly been concerned with avian leukosis based vector systems and safe packaging cell lines that produce high titer helper-free preparations of these vectors (Task 1). The safest packaging cell line has been constructed by separating the *gag-pol* and *env* genes on two transcriptional units, deleting the packaging sequences and the 3' non-coding regions including the 3'LTR. Moreover, genes for selectable markers have been inserted into the transcriptional units of viral genes allowing us to produce the packaging functions under selective pressure. This strategy of packaging cell line construction involving two separate plasmids has allowed the generation of packaging cells with subgroups A, B, C and E envelope specificities by using a semi-packaging cell line producing avian leukosis virus Gag and Pol proteins in which *env* genes from different origins were introduced. Superinfections of packaging cells by a *lac*Z retroviral vector were performed by co-cultivating packaging cells of two subgroup specificities (A and E). This 'ping-pong' process already described for production of mammalian viral vectors, did not result in increased retroviral vector titers but caused the emergence of replication-competent viruses which result from recombination between the viral sequences of the packaging cell lines and the cis-acting sequences of the *lac*Z retroviral vector. Replication competent viruses were shown to arise only in co-cultures of vector-producing packaging cells with two host ranges where multiple replication cycles are possible. However, recombinant viruses not competent for replication have been detected and characterized in vector-producing packaging cells cultivated alone. Similarly, some avian leukosis virus related sequences, present in the genome of a chicken cell line tested as a helper, were found to be packaged into virus particles. All these data allow us to reconsider the term of 'helper-free viruses' and lead to the choice of appropriate protocols in order to minimize the risks of emergence of unexpectable recombinant viral forms.

Experiments directed towards the analysis of long-term stability of expression of integrated proviruses (Milestone B) have been initiated by using both avian cells

from different origins and retroviral vectors carrying the *lac*Z gene and the selectable *neo* gene placed under *cis*-acting regulatory sequences from different origins. Experiments have been carried out either in the presence or absence of selective pressure and differences related to cell origins have mainly been observed.

Approaches of targeting vector expression to specific tissues have been continued by expression of double expression vectors that we have constructed. These vectors carry two selectable markers, one of them being expressed from the retroviral promoter present in the 5'LTR, the other one being placed under the SV40 promoter in an internal transcriptional unit. Moreover, an additional retroviral integration sequence has been inserted upstream of the internal transcriptional unit. Such a vector is expected to allow integration as a disorganized proviral structure and consequently allowing a specific activity of the SV40 promoter. Our results clearly demonstrate that vectors endowed with such an additional integration sequence lead to disorganized proviral integration in which the additional integration sequence is used for integration *in vivo*, thus allowing specific expression of the internal transcriptional unit.

UL: Personnel has been employed as foreseen in the contract. For model experiments both *'in vitro'* and *'in vivo'* in the mouse system, we are working with the Bag vector, a recombinant retrovirus derived from the Moloney murine leukaemia virus. This vector carries two marker genes of bacterial origin: the *lac*Z gene and the neo gene under the control of the viral LTR and the SV40 origin, respectively.

For viral production, we used an ecotropic packaging cell line and we cultivated several producing clones, resistant to geneticin. The β-Gal activity was measured for each clone both by staining of the cells and by measuring the enzymatic activity in the cell extracts. A comparison of the various producing cell clones revealed a very important integration site position effect on the expression of the acquired character. We have now made subclones and are following the long-term stability of the acquired gene expression (Milestone B). The best clone was kept and the recombinant viruses were produced in large amounts (Milestone S). It was verified that the virus preparation was free of replication competent, wild-type viruses. We set up conditions to concentrate the viruses and studied its stability both after freezing at -70°C and thawing as well as at 37°C in the mouse blood and in culture medium. The virus thus appeared very fragile under *'in vivo'* conditions (half-life: 2.6h). We are now propagating our viral preparation in another packaging cell line obtained from the Munich group, in order to further increase the viral titer for the *'in vivo'* experiments.

We have verified that the viruses possessing a murine ecotropic envelope have no infectivity for human cells in culture (Milestone V).

The SV40 early promoter is very frequently used in retroviral constructs to drive the expression of the gene of interest. We observed however, that this promoter is negatively affected by a cellular factor which is characteristic of mammary cells. We shall try to identify this factor as it is important in view of viral targeting to the mammary gland (Milestone K).
The *'in vivo'* fate of the viral vectors is currently studied (Milestones O and Q). Techniques were set up to localize the infected cells expressing the β-galactosidase gene on frozen sections (7 μm thick) and to detect the proviral DNA by specific amplification. We are using the double PCR technique and nested primers to overcome the problem of limited amplification of rare template sequences. Control tissues were totally negative in these PCR experiments. Viruses in various amounts were injected into a growing mouse tissue, i.e. mammary tumours, and searched

for after different time periods. The lowest virus dose ($2x10^4$) was detectable one week to three months after injection and no relation with the tumour growth rate was observed. We are currently following the viruses in the suckling animal after injection in the peritoneal cavity and after ingestion with the maternal milk.

HIGHLIGHTS/MILESTONES

We have been able to reach all the milestones planned for the reporting period, except for a delay in the generation of satisfactory integrase-deficient packaging cell lines necessary to reach Milestone D. A novel idea that has been included in our work programme after project initiation involves the use of artificial primers for reverse transcriptase as a means of biological containment.

WIDER CONSIDERATIONS

The importance of biosafety work on retroviral vectors has been stressed by the publication of Nienhuis' results: Helper virus induced T cell lymphoma in non-human primates after retroviral mediated gene transfer (J. Exp. Med. 176:1125, 1992).

COOPERATIVE ACTIVITIES:

In accordance with the time chart a workshop was held in Sandbjerg, Denmark on Nov. 15-18, 1992. Twenty nine members of the five groups as well as three external guest speakers participated in a productive schedule of presentations and discussions of results, research strategies and ideas for collaborative efforts. The five laboratories were represented by seven participants in the BRIDGE First Sectoral Meeting on Biosafety in Wageningen, NL, Dec. 6 — 9, 1992. F.S. Pedersen visited the Munich group for three weeks in July 1992 to set long-term directions for the collaborative activities of the five laboratories and to define the basis for a wider European network in the field of retroviral gene transfer. Seven members of the Munich group visited Aarhus for two days in November 1993. Three members of the Munich group visited Liège on December 9, 1992. In addition, members of the participating laboratories have met at other meetings in Europe and the U.S. Retroviral vectors, retroviral vector packaging cell lines, and vector transduced cell clones have been mutually exchanged.

EUROPEAN DIMENSION

The retroviral vector technology is relatively new and has potentially wide applications. In many ways this field is still in a 'trial-and-error' phase. For evaluation of specific procedures and their safety aspects it is therefore important to assure free exchange of research materials and protocols. In the longer run a transnational programme will be important to ensure a common standard for use of retroviral vectors in the EC-countries.

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Assessment of environmental impact from the use of live recombinant virus vaccines (BIOT CT-910289)

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PARTICIPANTS:
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OBJECTIVES

i) Construction and characterisation of the various recombinant virus vaccines.
ii) To determine recombination frequency using mixed infections with recombinant FPV's containing lacZ$^+$/gpt$^-$ or lacZ$^-$/gpt$^+$.
To determine the of the rate of recombination between heterologous poxviruses which contain a genomic region of homology. This constitutes an example of a 'worse case' scenario.
iii) Sera and tissue collection and screening for orthopox viruses and antibodies
iv) Pathogenesis of cowpox in mammals.

RESULTS

Coordinator

Recombinant capripoxviruses have been constructed containing the fusion or haemagglutinin genes from rinderpest virus. These were constructed using the Eco gpt gene positive selection system. (Tests within the high security facilities at the Pirbright Laboratory have shown that both of these recombinant viruses induce total protection against rinderpest and lumpy skin disease in cattle and sheep and goat pox and pestes des petits ruminants in sheep and goats.) The growth of the parent capripoxvirus and the derived recombinants are being examined in the standard permissive lamb testis cells and several cell lines non-permissive for the growth of capripoxvirus. Initial experiments indicate that although some stages of virus replication may be occurring the non-permissive cells do not appear to generate an on-going infection. We have problems with our tissue culture units in that we are experiencing frequent contamination and more importantly we have found that this years lamb testis cells appear to be refractory to infection. (Lamb testis cells are primary/secondary cells and are prepared during the spring lambing season). We are also characterising the recombinant viruses to determine whether the particles contain the F and/or H proteins as structural entities. Here again we have been somewhat held up by the problems with the lamb testis cells.
A recombinant capripoxvirus containing the gII gene of the pseudorabies virus is being developed. The group at CDI Lelystad have sent us a plasmid containing a segment of PRV DNA which includes the sequence of the gII gene). For an integrated non-poxvirus gene to be expressed in a recombinant poxvirus it must under the direct control of a poxvirus promoter. Thus it is necessary for the gII gene to be precisely excised or copied. There are no useful restriction endonuclease sites with which the gII can be excised it was thus necessary to amplify the gene using the polymerase chain reaction (PCR). Initial experiments highlighted the problems specifically associated with a nucleotide sequence of the gII type. This sequence is extremely high in G and C and is almost 3,000 bases

long. It proved impossible to produce any amplified product as PCR templates of such high G and C content and gene length are routinely very difficult if not impossible to amplify. Consultation with several research groups both in the U.K. and the U.S.A. indicated that it was not a realistic proposition to continue in this manner. Examination of the nucleotide sequence of the gII containing plasmid showed that digestion with *BamHI* and *Nhe*I would remove the DNA sequences from the multicloning site BamHI site to nucleotide 993 of the PRV DNA (i.e. of the gII gene). This being the location of a unique NheI restriction site. The nucleotide sequences from the start of the gII gene to nucleotide 993 could then be prepared using PCR and specific primers. We have prepared this short PCR product and in the process of incorporating it back into the modified gII containing plasmid. This will provide a plasmid in which the gII gene is located immediately downstream of the plasmid multicloning site, which will facilitate the insertion of a poxvirus promoter.

Dr. A.L.J. Gielkens, CDI.

Recombination between a vector virus that carries a foreign gene and a field virus may result in transfer of the heterologous gene to the field virus. To investigate whether recombination in vivo can lead to pseudorabies virus (PRV) recombinants with altered biological characteristics, the envelope glycoprotein E1 of hog cholera virus (HCV) was incorporated and expressed in the gX locus of PRV strain NIA3. The virulence and the pathogenesis of this 'worst case' recombinant (gX-, E1+) were compared with the parent strain. No increase in virulence was detected in pigs. In contrast we observed a longer survival time after infection with the recombinant virus (gX-, E1+). This observation suggests that insertion of E1 leads to a slight attenuation of the parent strain.
To study whether incorporation of foreign genes leads to an altered host or tissue tropism, the genes coding for the fusion protein (F) and haemagglutonin (H) protein of rinderpest virus (RPV) will be incorporated in a PRV vaccine strain (TK-, gI-). The H-protein is involved in attachment of the RPV to the host cell whereas the F-protein is involved in virus spread by cell fusion. For this purpose both genes were cloned in a plasmid which contains the E1 gene of HCV flanked by 500 bp of PRV sequences. The E1 gene was replaced by the F and H genes containing their own signal sequences, or the F and H gene were fused in frame with the gX signal sequence. The resulting plasmids will be used to generate recombinant viruses by homologous recombination.
To examine whether recombination can occur in vivo between a vector virus and a field virus, we developed a PCR assay to discriminate between field viruses and possible recombinants. Unfortunately, we were unable to amplify viral DNA in tissue samples of PRV-infected piglets. We therefore decided to construct mutant viruses that lack either the 5' part or the 3' part of HCV E1. Reconstitution of E1 in vitro can only occur after recombination between both mutant viruses. This recombinant event can be assessed in vivo by measuring the antibody response against a particular epitope

Dr. M.A. Skinner, AFRC.

Task 1: Recombination between fowlpox viruses using marker selection.
The task has been completed and the objectives achieved. Mixed infections of CEFs with different recombinant FPV carrying *E. coli lacZ* and *gpt* genes in different genomic locations has been performed. To our surprise and satisfaction it proved possible to select directly from the recombination mixture for FPV virus expressing gpt by plaquing in the presence of MXHAT. Recombinant FPV carrying

both *gpt* and *lacZ* genes have been isolated, allowing us to determine the frequency of recombination between the two markers in this otherwise homologous background.

Task 2: Recombination between heterologous viruses carrying a region of homology.
We have experienced difficulty in isolating the final stage plasmid construct to be used as a transfer vector for production of the recombinant FPV. The reasons for this problem, which appear to be due to DNA sequence instability, are not clear. We are continuing to try alternative routes to the same construct.

Additional task: Construction of FPV rinderpest recombinants.

Objectives: Construction of FPV recombinants to express F and/or H genes of rinderpest virus (the problems experienced with task 2 have allowed time to be spent on this task, which was in our initial application for a 3 year programme but which had to be removed when the programme was *abbreviated to 2 years).*

Results: In a collaboration with Dr. T. Barrett (IAH, Pirbright Laboratory) rinderpest F & H genes have been inserted into FPV transfer vectors. As the rinderpest gene cassettes are the same as used in constructing the capripox virus recombinants, any recombinants isolated will be comparable. Recombination experiments have been performed to introduce the rinderpest genes into FPV and screening is being performed to identify and isolate recombinants which express either or both of the rinderpest genes (one of the constructions was designed to generate a single recombinant FPV which would express both the rinderpest F and H genes).

Cooperative activities: FPV transfer vectors and rinderpest F and H gene cassettes (under vaccinia virus p11 promoters) have been exchanged between participants 01 and 03.

Dr. R.M. Gaskell, Univ. of Liverpool

Work has concentrated on trying to identify the reservoir host of cowpox virus and studies of the pathogenesis of cowpox virus in laboratory mice. Tests on sera from several small wild rodent species trapped in the UK have demonstrated antibody reactive with cowpox virus in 28% *Clethrionomys glareolus,* 53% *Microtus agrestis* and 16% *Apodemus sylvaticus*, but virus has not been isolated from any wild mammals as yet. This suggests that cowpox virus probably does not cause persistent infection in these species, but further attempts to isolate virus from wild animals will continue once spring makes trapping possible. Meanwhile, experiments in which two strains of laboratory mice were inoculated with various doses of cowpox virus have shown that laboratory mice are very resistant to cowpox and probably will not make good laboratory models for the reservoir hosts. This also explains the lack of antibody reactive with cowpox virus in house mice trapped in the same environment as seropositive voles and woodmice.
Breeding colonies of *Clethrionomys, Microtus* and *Apodemus* have been established at Liverpool and some preliminary dose-response infection trials have been carried out in *Clethrionomys* and *Apodemus. Microtus agrestis* have proved more difficult to maintain in the laboratory than the other two species, and adequate numbers of animals are not vet available for infection experiments. *Apodemus* appear very resistant to cowpox virus infection following footpad inoculation, but *Clethrionomys* are very susceptible to infection — animals given 200 pfu died within a few days of inoculation. Detailed examination of tissues from these

experiments is still in progress, and further experiments are planned in order to study the pathogenesis of cowpox in these species and in *Microtus.*

Prof. P.P. Pastoret, Univ. de Liège

The red fox is the main vector of rabies (a disease of major importance in Europe). Recent studies have shown that it may be possible to control wildlife rabies in Europe using baits containing a recombinant vaccinia virus which expresses the immunogenic glycoprotein of rabies virus. Field trials and laboratory experiments have shown this approach to be safe and efficient. However, before this vaccine can be used routinely in wildlife it is important to know how its efficacy and safety might be affected by possible recombination with poxviruses which are endemic in European wildlife. The aim of this work is first to study the pathogenicity of cowpox virus, an orthopoxvirus that could be maintained in a wildlife reservoir, in foxes and thus to determine whether foxes are susceptible to cowpox virus infection and to evaluate the risk of recombination between this virus and the recombinant virus.
In order to evaluate the risk of recombination of the recombinant vaccinia-rabies virus and cowpox virus the pathogenicity of cowpox virus in foxes has been studied. Ten foxes were orally inoculated with cowpox virus and two others intradermally. Control animals were inoculated with DMEM or with the recombinant vaccine. Neither clinical signs nor pyrexia were observed during this experiment. Animals inoculated intradermally with 10^5pfu or more (of either the recombinant and cowpox virus) showed depilated areas at the site of inoculation. Foxes were euthanised at different times and a series of organs were collected. Blood samples, oral and nasal swabs were also collected every two days. The presence of virus was detected by isolation on Vero cells and titrated. Cowpox virus was detected primarily in tonsils but also in oral swabs in orally inoculated animals during a few days after inoculation. To detect specific viral DNA, a PCR test was also performed. The PCR reaction were not sensitive enough to detect viral DNA and the technique will be modified using digoxigenine labelling to increase the sensitivity. Histological slides of skin samples have been stained with haematoxylin/eosin. Eosinophilic inclusions were observed their specificity will be assessed using the immunoperoxydase assay.
The results of this study show that, like the recombinant vaccinia-rabies virus, cowpox virus multiplies in restricted sites during a short period in foxes. The risk of recombination between these two viruses is therefore almost null.
We collaborated with the orthopoxvirus serological survey in wildlife, performed by the team of Liverpool, (by sending to them 147 foxes, 47 wild boars sera, 10 badger sera, 2 stone marten sera, 2 polecat sera, 2 vole sera and 1 field mouse serum) and also with the capripoxvirus serological survey, performed by the team of Pirbright, by sending 281 sheep sera, 284 bovine sera, 3 mouflon sera, 33 red deer sera and 52 roe deer sera from Belgium and 180 sheep sera and 189 bovine sera from France .

HIGHLIGHTS/MILESTONES

The preparation of the recombinant capripoxvirus vaccines marks a major step in the use of vaccination to control and hopefully eradicate capripox and rinderpest in livestock.

WIDER CONSIDERATIONS

The recombinant capripoxvirus vaccines have been chosen for a three year trial in Kenya. The demonstration of their successful and safe use in a country where both capripox and rinderpest are endemic will provide strong support for their world wide use.

COOPERATIVE ACTIVITIES

Two group meetings were held within the reporting year.

i) C.D.I. Lelystat October 12th. This meeting was limited to the groups with a more molecular based programme (The coordinator and participants 2 and 3). Although we did discuss our progress within the bridge programme we took the opportunity to sent discuss all aspects of the groups work and to exchange ideas and problems that we were encountering in the area of molecular biology of recombinant viruses.
   This was a very useful and successful meeting and the ability to have such get togethers are a major benefit of the Bridge programmes.

ii) The Group Meeting Liege November 6th.

This was attended by representatives from all the participating groups (2 to 3 from each group). In addition we were very fortunate to have Prof. Frank Fenner, one of the worlds leading poxvirus virologists join us for our meeting. All groups gave their reports and there was a consensus that we were achieving our programme objectives.
Prof Fenner also felt that the programme was addressing very important fundamental aspects within the area of recombinant vaccines and that the results obtained would provide a strong basis for future work and regulatory systems.

EUROPEAN DIMENSION

It is important to note the that the use, in specific areas of Europe, of the recombinant rabies vaccinia vaccine is proving to be very effective in the control of wildlife rabies.

Identification of genes involved in latency and reactivation of pseudorabies virus, use in biological containment study of viral genomes in pigs (BIOT CT-910297)

COORDINATOR:
A. JESTIN, CNEVA, Ploufragan, F
PARTICIPANTS:
1. R. THIERY, CNEVA, Ploufragan, F
2. H.J. RZIHA, FRCVDA, Tübingen, D
3. I. ALMEIDA, NVRL, Lisbon, P
4. P. SHELDRICK, IRSC, Villejuif, F
5. M. RIVIERE, Rhône-Mérieux, Lyon, F

OBJECTIVES

Two major points have to be mentioned participant 1 has reoriented his work in the quantitative approach of the genomic detection and participant 4 has reoriented his work in the expression of a cytokine involved in regulation in viral infection as it has been shown in poxvirus.

RESULTS

Participant 1: THIERY R. and JESTIN A.

Quantitative approach of PRV Latency

Aujeszky's disease is one of the major causes of economic lose in the swine industry. The causative agent of this disease is a virus belonging to the alpha herpes virus family, the pseudorabies virus (PRV), which can persist latently in the host. Latent infection is one of the main problems found for the eradication of this virus because the virus present in latently infected individuals can reactivate under different conditions and spread out in the environment. This is also a main concern, as inactive vaccine strains can also persist latently in vaccinated individuals which is an undesirable feature for a vaccine.

The Polymerase Chain Reaction (PCR), a major recent advance in molecular biology was successfully used to detect low amounts of viral DNA in latent and in productive infections. The aim of this study is to take advantage of the powerful PCR technology to quantify minute amounts of PRV virus present latently in different tissues. The high specificity of PCR will also allow us to distinguish different strains of PRV (i.e. wild type and vaccinal strains), and therefore it will allow the differential quantification of those strains present at a given time in a particular tissue. Such a tool should be very useful for monitoring vaccine efficiency.

Several methods have recently been proposed to make out of PCR a quantitative tool. Among those we have choosen to develop an assay based on the use of an internal standard. This standard is added at known concentrations to the components of the PCR reaction and co-amplified with the target DNA. In this method the internal standard only differs from the target by a small deletion (4-8 base pairs). In this case the efficiency of amplification by PCR is equivalent for the two species. The resulting PCR products are labeled with fluorescent primers and then separated and detected by the means of an automated sequencer.

A highly specific and sensitive PCR assay has then been developed in the laboratory. Two specific primers directed against the envelope glycoprotein gp50 were selected with computer assistance using the Oligo software (National Biosciences). This assay is highly reproducible with a detection limit of only one copy of PRV DNA. Then several internal standards were constructed using a strategy developed in the laboratory and used in preliminary experiments in collaboration with C. Pannetier and P. Kourilsky (Pasteur Institute, Paris). This team developped independently a powerful software designed for the quantification of PCR products using the method described above with an automated sequencer from Applied Biosystems. As a result from this preliminary experiments, we can confirm that the strategy that we have choosen is adequate for the quantification of low amounts of virus DNA present in latently infected pigs.

We also developed another PCR assay for the quantification of the endogenous porcine gene NF-1 (Nuclear Factor 1) using the same strategy. This last assay will be useful for the absolute quantification of the amount of virus present in a particular tissue expressed as the number of copies of PRV per porcine genome (or cell).

Participant 2: RZIHA HJ.

Analysis of LAT ('latency-associated transcript') region of PRV and Construction of viral mutants

The identification, DNA sequencing, and analysis of transcriptional organization of a PRV genomic region specifically transcribed during latent infection has been described in the last report. The results indicated the presence of a potential promoter region upstream of the cap site of PRV LAT (latency-associated transcript) RNA. Consequently we proceeded studies to elucidate possible latency-related functions of this LAT region focussing on the following two major issues.

***In vitro* analysis of the LAT-promoter (P-LAT) activity**

Defined parts of P-LAT were plasmid-cloned and fused to the functional promoterless luciferase gene, which could be used as an indicator for cis-acting promoter elements. Numerous experiments were necessary to establish the optimal transfection protocol for measuring transient luciferase expression, which differed for each individual cell species. in summary, the results obtained so far demonstrate comparable promoter activity in 4 different epithelial cell lines (of porcine, bovine and simian origin) and mouse neuroblastoma cell lines. In contrast, no activity at all of PRV P-LAT was found in human neuroblastoma cells, although successful uptake of DNA could be proven in those cells. A ca, 270 base pair fragment of P-LAT could be determined to achieve maximum *in vitro* promoter activity. Furthermore, we could show by PRV superinfection experiments that late viral gene products did not transactivate, but rather suppress the activity of PRV P-LAT. Studies are now in progress to localize possible enhancing and/or suppressing promoter elements. To answer the most crucial question, wether PRV-LAT is capable to promote neural-specific gene expression, transfection experiments of neural porcine cells remain to be done.

Construction of viral mutants

As already reported, a new non-essential, non coding intergenic site was localized on the PRV genome and coulb be used to insert and express foreign genes (Fuchs et al., in press). Such a recombinant wild-type PRV, expressing e.g. the functional E. coli lacZ gene, can be now not only used to trace PRV replication for cell tropism studies, but also for production of other PRV mutants. Therefore, we

haven taken advantage of this lacZ-PRV to construct a first LAT-virus mutant. From the results described in (1) we were able to obtain a viable lacZ-PRV which is lacking the complete P-LAT. Analyses of viral gene expression and *in vitro* growth characteristics of this LAT-mutant have to be finished, before starting with first *in vivo* studies.

HIGHLIGHTS/MILESTONES

The briefly described results demonstrate for the first time the principal promoter activity of PRV P-LAT, and now enable defined studies for the biological function of LAT in the natural host. The already initiated and further planned experiments should not only help in elucidation of mechanisms leading to latency of alphaherpesvirus, but also open new tools and strategies in vaccine developments and targeted gene delivery.

Participant 3 — Dr ALMEIDA

Use of vDNA hybridization and immunohistochemistry to study the mechanism of PRV infection in embryonic neuronal cultures

The neuropathogenic properties of a number of different strains of swine PSEUDORABIES Herpesvirus were evaluated in primary embryonic porcine neuronal cell cultures derived from dorsal root ganglia and cerebral cortex. Rat sensory neurons were used for comparative proposes.

Our wild type strains of PRV (KOJNOK and PHYLAXIA) are virulent field isolates, whereas GI — is a vaccine strain with the deleted glycoprotein GI. The pattern of infection of a recombinant PRV containing the functional E. coli lac Z gene (PHYEBG, FUCHS et al.) have been studied to examine strain-specific differences. The course of recombinant PRV *in vivo* infection were checked for B-gal expression.

Combination of multiple immunoenzymatic and immunofluorescent staining for the analysis of plural PRV antigen production by infected neuronal cells and *in situ* hybridization for localizing vDNA autoradiographically in the same cell culture were performed.

The location and temporal appearence of infected neural subpopulations was followed either by the application of avidin-biotin-peroxidase-complex-method with a monoclonal antibody to gII PRV glycoprotein or by direct immunofluorescence with a polyclonal pig anti-PRV serum FITC-labeled.

Histological distribution of cell-type-specific antigenic markers: glial fibrillary acidic protein, tetanus toxin, and, neurofilament protein, was simultaneously demonstrated by an indirect immunofluorescent method in which the binding sites were revealed with TRITC.

PRV — infected neuronal monolayers stained for PRV proteins, and, or, for cell markers were further hybridized *in situ* with (32P) cloned restriction endonuclease fragment of PRV (400 bp long of gIII DNA) labeled by multipriming. The localization of probe hybridization to individual cells was done by light microscopy.

Immunohistochemical localization of PRV results

The distribution of PRV containing neurons was examined at 12 to 24 hours intervals.

Several aspects of the cellular localization of viral antigens were commun to both wild type and vaccine strain infecting porcine embryonic central or sensory neurons. First neuronal labeling always followed astroglial labeling in the temporal sequence of infection. Dark antigen staining is noted in a minority of neuronal cells while sorrounding cells are indistinguishable from control 12 hours after infection. The number and staining intensity of infected neurons increased progressively with time and by 72 hours axonal antigen staining is prominent with a beaded appearence. Loss of integrity of these neuronal somas was delayed in primary cerebral cortex cultures GI-infected.

The principal feature which distinguished patterns of PRV *in vitro* infection was noted following infection of rat sensory neurons with vaccine strain. The distribution of immunoreactive cells was restricted to small foci of ganglion cells (SCHWANN or glial lineage) early in the infection (18 hours). Our finding also indicate that selective infection of subpopulations of DRG cells persisted through at the latest stages of infection (96 hours). In every instance the identity of infected cells were examined with a simultaneous immunocytochemical assay for cellular marker antigens.

In situ assay for vDNA

vDNA was visualized by standard autoradiographic techniques after annealing a strand of radiolabeled DNA over monolayers of infected neuronal cultures. Morphological details of both cultures were well preserved and autoradiographic grains were readily detectable as early as 18 hours after infection. Silver grains were present in the cytoplasm of PRV — containing neurons as well as in unlabeled cells. The majority of control studies have been conducted with probe and with mock-infected cells.

Conclusions

(1) The data demonstrated that either strain of PRV readily infects sensory and central neurons of porcine, or, rat origin without requiring prior adaptation which points to the natural susceptibility of these cells to the virus.

(2) Our results also indicate that the replication pattern of wild type strains were indistinguishable from that of recombinant PHYEBG strain.

(3) By contrast, the vaccine strain of PRV selectively infected subgroups of the total population of ganglion and cortical cells at the earliest time point of infection (24 hours). There was also a clear temporal aspect to the infectious virus particle production as the plaque titration of extracellular virus clearly showed. However the circonscribed character of PRV immunoreactivity was more pronounced in rat sensory neuronal cultures in which is consistent with the heterogeneity of these cell substrates.

(4) Furthermore kinetics of replication at various multiplicities of infection led us to the conclusion that the size of viral input has important implication for the temporal cytophatic events.

(5) Growth curves from titrated progeny virus released in the supernatant fraction indicate that titers in the cultures rose steadly up to day 3 after which they declined while with vaccine strain a rise in the titer was delayed, indicating slower initiation and progression of the infection. Although the cytopathology was well developed at the onset of the infection the increase in virus titers is negligeable.

Participant 4 — SHELDRICK P. and FOULON Th.

Projected development of recombinant PrV carrying a gene for porcine tumor necrosis factor alpha (TNFα)

Since results obtained by the Tubingen laboratory (Partner 2 ; Progress Report 1992) permitted a detailed description of major mRNA species present in latently infected bovine tissue, we have provisionally put aside the exploitation of a cDNA library (Progress Report 1992) in favor of the development of recombinant PrV.

Recently, Sambhi and co-workers (1991, *Proc. Natl. Acad. Sci. USA88, 4025-4029*) demonstrated the antiviral effects of TNFα *in vivo* using recombinant vaccinia virus in a mouse model. Their experiments indicated that a localized production of TNFα during infection could lead to enhanced clearance of the virus. These results encouraged us to explore the feasability of modifying the pathogenicity of PrV by a similar approach, and to develop recombinant PrV carrying the porcine TNFα gene under the control of selected viral promoters.

We have used a genomic DNA fragment (4 Kbp) containing the entire porcine TNFα gene, cloned into a pUC18 cloning vector (CHARDON, P. et al, 1991, *Immunogenetics 34, 257-260*). The published porcine TNFα sequence is that of the mini-pig, our partial sequence data show that there are only few differences between the two DNA sequences. The TNFα gene codes for a 232 amino acid protein, and is composed of exons and 3 introns. The 3' non-translated region (approximatively 1 Kpb long) contains a putative PolyA site and an AT-rich sequence. The latter sequence is found in the 3' region of several cytokine genes and is supposed to be a tissue specific regulatory sequence, probably influencing mRNA stability. We have deleted both sequences in our first construction pTFH, leaving only 300 bp downstream of the stop codon. The 5' non-translated region contains a TATA-box and several binding sites for other transcription factors. We have removed this region in the pTFHX recombinant vector, leaving 55 bp upstream of the ATG codon.

The viral glycoprotein gX promoter was chosen to drive the expression of the TNFα mRNA. Glycoprotein gX is expressed early in the lytic cycle. The promoter has been obtained from the Phylaxia strain genome in the form of a BamHI restriction fragment. A portion of this fragment containing the promoter has been cloned in the pSPT18 vector to construct pRZPgX (Tubingen). We have constructed pRZPgX/TFHX, in which the TNFα gene is inserted in the orientation which puts it under the control of the gX promotor and pRZPgX/TFHXinv which carries the fragment in the opposite orientation to provide a negative control in tests of TNFα activity.

To permit eventual insertion of the PgX-TNFα cassette into the PrV genome, we used the plasmid pRZ12.1 (Tubingen) which contains the fragment KpnI-F and a multiple cloning site from M13mp18. We have cloned the PgX-TNFα gene into the EcoRI site of pRZ12.1 to obtain the pRZ12.1/PgXHK vector, which will be used for the recombinational insertion of TNFα into the PrV genome. In order to facilitate the recognition of the recombinant viral genome, we will use the recombinant PHYEBG PrV virus (Tubingen), which carries the LacZ gene under the control of the PgX promoter.

The competence of PrV expressing biologically active to replicate and to establish a reactivatable latent state in animals will be studied by the Ploufragan and Tubingen laboratories and in neural tissue culture by the Lisbon laboratory.

COOPERATIVES ACTIVITIES

— *Meeting*
  - Wageningen meeting: 6-9 December 1992
  - Paris meeting: 3 Mars 1993

— *Probationer*
  - Dr ALMEIDA probationer in Tubingen one month in November
  - Dr ALMEIDA probationer in Pasteur Institute (Service Dr Henri TSIANG, Service Rage)
  - Dr ALMEIDA collecting dorsal root ganglion in fetuses at Ploufragan on 28-04, 19-05, 9-06, 30-06, 21-07, 1-09, 22-09, 13-09, 22-12, 12-01, 2-02, 2-03, 23-03.

— *Exchange of material*
  - strain and probes from Tubingen to Lisbon Team
  - strains from Tubingen to Ploufragan
  - Samples from Ploufragan to Tubingen
  - Dorsal root ganglia from Ploufragan to Pasteur Institute
  - Shuttle navette from Tubingen to Villejuif

Biosafety of genetically modified baculoviruses for insect control (BIOT CT-910291)

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OBJECTIVES

The objectives of the project are to construct baculoviruses with enhanced insecticidal activity, but predicted reduced survival in the field and to develop systems to model this behaviour in the laboratory prior to release experiments. This will be achieved by introduction of a 'built-in' suicide mechanism or gene deletions in baculoviruses that would impair their survival, but not their short-term efficacy. After construction these genetically modified, biologically contained viruses, will be tested in the laboratory (biological activity and host-range) and in a microcosm (persistence and spread). In this way information can be obtained about the biological behaviour of such 'biosafe' viruses, which allows a prediction to be made on the consequences when released in the field.

In the reporting period the objective has been to complete the construction of the various recombinant viruses (1 and 3), to determine their biological activity (2 and 3) and host range (3) and to test those recombinants already available in a microcosm (1 and 2).

RESULTS

Construction of recombinants

The strategy employed to produce a 'suicide' *Autographa californica* nuclear polyhedrosis virus (AcNPV) insecticide has utilized the lac operon and repressor from *Escherichia coli* to render an inducible baculovirus gene promoter. This permits the construction of a virus with the ability to produce polyhedra in insects only in the presence of IPTG. Normal virus could be produced in the laboratory but after release in the field, only non-occluded virus would be made, severely limiting virus survival.

The 39K gene promoter of AcNPV, which has a region of sequence homology to the *lacO*, was mutated to more closely match the bacterial sequence and then fused with a copy of the chloramphenicol acetyl transferase (CAT) gene. In the same plasmid, a copy of the lac repressor (I) was placed under the control of the *Drosophila* heat shock promoter (hsp). This plasmid was then co-transfected with AcNPV DNA to derive a recombinant virus. Subsequent tests have confirmed that expression of the CAT gene under the control of the 39K-*lacO* is repressed at least six fold in the absence of IPTG. The next step is to add a second *lacO* to the 39K gene promoter to improve repression and to replace the CAT gene with the polyhedrin gene coding region.

The construction of baculovirus recombinants with deletions of genes that affect virus persistence in the field has been focussed on four genes, which have been

described not to be essential for viral replication, increase virulence and reduce persistence of the virus. The genes under study in Wageningen are p10, the polyhedron-envelope gene (pp34) and the ecdysteroid UDP-glycosyl transferase (EGT), respectively. The chitinase gene, identified by the group of Oxford, causes rupture of the larval cuticle, thus enhancing the release of the progeny polyhedra. Spread of recombinant viruses lacking this gene should be limited.
Due to the fact that viruses containing deletions in these genes cannot be detected phenotypically, a marker gene is being inserted in or substituted for each of these three genes. The marker gene, the bacterial β-galactosidase gene (lacZ), allows convenient screening of recombinant viruses by blue coloration of their plaques using the chromogenic indicator X-gal. Subsequent elimination of the lacZ gene by recombination with a transfer vector with a deletion in the gene of interest then leads to the desired deletion mutant. The deletion of a gene thus requires a two-step procedure.

A wide variety of the deletion mutants (single, double and triple) are now available. The viruses were characterized by DNA restriction enzyme pattern analysis. Preliminary experiments showed that all the deletion mutants tested are still infectious to *Spodoptera exigua* larvae.

Investigation of the fitness of recombinant viruses

This line of research is focussed in three areas: a/ assessment of the fitness of genetically modified baculoviruses, b/ investigation of their behaviour in hosts with different levels of permissiveness and c/ assessment of the behaviour of deletion mutants in mixed infections.

Initial studies have focussed on the first area, assessment of fitness. The four viruses selected to test were *wild-type* AcNPV, AcNPV containing a 90 base pair inert marker sequence, AcNPV expressing the lacZ marker gene and AcNPV expressing an invertebrate specific scorpion toxin. These constructs were compared both singly and in mixed infections in order to see how selected fitness parameters varied and to see what the outcome of multiple passaging mixed infections of *wild-type* plus engineered virus would be. An assay system using both in vivo and in vitro techniques was set up. Parameters such as replication rate and yield have been measured in single infections in insects, which have shown that the AcNPV plus lacZ both reproduced less rapidly and produced less virus than the *wild-type*. The viruses were then set up in mixed infections with different proportions of each virus (*wild-type*: AcNPV lacZ, 1:10, 1:1 and 10:1) and passaged in cohorts of insects. After each passage the proportion of each virus type was estimated and the remainder of each virus repassaged in further hosts and so on. The first few passages using *wild-type* AcNPV and AcNPV plus lacZ show that the lacZ gene is being rapidly lost from the baculovirus population. This process will be repeated with other virus constructs.

A model system has been identified for the work on hosts with varying degrees of permissiveness, based around various *Spodoptera* species and their NPVs. Techniques are currently developed for this work. The first set of single and double deletion mutants have now been sent from the Wageningen to the Oxford group and studies on their biology have recently been initiated.

Behaviour of recombinants in microcosms

The AcNPV recombinant DZ5 and *wild-type* AcNPV were analyzed in the laboratory to test biological activity and UV-persistence and in a microcosm to analyze virus propagation and spread. The recombinant AcNPV-DZ5 has an inser-

tion of the bacterial marker gene lacZ into the polyhedron envelope gene (pp. 34). The absence of the pp34 gene renders the polyhedra more sensitive to alkali. An enhanced virulence and a reduced persistence is, therefore, anticipated.

The UV-persistence was studied by inactivation of dry virus deposits to direct exposure of artificial UV-sunlight. Comparison between the *wild-type* virus and the recombinant showed no significant difference in their half lifes after illumination with solar UV (266 and 253 minutes, respectively) as well as with 10-times higher UV-intensity (22.6 and 21.6 minutes, respectively). The loss of the polyhedron envelope does, therefore, not result in a increased UV sensitivity. The biological activity in terms of LC_{50} and LT_{50} indicated that the LC_{50} value of the recombinant was significant higher (factor 1.6) only with Autographa gamma neonates 6 days post infection and that the LT_{50} value was slightly smaller (7.9 days for the recombinant, 8.5 days for the *wild-type*).

A field release of the genetically manipulated AcNPV-DZ5 and *wild-type* AcNPV was simulated in a contained model-ecosystem, or microcosm. This microcosm, constructed during BAP, simulated the situation *in vivo*, including rain, solar UV, soil, sugar beet plants, and second instar larvae of the beet army worm *S. exigua* as much as possible. Equal quantities of both viruses applied to 100 larvae gave only minor differences in response (39 and 48% larval mortality, respectively). Low differences in virus distribution among the components of the microcosm were detected between *wild-type* virus and AcNPV/DZ5. Both viruses were washed into the soil but only the *wild-type* virus penetrated the whole soil profile of 15 cm depth and led to a contamination of the infiltration water, whereas the recombinant virus was only detected to a depth of 0-6 cm. This suggest a higher retention of recombinants in the upper layers of the soil.

HIGHLIGHTS/MILESTONES

1. Baculoviruses (AcMNPV) can have multiple deletions in the genome without loosing their biological activity. Their competitiveness need to be assessed (see 2).
2. A novel system for the assay of the fitness of genetically engineered viruses has been developed which can be used to assess the competitiveness of genetically modified baculoviruses and could provide information about their likely fate after release in the field.
3. A baculovirus with a 'built in' suicide mechanism is on its way to successful completion.

WIDER CONSIDERATIONS

Baculoviruses are insect pathogens which are successfully used as biological control agents of insect pests in agriculture and forestry. Although baculoviruses are effective, there is a necessary period when disease must develop in the pest. During this time severe damage to the crop may occur. This slow speed of action is a major drawback to a much wider commercial application of these viruses and is most relevant for crops with low damage thresholds. Genetically modified baculoviruses with improved insecticidal properties could overcome this drawback and may lead to a wider use of these viruses. It is important to realize, however, that risks may be associated with the release genetically modified viruses. Studies of the type undertaken in this project (construction of biosafe baculoviruses and controlled field testing) address this issue and will aid in the decision making process on the large scale release of genetically engineered organisms.

COOPERATIVE ACTIVITIES

All participants frequently exchanged materials (transfer vectors and recombinant viruses). Detailed discussion of current progress and future initiatives took place between all consortium participants during the EC meeting at Wageningen in December 1992. I.C Hauxwell visited both the Darmstadt Institute (1 day) and the Wageningen laboratories(3 days) in August 1992 to discuss the work plan and exchange ideas and techniques. A promotional pamphlet (Blue Brochure) is being prepared for public interest to explain the activities of the group.

EUROPEAN DIMENSION

The exchange of ideas and the novel expertise not available locally are the major benefits of this transnational research. Further extension of the platform is sought with the EC as well as in Eastern Europe.

LIST OF JOINT PUBLICATIONS / PATENTS WITH TRANSNATIONAL AUTHORSHIP

Gloger, U., Undorf-Spahn, K., Huber, J. and Vlak, J.M. 1992. *UV-persistence of a wild-type and a recombinant Autographa californica nuclear polyhedrosis virus.* Abstract Book of the 1st EC Sectoral Meeting on Biosafety, Wageningen, The Netherlands, **82**.

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Undorf-Spahn, K., Huber, J. and Vlak, J.M. 1992. *Biological behaviour of a genetically modified Autographa californica nuclear polyhedrosis virus.* Abstract Book of the 25th annual meeting of the Society for Invertebrate Pathology, Heidelberg, Germany, **141**.

Undorf-Spahn, K., Huber, J. and Vlak, J.M. 1992. *Simulated field release of a recombinant Autographa californica nuclear polyhedrosis virus in a model ecosystem.* Abstract Book of the 1st EC Sectoral Meeting on Biosafety, Wageningen, The Netherlands, **82**.

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Hauxwell, I.C., Possee, R.D. and Cory, J.S. 1992. *Genetically engineered baculoviruses and gene survival.* Abstract Book of the 1st EC Sectoral Meeting on Biosafety, Wageningen, The Netherlands, **81**.

Mans, R.M.W. and Vlak, J.M. 1992. *Engineering of biosafe recombinant baculoviruses.* Abstract Book of the 25th Annual Meeting of the Society for Invertebrate Pathology, Heidelberg, Germany, **139**.

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Vlak, J.M. 1993. Genetic engineering of baculoviruses for insect control. *In:* Molecular approaches to pure and applied entomology (M.J. Whitten and J.G. Oakeshot, eds). Springer Series in Experimental Entomology, 90-127.

Risk evaluation for genetically modified microbial inoculants (BIOT CT-910283)

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OBJECTIVES

1. Development of highly expressed reporter genes and their evaluation at microcosm and field level

2. Development of alternative vector systems and their evaluation under greenhouse and field conditions

3. Construction of a suicide system for *Rhizobium meliloti*

4. Development of physical and biochemical techniques for studying microorganisms which can be used to monitor populations in the soil and characterization of isolates of *Bradyrhizobium japonicum*

5. Assessment of population dynamic of rhizobia released as soil inoculant and the effects of agronomic factors on their survival

MAJOR PROBLEMS

ENCOUNTERED:The availability of a pyrolysis mass spectrometry facility in a place different from that of one participating lab, along with cultural traits of bacteria and the cost of having samples analysed has limited the number of samples which could be handled. The detailed legislation implementing the Directive EC 90/220 has been installed in Italy on April 3rd, 1993. Field releases with GMOs have been virtually impossible in this country till then.

RESULTS

1. Highly expressed reporter genes

The regulated *lacZ* + *lacIq* genes and the mercury resistance genes have been assembled into a cartridge. This structure, which is about 10 Kbp long, is flanked by unique restriction sites to facilitate cloning procedures. The *lac* operator, which is located within a 100 bp *HindIII* fragment, can be easily removed, leading to high-level constitutive expression of the *lacZ* gene. Three different chimeric plasmid have been prepared, based on the lac-mer cartridge. Their features are as follows:

a) ***pDG3***; the cartridge is inserted in a broad host range vector which is a RSF1010 derivative. It is devoid of any antibiotic resistance gene. Bacteria carrying this construct will have the reporter genes located on a plasmid, i.e. in a unstable form.

b) ***pDG4***; this plasmid is the same as pDG3 except that the lac operator has been removed. It will allow to study the effects of a high level expression of the reporter gene on its stability within the host microorganism.

c) ***pIRL1***; this pSUP102 derivative contains a fragment of *R. leguminosarum* DNA which carries the *recA* gene sequence. The cartridge is inserted within the coding region thus inactivating the recA gene. Since the plasmid is not able to replicate in *R. leguminosarum*, the marker genes can be mantained in the host bacterium only if homologous recombination occurs. In this way it is possible to have the reporter genes located on the chromosome, i.e. in a stable form.

All the constructs are available in Escherichia coli and in Rhizobium leguminosarum bv. viciae strain 1003. The expression levels are as follows:

a) ***lacZ gene***
The expression of the *lacZ* gene was assessed by the β-galactosidase assay proposed by Miller. We have found that in *E. coli* the activity is of about 3600 Miller Units for the regulated promoter, and reaches 15.000 Units when the non-regulated promoter (devoid of the *lac* operator) is used.

In *R. leguminosarum* biovar viciae 1003 the β-galactosidase activity is about 5000 U and 9000 U for the regulated and the non-regulated promoter respectively. The ß-galactosidase activity of the wild type strain is less than 50 U.

b) ***Mercury resistance genes***
In E. coli the presence of the resistance genes on a pBR322 derivative confers resistance to 10 μg $HgCl_2$/ml in the growth medium, while the wild type fails to grow at 4 μg/ml.

We have found for *R. leguminosarum* a different response depending on the composition of the growth medium. In Yeast Mannitol Agar the strain can grow on 1 μg $HgCl_2$/ml while wild type can not. In Tryptone Yeast the resistance increases up to 7 μg/ml and the wild type does not grow at 3 μg/ml. The assessment of the above genetically modified rhizobia (containing unstable and stable *lac* or *lac-mer* reporter cartridges) vs. non-modified parental strain(s) in commercial-type inoculants is under way and final results will be available by autumn '93.

2. *Development of alternative vector systems*

The *thy* vector system has been evaluated for *R. meliloti* under field conditions as reported previously. It was found to perform exceptionally well, even though the *R. meliloti* thy mutant strain was found to revert back to wild-type. No further work has been done on this aspect of the project.

3. *Development of a suicide system for R. meliloti*

There are two aspects which are crucial for the success of a suicide system, based on the *thyA* gene. Firstly, the *R. meliloti thy* mutant has to be stable and unable to revert back to wild-type. A reverse genetics approach is well underway to generate insertion and deletion mutants in *R. meliloti* and possibly other *Rhizobium species*. Secondly, we have to assess the capability of the *thy* mutants to survive in the soil, in the presence and absence of the host plant. The latter is also important to be able to decide on the nature of the promoter that will be used to conditionally express the thy gene. The field data suggested that the alfalfa host plant might be able to sustain the *R. meliloti thy* mutants in the rizosphere. This possibility was further investigated in the laboratory. The survival of the wild-type and mutant in rooting solution was monitored. After 35 days, significant numbers of the wild-type strain were found to survive in rooting solution, either in the presence or absence of thymidine. In contrast, the thy mutant only survived when

thymidine was provided and disappeared when thymidine was not provided. Also, in the presence of thymidine, the mutant was found to be 100% stable and no revertants (able to grow on MSG) were detected.

In the presence of the host plant, the *R. meliloti thy* mutant was found to survive the entire length of the experiment (35 days), without the addition of thymidine to the rooting solution. This indicates that the alfalfa host plant can sustain the *thy* mutants on their root surface. However, the reversion rate of the mutants back to wild-type (26% reverted), indicates that the amount of thymidine supplied is marginal.

Since alfalfa was able to sustain the *thy* mutant on their root surface, it was of interest to evaluate if other plants would be able to provide thymidine at their root system. In an experiment, conducted simultaneously with the previous two, sugar-beet seedlings were shown to be unable to sustain the *thy* mutants for the length of the experiments, the survival being essentially the same as that found in rooting solution only.

In the colonisation experiment described, the nodulation frequency was determined. The nodulation of the alfalfa plants by the *thy* mutants, in absence of thymidine, was severely delayed. Nodule occupancy analysis showed that 100% of the *R. meliloti* bacteroids were reverted to wild-type. This indicates that the *R. meliloti thy* mutant cannot nodulate, even though it can survive on the root surface of the alfalfa plants. In contrast, when thymidine is provided in the rooting solution, nodulation proceeds at normal rate and no reversion of the reisolated bacteria is observed.

We have shown that the complemented *R. meliloti thy* strain (RM42T/pGDT11) has similar survival and nodulation characteristics as the wt strain (RM42). Its competitiveness against the wt strain was assessed in mixed inoculum experiments. Alfalfa plants were inoculated with an equal ratio of RM42 (parent) and RM42T/pGDT11 (mutant). The survival in the rooting solution and on the root surface was monitored and the ratio between the parent and the mutant was found to be changed in equal amounts (from 50:50 to 65:35) in favour of the parent strain. When bacteroids were reisolated from the nodules, the shift towards wt was found to be more pronounced.

These preliminary experiments indicate that the *thy* vector system can affect the competitiveness of the *R. meliloti* strain.

4. Development of physical and biochemical techniques for studying microorganisms, which can be used to monitor population in the soil,andcharacterization of isolates of Bradyrhizobium japonicum.

PYMS (pyrolysis mass spectrometry) and PCR-based techniques have been so much more successful than anticipated two years ago that we have concentrated virtually all our efforts on them. Because we have not been able to correlate population in soil with differences in the chemical or physical properties, there is no need or advantage in taking samples from elsewhere as was originally expected.

Last year we were concerned because the above techniques were typing many of the Italian isolates as a strain that was not expected in the fields we were studying. The isolates were characterised as a strain used elsewhere, and we were trying to understand how the fields we were studying were contaminated. After checking carefully in Italy we were informed that the strain we were told was in an inoculant used in these fields was incorrect and that the strain we thought was there has

indeed been introduced. This provided a very rigorous test of the value of our techniques.
In one field, inoculated with a single strain, we have shown that the strain has persisted because all isolates have the same PCR-based fingerprint, and that it has split into two populations bacause there are two groups of isolates discriminated by PYMS.

5. Assessment of population dynamic of rhizobia released as soil inoculant and the effects of agronomic factors on their survival

In order to assess how the survival of rhizobia is affected by the water-table dethp, a soil site with pH 7,76 was selected and during 10 months the water-table was artificially raised twenty-two times between 1 and 2 meters below soil level.

Soybean was planted in some plots, and *B. japonicum* was used as soil/seed inoculants. The survival was determined by using the sensitive technique MPN (most probable number), having a lower detection limit of ca. 1 cell x g of soil. After 90 days from sowing the number of soil-inoculated rhizobia was greatly reduced (60%) where water table was artificially raised, and a remarkable effect was also given by the presence of plants. After six months from sowing, the rhizobial populations seem to have established at 10^1 -10^2 CFU × g of soil, irrespective of treatment.

MILESTONES

The *lac-mer* cartridge represents an appropriate reporter system to monitor the fate of soil microbial inoculants for legumes
The *R. meliloti* thy mutants were shown to be able to colonise, but not to nodulate the alfalfa host plant. This indicates that a promoter which is expressed during symbiosis only (such as many *nif* or *fix* promoters), will be suitable for the construction of a suicide system based on conditional expression of the *thy* gene.

Pyrolysis-mass spectrometry and PCR-based fingerprinting techniques, along with DNA fingerprinting through pulsed- field gel electrophoresis, provide an accurate identification of soil microbial inoculants.

WIDER CONSIDERATIONS

Combined molecular (genetic-biochemical) approaches prove to be extremely useful to (1) offer reliable methods to monitor the fate of microbes in soil environment (2) start the construction of genetically modified intrinsically biosafe soil microbial inoculants (3) identify microorganisms at strain level.

COOPERATIVE ACTIVITIES

One coordination meeting has taken place during the Sectoral Meeting in Wageningen (Dec. '92). Strains are regularly exchanged among participating laboratories and common experiments are undertaken by using the same biological material.

LIST OF JOINT PUBLICATIONS WITH TRANSNATIONAL AUTHORSHIP

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H. E. Kay, H. L. C. Couthinho, M. Fattori, G. P. Manfio, R. Goodacre, M. P. Nuti, M. Basaglia, J. E. Beringer (1993) Investigating the survival of Bradyrhizobium japonicum strains released into italian soils (to be submitted).

Methodology for the fast design of fungal DNA probes and PCR primers (BIOT CT-910301)

COORDINATOR:
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OBJECTIVES:

A general methodology combining DNA amplification and sequencing was proposed for the fast identification of microbes in the case of filamentous fungi.

During the first reporting period, the concerned DNA techniques were set up for fungi and standardised using representative fungal genera and species involved in several areas of biotechnology. A coordinated quality insurance system between the participating laboratories was established. Two protocols of direct sequencing of amplified ribosomal 18S fragment were carried out in two of the participating laboratories. Several fast DNA extraction protocols were evaluated. The 18S ribosomal subunit was found to be a poor source of probes. However, 2 genus specific probes and 4 species specific probes of very good specificity for human mycopathogens were found in that region and validated against a DNA bank of about 25 fungal species.

During the present reporting period, four objectives were carried out:

1) Standardisation of simplified protocols for the preparation of PCR-quality fungal DNA.
2) Assessment of fungal nuclear consensus PCR primers (NS and ITS series) for a broad spectrum of fungal species.
3) Amplification and direct sequencing of the ITS region using ITS5 and ITS4 primers under high stringency PCR conditions.
4) Standardisation of arbitrary primed-PCR (AP-PCR) under low stringency conditions and study of the generated Amplification Fragment Length Polymorphisms (AFLP) for studies of genetic relatedness between strains of the same species or genus.

RESULTS

1) Quick DNA preparation methods

A first protocol was set up in the Danish laboratory. It is based on disruption of spores and mycelium by freeze-thawing without the use of enzymes followed by phenol-chloroform extraction and DNA precipitation. It was found convenient by all participants for small cultures of most fungi tested including those with very resistant cell walls. The resulting DNA could be used for PCR and direct sequencing of PCR products. A second protocol was further derived by the Belgian laboratory which is based on glass beads disruption of spores and mycelium in the presence of phenol and SDS followed by DNA precipitation. The last protocol was faster than the previous one and had a better DNA yield. It was found suitable

for both PCR, AP-PCR, direct DNA sequencing and the management of a bank of fungal DNA's. The resulting DNA's bank presently includes certified DNA's of about 560 fungal strains prepared according to the second protocol.

2) Assessment of fungal nuclear consensus PCR primers (NS and ITS series)

The universality of the consensus primers for the 18S ribosomal subunit (NS primers) and for the ITS region (ITS primers) was assessed by all participants on the DNA's isolated from 97 species representative of *Absidia , Acremonium, Aspergillus, Candida, Cladosporium , Cryptococcus, Epidermophyton, Fusarium, Geotrichum, Microsporum, Mortierella, Mucor, Paecilomyces, Penicillium, Phialosphora, Pityrosporum, Rhizoctonia., Rhizomucor, Rhizopus, Scopulariopsis, Synecephalastrum , Trichoderma, Trichophyton and Trichosporon.*
A standard high stringency PCR format was validated by all participants. This format was suitable for all strains tested including those producing PCR inhibitors or whose DNA sequence beared secondary structures interfering with the processing of Taq polymerase. Primers of the NS series were working with all fungi tested whereas only ITS5 and ITS4 primers were able to prime the amplification of the ITS ribosomal region in all species tested so far.

3) Analysis of the DNA sequences of the fungal ribosomal genes

Several parts of the fungal ribosomal genes from various fungal genera, species, varieties and types have been directly sequenced and analysed after DNA amplification using consensus primers. Two common protocols for the direct sequencing of PCR products were standardized by the Irish and Danish laboratories and are now used by the other participants.
Due to patent application procedure, complete report will not be possible prior to the final report

4) Arbitrary primed-PCR (AP-PCR) under low stringency PCR conditions and analysis of the generated Amplification Fragment Length Polymorphisms (AFLP) for studies of genetic relatedness between strains of the same species or genus.

When DNA sequencing of some genomic region is considered for microbial phylogenetic studies and DNA probing applications, there is a requirement to ascertain whether a given strain of a given species is representative of the whole population of the strains of the species considered.
Such an important question is most often skipped in the litterature or in gene banks records.
In order to address this question and to reduce the number of isolates that needed to be analysed, a fast screening method had to be used prior to selection, amplification and sequencing of PCR products from specific strains.
Amplification Fragment Length Polymorphisms generated by RAPD or arbitrary primed-PCR (AP-PCR) techniques were prefered to polymorphisms derived from enzymes patterns, western blotting of glycomannanes, probing of repetitive sequences or restriction analysis (RFLP).
The generation of AFLP's was found to be faster, cheaper and more easily standardisable than the others methods and fitted with the already available DNA preparation protocols.
The Belgian laboratory has found that some NS and ITS fungal consensus primers (20 bases) were individually able to prime the generation of AFLP's from fungal DNA under low stringency PCR conditions. From a practical viewpoint, the same

set of laboratory primers could then be used for both PCR and AP-PCR. On basis of that observation, objective criteria and controls were further established to distinguish 'informative' primers from 'non informative' and 'false positive' primers. Using different informative primers, rules to establish a standard AP-PCR format were defined in order to generate reproductable AFLP's and -most importantly-fungal species-, variety- and type-specific AFLP's. At time of reporting, ribosomal consensus primed-PCR has been used to generate a database of 473 strain-specific fingerprints. Preliminary results have been presented at the first BRIDGE sectorial meeting in Wageningen (Dec. 92) Final results are under publication and will be reported at next sectorial meeting (Grenada Oct 93).

HIGHLIGHTS/MILESTONES

The concerted action has provided the opportunity to set up and assess a coherent methodology based on the standardised combination of classical DNA techniques for the following purposes:

1) Description of the genetic relatedness of large populations of strains belonging to the same genus or species
   (Applications: fast automated taxonomical identification, population diversity and structure, process quality control, Culture collection management)

2) Phylogeny based on DNA sequencing of amplified regions of the fungal ribosomal genes
   (Applications: Phylogenetics, Taxonomy, fast identification and validation of ribosomal genes-based DNA probes, probing fungi by PCR, sandwich-PCR, LCR or flow cytometry. DNA probes are destinated to agro-food-fish research and industries, medical and veterinean research and clinical diagnostics, biosafety and intellectual property protection).

Although the methodology was specifically adapted to fungi, similar approaches are reported for animals, plants, insects, bacteria and viruses in the litterature.

WIDER CONSIDERATIONS

1) Call for collaborations

The ITAMOPIA approach is essentially prenormative. Although initially generated with reference to the traditional taxonomic classification, the AFLP, the data of the ribosomal DNA sequencces and of the DNA probes become afterwards independent of any taxonomic or phenotypic criteria: in other words, our data are independent of a continuously evolving taxonomy and can be used as standards.
The ITAMOPIA technology has been designed in such a way allowing its adaptation to the ISO9000 norm.
Such objective still requires further normalisation work on the methodological standards developped- and the data generated- in the course of the concerted action. Therefore, we are willing to diffuse our technology in both academic and industrial areas through scientific and R&D collaborations.

2) Call for European harmonisation

The principle of non patentability of natural DNA sequences is widely accepted in Europe. However, the sharing of DNA sequences *databases* rises new problems because of the added value resulting from the DNA sequence alignment and the intellectual and financial investments represented by DNA sequencing studies.

Local ribosomal DNA sequences databases are growing in several places of Europe whose access is either private, very limited technically or restricted to a small number of selected scientists.
There is thus a need to define a sound European agreement on the sharing of intellectual and financial investments represented by DNA sequences databases destinated to the public domain.
Without an agreement on the access conditions to ribosomal DNA sequences databases within the EC, duplication of efforts and lack of European competitiveness will remain the rules.

COOPERATIVE ACTIVITIES

Contact group meetings: June 92-Madrid, September 92-Brussels, November 92-Copenhagen.

Exchange of certified material: fungal strains and DNA batches, plasmids, DNA sequences.

Internal collaborations: BE + DK + IE for the sequencing of *Aspergillus niger* ribosomal gene, DK + SP for the sequencing of *Rhizoctonia spp.*, BE+ DK for the correlation between AFLP typing and chemotyping of Penicillium spp., BE+SP typing of dsRNA containing *Aspergillus spp.*

External collaborations: to be reported later.

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T-PROJECT

“SEQUENCING OF THE YEAST GENOME”

Sequencing of the yeast genome (BIOT CT-900167)

COORDINATOR:
Université Catholique de Louvain, Louvain-la-Neuve, B
PARTICIPANTS:
(see reports from H. Feldmann and B. Dujon in the following pages)

OBJECTIVES

- Decentralised administration of the European Yeast Genome Sequencing Network: subcontracting the work to 5 DNA coordinators (Chromosomes II, VIII, X, XI and XIV) and 36 sequencing laboratories.
- Initial advance payments to each subcontractor.
- Detailed accounting of the base pairs produced by each sequencing laboratory.
- Subsequent payments on the basis of 2ECU per final base pair sequenced as approved by the DNA and informatic coordinators.
- Subsequent payments of the DNA coordinators according to progress.
- Continuous interaction with each DNA coordinator, the informatic coordinator and the sequencing laboratories.
- Organisation of contractors meetings.

MAJOR PROBLEMS ENCOUNTERED

Insufficient progress was made on the chromosome VIII library and therefore this chromosome is not going to be covered in the framework of the BIOTECH programme (1993-1995: Sequencing of chromosomes VII, X, XIV and XV as well as 740Kb of chromosome IV).

RESULTS

- All subcontracts have been implemented in 1992.
- During the year 1993 all sequencing laboratories and DNA coordinators have received additional advance payment according to the rules mentioned above.
- Two plenary contractor's meeting were held in Munich (October 1992) and Louvain-la-Neuve (April 1993) where progress was assessed and decisions were taken concerning the organisation of verification procedures.

HIGHLIGHTS/MILESTONES

On May 12, 1993 about two years after the start of the BRIDGE programme a total of 1,739,779 from Chr. II and XI have been submitted in final form to MIPS.

WIDER CONSIDERATIONS

The complete sequencing of the entire Chromosomes II (830Kb) and XI(630Kb) (including about 20% of verification sequences) will be finalised before March 1994.

Sequencing of yeast chromosome II (BIOT CT-900167)

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F. FOURY, Université Catholique Louvain, Louvain-la-Neuve, B
(with C. JACQ, Ecole Normale Supérieur, Paris, F)
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F. POHL, Universität Konstanz, Konstanz, D
P. SLONIMSKI, CNRS, Gif-sur-Yvette, F
H.Y. de STEENSMA, University of Leiden, Leiden, NL
K. WOLFE, University of Dublin, Dublin, IRL

OBJECTIVES

In the first round, each contractor received a (non-overlapping) αS288 cosmid clone of chromosome II containing an insert of 30-40 kb of yeast DNA to undertake restriction fine mapping and sequencing, whereby the sequencing strategy was left to his discretion. In the following, extending cosmids should be supplied, whenever the contractor had submitted > 25 kb of final data from his first cosmid. All data (final segments > 5kb) should be entered into the dataset administered by the Martinsried Institute of Protein Sequences (MIPS) under the supervision of the DNA coordinator. A major goal was to build larger contigs from the data obtained by one group as well as by neighbouring groups, and finally to overlap the remaining 'gaps'. The data should be consecutively analysed for the occurrence of genetic entities and other features by MIPS and the results be communicated to the contractors.

MAJOR PROBLEMS ENCOUNTERED

No problems were encountered with the stability and the tractability of the clones. By contract, the sequencing laboratories had committed themselves to submit 25 kb of sequence/year at minimum. This goal has been achieved by only six of the 14 laboratories initially engaged. Fortunately, several new laboratories joined the chromosome II sequencing team in 1992. In part, however, it was necessary to reassign to some of the groups short segments of cosmids that had formerly been distributed to other contractors.

RESULTS

Prior to this project, we had prepared an ordered cosmid library covering yeast chromosome II (strain C836). A set of ca. 40 overlapping clones was needed to assemble a physical map of chromosome II, with an average resolution of 2 kb using the four restriction enzymes, *BamHI*, *SalI*, *XbaI*, and *XhoI*. The physical map of chromosome II was found to be largely co-linear with the genetic map known at that time. On this basis, a cosmid library of strain αS288 was established and clones for chromosome II (830 kb) were selected and mapped. A schematic overview is presented in Figure 1. During the course of sequencing, it was found that the restriction maps of chromosomes II from αS288 and C836 were congruent, except for those regions which have become highly polymorphic through transpositional events.

The submissions of data by the contractors (as of March 1993) is documented in Table 1. A total of 610 kb of unique sequence was obtained; several larger contigs could be built from overlapping sequences.

Analysis of the sequence data was carried out by MIPS in collaboration with the DNA coordinator. The results are schematically listed in Figure 2. To date, 424 open reading frames have been encountered including 12 genes for tRNAs, several singular delta, sigma and tau elements, a complete Ty2 and two Ty1 elements. Of the total ORFs, 66 are internal to other ORFs and may not represent functional genes. Of the remaining 358 ORFs, 71 are representing known yeast genes, 53 showed high similarity (significant FASTA scores) with known genes from yeast or other organisms, and 28 had a FASTA score < 200. The rest of the ORFs did not reveal any similarity with known functions.

HIGHLIGHTS / MILESTONES

The analysis of the larger contigs allows to conclude that the genetic information on yeast chromosome II is tightly packed: on the average, one genetic entity is encountered every 1.8 kb. Similar figures have been obtained for yeast chromosomes III and XI. The average GC-content of chromosome II is extrapolated to be 38.7 %. This figure is similar to the one obtained from total chromosome III (38.5%). Normally, the coding regions on chromosome II exhibit a higher GC-content (up to 46.9%) than the non-coding regions (as low as 27.8%); this seems to be a general rule for the yeast genome.

Cosmids covering the whole chromosome II have been distributed, except for the rightmost telomeric region. If the sequencing work is progressing well, the complete sequence of chromosome II should be available before the end of 1993. The following predictions can be made by extrapolating the results of the current analyses.

Altogether, some 460 open reading frames are expected on chromosome II. About 20% of these should correspond to genes which are already known to reside on chromosome II, and another 15% should exhibit significant similarity with other yeast genes or known genes from other organsims, so that putative functions may be attributable to them. About two thirds of the ORFs on chromosome II will remain truly 'novel' genes.

A detailed documentation has been set up that comprises any information collected during the analysis of the data (such as results of data base searches, comparative screenings, cross-references, etc.). It is intended to finally incorporate this information into a relational yeast data base.

We have also tried to group the putative genes occurring on chromosome II by function or other criteria, but no rules became obvious at present. However, data

base searches indicated that similar or 'redundant' copies of genetic entities do occur within the yeast genome. In several cases it appears that these are simply 'duplications' of particular functions. Particularly on chromosome II, two regions are apparent which seem to harbour similar sets of related genes. From our own work, we have additional proof that also multigene families with functionally redundant members are existent in yeast. These points have to be considered during functional analyses involving gene disruptions.

WIDER CONSIDERATIONS

The complete sequences of yeast chromosomes II and XI will render information on the genomic organisation and the structures of more than 800 genes. Together with the 1500 or so sequences in the databanks and data from activities outside the EC, the repertoire of known yeast genes should amount to some 2300-2500 by the end of 1993. We are confident that the final goal of the yeast genome project can be reached within a reasonable period of time, thus establishing the entire DNA sequence of this eukaryotic organism with a coding capacity for some 7000-8000 different functions. This will contribute a wealth of information to understand the molecular biology of a living cell and, at the same time, be of benefit for any other genome project. There is accumulating evidence that all of the basic functions, such as enzymes, structural proteins and regulatory factors, in yeast share high similarity with their counterparts in higher organisms. In a large variety of cases, it has already been possible to functionally exchange such entities between yeast and other species. In fact, yeast offers a unique opportunity to study functional aspects by concomitant genetic and biochemical approaches. We therefore feel it timely to start appropriate programmes along these lines. Furthermore, techniques experienced in the yeast genome project will be of invaluable help in other genome projects. At the same time, yeast can be used to clone and to propagate large pieces of genetic material from other organisms. Finally, it should be emphasized that yeast is of outstanding industrial importance, both as a source of interesting novel protein products as well as a potent instrumental system in biotechnology.

COOPERATIVE ACTIVITIES

In general, interaction between the contractors, the coordinator, and MIPS was good; communication and feedback was mainly achieved through E-Mail or Fax. In addition to the cosmids, the contractors received probes of the vector and the following information material:

(1) a continuously updated map of chromosome II;
(2) a continuously updated list of all elements genetically mapped and physically characterized on chromosome II;
(3) catalogues of chromosome II sequences, special sequence entries for Ty-derived elements and tRNA genes;
(4) documentation on yeast regulatory proteins and sequences.

A meeting for all contractors of chromosomes II and XI was organized by MIPS in collaboration with the Yeast Industrial Platform (YIP) in Munich, October 18-20, 1992, where any relevant information concerning the project was exchanged and future strategies were discussed.

Sequencing of yeast chromosome XI (BIOT CT-900167)

COORDINATOR:
Bernard DUJON, Institut Pasteur, Paris, F
PARTICIPANTS:
W. ANSORGE, EMBL, Heidelberg, D
B. DUJON, Institut Pasteur, Paris, F
M. BOLOTIN-FUKUHARA, Univ. of Paris XI, Orsay, F
F. FOURY, U.C. Louvain, Louvain-la-Neuve, B
H. FUKUHARA, Institut Curie, Orsay, F
F. HILGER, Fac. sc. agro., Gembloux, B
C. HOLLENBERG, Univ. of Düsseldorf, Düsseldorf, D
J.C. JAUNIAUX, U.L.B. Bruxelles, B
A. JIMENEZ, Universidad Autonoma, Madrid, E
S. OLIVER, University of Manchester, Manchester, UK
P. PHILIPPSEN, Universität Gießen, Gießen, D
R.J. PLANTA, Vrije Universiteit, Amsterdam, NL
F. POHL, Universität Konstanz, Konstanz, D
M. RIEGER, Biot. M. Bio. Forsch., Wilhelmsfeld, D
C. RODRIGUES, Instituto Gulbenkian, Oeiras, P
G. THIREOS, IMBB, Iraklio, GR
D. VON WETTSTEIN, Carlsberg Lab., København, DK

OBJECTIVES

Construction of a library of overlapping clones to cover the entire yeast chromosome XI as a unique contig. Sorting of clones and construction of a high resolution ('sequence-ready') physical map of chromosome XI. Distribution of clones to participants for sequencing until the entire chromosome is finished. Application of 'quality controls' to the sequences determined by the participants. Assembly of the complete sequence of chromosome XI from the set of overlapping clones and interpretation of the sequence.

MAJOR PROBLEMS ENCOUNTERED

The responsibility of each participant in terms of the quality and minimal length of sequences submitted to MIPS was not sufficiently well defined at the beginning of this work, resulting in too many unecessary submissions of partial or preliminary results. To solve this problem, operating rules, agreed upon by all participants, were defined during the program. They may help operate subsequent european programs of systematic sequencing which involve large numbers of laboratories.

RESULTS

Coordination:

Three cosmid libraries covering, altogether, 380 times the yeast genome, have been constructed and stored @ −80°C. They have been sorted in parallel for chromosomes XI and X, by hybridization. As judged from these two chromosomes, the libraries are complete and allowed the isolation of the entire chromosomes as single contigs. The average insert size is 35 kb.
The set of chromosome XI-sorted cosmids has been mapped by four standard methods (fingerprinting, terminal RNA probe hybridizations, EcoRI digestions, cosmid to cosmid cross hybridizations) and by systematic application of a new

method (the nested chromosome fragmentation) developped for this purpose (see below under *Mapping strategies*).

The physical map has a 3 kb average resolution and shows complete internal consistency. A total of 30 partially overlapping cosmids, or fragments thereof, was distributed to the contractors together with their precise map location and indications of the segments assigned for sequencing. Colinearity between inserts of distributed cosmids and the native chromosome XI itself has been checked by restriction mapping and hybridization. Altogether, the material presently distributed covers the entire chromosome (ca. 665 kb).

The very end of the chromosome, which contain the $(C_{1-3}A)_n$ telomeric repeats, have been amplified by PCR from total yeast DNA, using an oligonucleotide made of $(C_{1-3}A)_n$ repeats, and specific oligonucleotides synthesized from the terminal-most available sequences.

Original Sequencing: Each participant has the choice of its sequencing strategy, sequencing methods and internal organization of his/her own sequencing team. All participants have received the overall plan of the distribution of tasks as well as the EcoRI map of the entire chromosome. Participants with neighbouring or overlapping fragments have been individually informed. The table given in annex shows contribution of each participant, calculated as the number of base pair of final sequence submitted to MIPS as of March 26th, 1993. Most participants have sent detailed strategy protocols and quality statements to the coordinator.

Finalization of sequencing: At the Munich meeting in october 1993, when 80 % of the entire chromosome sequence was already available, a round table discussion with all participants has permitted to examine gaps, uncertainties or problems and to share the remaining task between labs.

Sequence verification: As soon as all chromosome fragments were distributed and participants were available for additional sequencing work, DNA material was distributed for sequence verification. This consisted of: *(i)* a set of 1100 random clones from a shotgun cloning of the entire chromosome (distributed to 6 labs as a pilote experiment); *(ii)* 8 fragments (ranging in size from ca. 3 to 6 kb) chosen by the DNA coordinator and distributed anonymously to 4 labs for resequencing; *(iii)* a set of 60 oligonucleotide pairs to serve as primers to redetermine the sequence of putatively uncertain regions as judged by the DNA coordinator upon examination of the entire chromosome sequence.

Sequence quality: From a total of 59,804 bp (8.9 % of total chromosome) that were determined twice from two different laboratories having partially overlapping cosmids, a discrepancy of 0.023 % was found. From a total of 18,778 bp (2.8 % of total chromosome) resequenced from the random clones, a discrepancy of 0.005 % was found. From a total of 11,616 bp (1.8 % of total chromosome) submitted to deliberate resequencing, a discrepancy of 0.08 % was found.

HIGHLIGHTS / MILESTONES

Cosmid libraries: Three cosmid libraries covering, altogether 380 times the yeast genome, have been constructed and stored, and have now been used for the continuation of the european sequencing projects on other yeast chromosomes (X, XIV, VII, XV).

Mapping strategies: A new mapping strategy with potential application to the entire yeast genome, as well as to mapping YAC inserts, has been developed on chromosome XI. It is based on the possibility to cleave total yeast genome at a

single artificial site using the new endonuclease I-*Sce* I. A set of transgenic yeast strains with artificial I-*Sce*-I sites is constructed and used to sort cosmid libraries and build physical map using nested chromosomal fragments as probes. This method has now been applied to other yeast chromosomes for continuation of the european sequencing projects. It permits to purify any subfragment of yeast chromosomes, as desired.

Physical map of chromosome XI:
The complete EcoRI maps of chromosome XI with a 3 kb average resolution shows complete internal consistency. Placing genetic markers on the physical map by hybridization and sequencing, reveals that the published genetic map used so far by all yeast researchers contains major inversions/ translocations in the left arm of chromosome XI.

Sequence analysis:
With the exception of the very ends of the chromosome (1.4 and 0.5 kb, respectively), all sequences from chromosome XI have now been determined and can be unambiguously assembled. More than 95 % of the entire chromosome sequence has been submitted to MIPS and analyzed with respect to open reading frames, presence of tRNAs, delta sequences, Ty elements or consensus regulatory signals.

WIDER CONSIDERATIONS

The coordinated effort of 17 labs from 9 different member states of the E.C. will have resulted in completing the sequence of a new yeast chromosome (665 kb long) within a little more than two years. This is the second yeast chromosome to be entirely sequenced, and so far the second eucaryotic chromosome ever sequenced. It is roughly 2 times larger than the chromosome III, published in 1992. Work on chromosome XI has helped to launch new strategies for efficient and reliable cooperation in a large network of laboratories.

More than 300 putative protein coding genes have already been discovered (excluding last submissions), among which only 24 % were already known from classical genetic and molecular studies, 17 % have significant homologs in data bases and the remaining 59 % have either no homologs or only borderline cases of homology, hence represent new types of genes, not discovered before in any other organism and not belonging to any of the known protein families.

COOPERATIVE ACTIVITIES

Plenary meeting: Second BRIDGE meeting at Munich (DE) September 18-20th, 1992. All participants of the sequencing program for yeast chromosomes XI and II.

Other meetings at which participants presented results of the present program:
XVIth International Conference on Yeast Genetics and Molecular Biology, Vienna, 15-21st August 1992 (Members of following laboratories: Ansorge, Dujon, Foury, Hilger, Hollenberg, Philippsen, Planta)
Staden XDAP course (Harrow, GB, 13-14 th april 1992) (Members of Dujon's lab and Foury's lab)
Genome mapping and sequencing Meeting, Cold Spring Harbor (New York) 6-10 May 1992 (Dujon)
Second Nordic Hugo Workshop 14-16 may 1992 Oslo, Norway (Dujon)
FASEB Summer Research Conference, Snowmass (Colorado) 5-10 july 1992, (Dujon)

IV Portuguese-Spanish Biochemistry Congress 29/9 - 2/10, 1992, Povoa de Varzim (PL) (Members of Rodrigues-Pousada's lab)
Biocenter Symposium, Basel, CH, 15/10/92. (Philippsen)
EMBO DNA Sequencing Course November 1992, Heidelberg (DE) (Members of Ansorge'lab and Dujon)
ABI user group meeting February 1993, Oslo, Norway (Member of Von Wettstein's lab)
ABI user group meeting of UK. (Member of Oliver's lab).

Information exchanged:
Several reports with ongoing results were prepared for YIP. Newsletters with maps, protocols, progress reports and other relevant data were sent to all participants.

EMBO Sequencing course manual prepared by Ansorge's lab available on request (sent to Thireos's lab and Foury's lab). Protocol for sequencing directly on cosmids prepared by Dujon's lab available on request (sent to Foury's lab). Protocols for automated sequencing on ABI373A prepared by K. Wolfe and T. Hawkins (Cambridge, GB) sent to Oliver's lab. Mapping procedures prepared by P. Hieter (Baltimore, MD) and R. Davis (Stanford, CA) sent to Philippsen's laboratory.

Unpublished sequence data were exchanged between the following labs: Ansorge - Thireos; Bolotin-Fukuhara - Oliver; Dujon - Pohl; Hilger - Hollenberg; Oliver - Von Wettstein; Oliver - Pohl; Planta - Bussey (Québec); Planta - Gibbons (Hawaii); Phillipsen - Pohl (this list excludes sequences sent by participants to the DNA coordinator).

Material exchanged: (this list excludes distribution of DNA by the DNA coordinator to participants).
Yeast DNA shotgun library prepared by Dujon's lab sent to Philippsen's lab.
Set of cosmid clones covering chromosome XI sent by Dujon's lab to N. Kleckner'lab (Harvard, Massachusetts). Set of isogenic transgenic yeasts with I-Sce I sites sent by B. Dujon's lab to E. Louis (Oxford, GB). Set of isogenic series of yesat strains sent by Dujon's lab to Bolotin-Fukuhara, Foury, Hilger and F. Lacroute (Gif sur Yvette, FR). Cosmid pEKG119 sent by Foury's lab to J.M. François (Toulouse, FR). Part of cosmid pEKG121 sent by Oliver's lab to Dujon and Hilger. Part of cosmid pEKG080 sent by Von Wettstein's lab to Oliver. Shotgun library of yeast sent by Pohl's lab to Philippsen. Part of cosmid pEKG011 sent by Rieger to members of DKFZ and Molecular Genetics Center (Heidelberg, DE). Part of cosmid pEKG019 sent by Rodrigues to Slonimski (Gif sur Yvette, FR) and Fukuhara.

Staff exchange:
M. Haaseman and I. Becker from MIPS visited Dujon's lab (August 26-28th, 1992) for coordination of sequence analysis
B. Purnelle (Foury's lab) visited Jauniaux's lab for 3 weeks to learn and import ALF technique, shotgun strategy and software utilisation.
J.C. Jauniaux visited several times Foury'lab for discussions about DNA sequencing strategy and software analysis.
Member of M. Tuites' lab (Kent, GB) has visited Oliver's lab for production of DNA.
L. Gaillon (Dujon's lab) visited Grivell's lab (Amsterdam, NL in october 1992, for software utilisation).
J.C. van Vliet Reedijk (Planta's lab) attended EMBO course on DNA sequencing

(Heidelberg, DE, Nov. 13-23, 1992) and visited Unilever Res. Lab. (Vlaardingen, NL, 11 nov. to 24 dec., 1992) and Slonimski's lab (Gif-sur-Yvette, FR, 1 jan. to 25 feb. 1993).
T. Pohl visited Philippsen' lab in dec. 1992.
B. Dujon and collaborators (L. Gaillon and A. Thierry) visited MIPS (Martinsried, DE) March 6-11th, 1993 for assembly of entire chromosome sequence and coordination of publication of the work.
H. Tettelin (Foury's lab) works in Dujon's lab since october 1992 for mapping new yeast chromosomes and sorting the cosmid libraries.
P. Philippsen and a member of his lab (R. Pöhlmann) visited D. Düsterhöft (Düsseldorf, DE) in february 1993 for finalizing sequence data.

EUROPEAN DIMENSION

This second phase of the european yeast sequencing network of laboratories has largely benefited from experience with the first phase, devoted from chromosome III. In particular, the decision to built new recombinant libraries, sort them and to construct high resolution physical maps prior to sequencing has been of tremendous help to coordinate the activities of the network, redistribute materials when needed and assemble the results. It is expected that experience of this phase will also help operate subsequent phases, in particular with respect to more precisely defined responsibility of each member of the network and to application of quality controls over the common work.

Exchange of staff, materials, techniques or information between the various laboratories of the network has generally been good but could probably be improved in the future for the benefit of all. Communications between the participants and the coordinator have generally been very good and have improved with time, as responsibility became better defined.

Unequal achievements and working efficiencies still exist between laboratories but tend to diminish, as the work is progressing. With its network structure, the european effort is unique in many respects, not the least of which being the educational part, and has created an impetus for participating to the next phases.

LIST OF JOINT PUBLICATIONS / PATENTS WITH TRANS-NATIONAL AUTHORSHIP

D. Eshel, L.A. Urrestarazu, S. Vissers, J.C. Jauniaux, J.C. van Vliet-Reedijk, R.J. Planta, and I.R. Gibbons (1993) Nature (submitted).
General publications of the entire chromosome sequence and interpretation (in preparation).

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B. Dujon (1992) Current Biology **2**, 279-281
A. Düsterhöft and P. Philippsen (1992) YEAST, **8**, 749-759.
A. Thierry and B. Dujon (1992) Nuc. Acids Res. 20, 5625-5631
B. Purnelle, J. Skala, L. Van Dyck, and A. Goffeau (1992) YEAST **8**, 977-986
S. Pascolo, M. Ghazvini, J. Boyer, L. Colleaux, A. Thierry, B. Dujon (1992) YEAST **8**, 987-995
J. Boyer, S. Pascolo, G-F. Richard, B. Dujon (1993) YEAST (in press)
C. M. James, M. E. Gent, K.I. Indge, and S.G. Oliver (1993) YEAST (in press)
S. Wiemann, H. Voss, C. Schwager, T. Rupp, J. Stegemann, J. Zimmermann, H. Erfle, N. Hewitt and W. Ansorge (1993) YEAST (submitted)
C. Pallier, M. Valens, V. Puzos, H. Fukuhara, G. Chéret, F. Sor, M. Bolotin-Fukuhara (1993) YEAST (submitted)
G. Chéret, C. Pallier, B. Daignan-Fornier, H Fukuhara, M. Bolotin-Fukuhara and F. Sor (1993) YEAST (submitted)

Informatics network (sequencing the yeast genome) (BIOT CT-900161)

COORDINATOR:
H.W. MEWES, Max-Planck-Institut für Biochemie, Martinsried, D

OBJECTIVES

The overall objective of this project is to provide informatics support for the collection, organisation and analysis of data from the sequencing of the yeast genome in the framework of the BRIDE programme. MIPS actively supported the sequencing of yeast chromosomes II and XI. The objectives met for the ongoing period have been fully met.

RESULTS

As the informatics coordinator, MIPS has been responsible for the collection, storage and analysis of all sequence data submitted by the 33 laboratories involved in sequencing chromosomes II and XI (620 and 830 kb, respectively). As of April 1993, 1.2 Mbases of non-redundant DNA have been submitted to MIPS, both chromosomes are near completion and expected to be published within the year 1993.

The work involved close collaboration with the DNA coordinators (B. Dujon, Paris and H. Feldmann, Munich) for data analysis and quality control. The analyses were performed using the PIR-NBRF Experimental Query System (ATLAS/XQS) and the Genetics Computer Group (GCG) Sequence Analysis Software, as well as several programs for sensitive sequence comparisons obtained from the EMBL file server. Preliminary sequence data were also sent to MIPS; they provided valuable information about clone orientation and overlapping regions, and helped in the rapid identification of known genes. Detailed plots displaying the clones used in sequencing, all open reading frames as well as previously mapped genes, and other elements (Ty, δ, tRNA) were constructed. All sequences were inspected for consistency with the physical map, detection of frameshifts, and verification of overlapping sequences.

Homology searches were carried out at the nucleotide level in order to detect possible vector contaminations, indication of non-coding regions of significance (tRNAs, δ and Ty elements) and regulatory sequences like splicing signals, promotors and enhancers.

A total of 660 putative open reading frames have been yet identified. 136 are known yeast genes (20%), 107 (16%) showed significant homologies to published sequences whereas 293 (44%) did not reveal any significant similarity to known protein sequences. The rest displays low milarities requiring more detailed analysis to uncover possible functions.

Data obtained from overlapping regions allow an estimate of the sequencing accuracy. In chromosome XI a random library (SER-clones) was generated to sequence short fragments inserted in the pBluescript vector. The error rate of the overlaps is 0.023% (in 59 kb), 0.09% discrepancies have been observed in comparison with the SER sequences (18 kb).

A database of sequence similarities (FASTA-database) has been developed and set up to allow the comparison to the latest sequences in the public data banks. The system allows the inclusion of the yet unpublished, confidential sequences. The matrix of sequence similarities is continuously updated to include all sequence data available through the MIPS database. Only similarity scores and sequence length are displayed, the sequence itself remains hidden and confidential.

Regulary summaries have been forwarded to the contributing laboratories and the yeast industry platform (YIP). These reports mirror the progress of the sequencing work.

An on-line computing facility provided by MIPS enables the participating laboratories to analyze their data and perform database queries.

Summary of sequence data processing

Any new sequence submission to MIPS is compared to previous submissions of the same contractor in order to find the overlaps needed to assemble the contig for the particular region. Restriction fragment data of the resulting contig are compared with the physical map provided by the DNA coordinator for consistency. Subsequently the new sequence is compared to the adjacent sequences to detect overlaps for the stepwise assembly of the entire chromosome.

On the DNA level systematic searches are performed to detect:

— known yeast sequences
— rRNA genes
— Ty elements
— regulatory elements (UAD, ARS, promotors, repeats, etc.)
— introns

Open reading frames (ORFs) are extracted with a cutoff value of 100 amino acids, correlated to regulatory elements and examined for codon usage and codon adaptation index to assess the probability of expression.

Hypothetical proteins translated from putative open reading frames are further analyzed by similarity comparison against the most recent version of the protein sequence database. Proteins are classified in three categories: strongly homologous, possibly related, and unrelated proteins (orphans or EEC proteins). Strong homologs may be published yeast proteins with known function or homolog proteins that allow an enambigous functional asignment by family classification. Weak similarities are analyzed in more detail with sensitive sequence comparison programs (e.g. ISSC Interactive Sensitive Sequence Comparison, Algorithm, P. Argos, EMBL). Pattern searches are performed against the ProSite Dictrionary of Protein Sties and Patterns (compiled by A. Bairoch, Geneva). The ORFs are also inspected for internal repeats, putative trans-membrane segments etc. Local homologies to other evolutionary related proteins are analyzed with the BLAST program by Altschul et al.

Work in progress

MIPS is currently developing a user informations system for yeast chromosome data. Existing software (ACEDB), developed for the C. elegans project, will be used to provide a suitable medium for data distribution and access. Sequence similarities of open reading frames will be updated dynamically through the FASTA database.

HIGHLIGHTS / MILESTONES

Up to March 1993, 630 kb (95% of the entire chromosome) have been submitted from chromosome XI; 577 kb were received from chromosome II. We have analyzed all putative open reading frames longer than 100 amino acids by comparison to the latest available sequence databanks. Results including matches within the confidential database have been forwarded to laboratories. With the help of careful sequence analysis, sequencing errors have been corrected and a number of problems has been solved.

In collaboration with the protein sequence databank group, MIPS prepares annotated, merged protein sequence databank entries for all ORF's found on the chromosomal DNA sequence. These entries will be integrated into the protein sequence database at the time of publication.

WIDER CONSIDERATIONS

A current estimate expects yeast to convey approximately 6800 open reading frames (Goffeau et al., Yeast (1993), in the prtess). Nearly 1000 of those have been discovered by the EEC yeast sequencing programme. In case of about half of the hypothetical proteins found no functional characterization is possible with the help of sequence comparison methods.

The current sequence data flow processed by MIPS increased 5fold since the publication of chromosome III (from ca. 100 kb to 500 kb/year). This data turnover rate requires efficient standardization of data processing procedures to cope with the increased workload. Electronic communication and data exchange is an absolute requirement for the successful completion of the project. Bandwith and connectivity of European academic networks are still lacking behind US standards. Efforts of other EC projects in the BRIDGE programme to improve the situation ought to be supported (e.g. EMBnet).

COOPERATIVE ACTIVITIES

The coordination of the informatics support and evaluation of the data is a highly interactive process. Frequent communication with each of the laboratories by all telecommunication, meetings and visits with the DNA-coordinators. Also the ongoing A. thaliana sequencing project started to collaborate with the yeast informatics coordinator.

LIST OF JOINT PUBLICATIONS

A detailed report for the Yeast Industry platform is issued quarterly. 'Chromosome' Newsletters were distributed to the contractors in collaboration with the DNA-coordinators. Also the final complete sequence of chromosome III was published in the report period (Oliver et al., Nature (1992), **357**: 38-46).

T-PROJECT

“IDENTIFICATION OF NEW PLANT GENES”

Identification of new plant genes (BIOT CT-900207)

COORDINATOR:
M. BEVAN, AFRC, Norwich, UK
PARTICIPANTS, OBJECTIVES, RESULTS
see in the following pages

ABSTRACT

The second year of the BRIDGE T Project 'Molecular Identification of New Plant Genes' has seen remarkable progress in several areas. The sharing of activities by groups to solve problems not approachable by the efforts of smaller labs was one of the principal goals foreseen in T programmes, and it is pleasing to see networks of labs in the T projects achieving their joint goals. This was evident from the second meeting of the Programme in Gent in Sept 1992, where we were fortunate enough to meet several members of the Plant Industrial Platform. Thanks to the efforts of Arlette Uytterhaegen, Marc van Montagu and colleagues the meeting not only acheived the necessary exchange of data and views, but gave us all a glimse of life in the Mecca of plant sciences, and of the many culinary and bibulous delights of that fine city. Major accomplishments of the Programme in its second year include the excellent function of the Nottingham Arabidopsis Stock Centre (NASC), which has more than fulfilled everyone's expectations. Thanks to the dedication of the Director, Mary Anderson, and Bernie Mulligan, NASC is the first place one thinks of for advice and seed stocks, and for depositing interesting mutants etc. Moreover, it provided a world-wide service while its sister organisation in Ohio was being set up. It has dispatched more than 6000 samples, and has notably incorporated recombinant inbred lines and become the Strain Curator for AAtDB. But obtaining funding over a longer period is now a major challenge. Despite the relatively modest resources allocated to Physical Mapping, all major goals have been achieved by Caroline Dean and her group, with 80% coverage of chr. IV and 50% of the top of chr.V. The international collaboration devoted to establishing a complete physical map has undergone some changes of emphasis and participants, and one wonders why such an important topic has not been the focus of more extensive funding as it has been in several other organisms, notably rice and wheat. Another major achievement has been the arrangement of YACs in a 3MB contig on the top of chr.IV. The forthcoming ESSA sequencing project aims to scqucncc half of this region. Future goals include mapping a large number of new markers to complete the alignment of YACs. It is predicted that a complete physical map of chr. 4 and 5 will be available in 1-2 years. The collaboration of Chris Cobbett, Brian Hauge and Howard Goodman has been invaluable in establishing cosmid contigs for sequencing. The third year of BRIDGE sees two new participants, Marc Zabeau from Keygene in the Netherlands who will make a megaYAC library, and Tony Kavanagh from Trinity College Dublin who will make a P1 phage libary. These libraries will be a valuable resource for gene isolation and large-scale sequencing. Transposon tagging of genes is now a viable technique; the reports of George Coupland describing a two element system based on Ac and the report of Andy Pereira on En are the culmination of large efforts which have provided the plant community with a powerful and versatile new way of isolating genes. In the next year emphasis will be placed on increasing the number of lines with mapped launching pads, and exploiting the large number of interesting mutants arising from the screens. Close involvement with the PIP in screening these lines could be mutually beneficial. The Flowering programme epitomises the

value of networks espoused by BRIDGE. Isolation and characterisation of genes conditioning flowering time and vernalisation responses are progressing well, with at least 3-4 genes having been mapped and corresponding cosmid and YAC clones identified that carry the genes of interest. The coming year will see the molecular characterisation of several of these genes.

Work on seed development has been highlighted by the isolation of ABI3, mutants of which confer extreme abscisic acid insensitivity. Work by Jerome Giraudat and colleages, in a text-book example of map-based cloning, identified and sequenced the gene. Interestingly, it has a good deal of similarity to the VP1 gene of maize which integrates the activity of different sets of genes during maize seed development. It is likely that the ABI1 gene will also be isolated in the coming year. Work in Maarten Koornneef's lab. continues to identify new mutations effecting seed dormancy and shape, which will provide a rich source of variation for those interested in isolating the genes involved. Work on embryogenesis, using enhancer trapping T-DNA constructs, has identified a range of inserts which show different patterns of seed-specific gene expression. The regions adjacent to the GUS gene marker in these transformants are being cloned and it is possible that the genes involved will be isolated in the coming year. A search for mutations caused by the T-DNA insertions is underway. Work on gene replacement was never thought to be straightforward, and work in this by BRIDGE participants show how remote this goal remains. Several promising approaches have proved to be futile, and even past successes have been subsequently shown to be incorrectly identified. Clearly some focus has to be brought to bear on the remainder of the programme if it is to achieve worthwhile advances. Important areas include the identification of new and effective negative selection agents, and establishing the effect of increased DNA homology on the frequency of site-specific integration.The ESSA programme, aimed at large-scale sequencing, is due to start in June pending the outcome of Contract Negotiation with the CEC. A number of participants in BRIDGE are also in ESSA, and the Physical Mapping programme of Caroline Dean in BRIDGE will provide a contig of 1.5Mb of cosmids for sequencing by a group of 6 labs. The data will be amalgamated, analysed and annotated at MIPS in Martinsried, who also provide similar services to the yeast sequencing programmes.

Finally, the third and final BRIDGE meeting will be held concurrently with the first ESSA meeting in Cambridge on the 26-29 July, 1994.

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Flowering

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DNA RESOURCE CENTRE

J. DANGL, Max-Planck-Laboratorium, Köln, D (participant No. 7)

OBJECTIVES FOR THE REPORTING YEAR

Continue to accumulate clones and libraries relevant to the needs of the EC *Arabidopsis* community.

MAJOR PROBLEMS ENCOUNTERED

These goals have been met, but not without a certain level of frustration with the lack of enthusiasm from the BRIDGE program participants it is meant to serve.

RESULTS

The following phage libraries are being distributed:
Genomic banks From ecotype Col-0 (2 sources, in different vectors), from ecotype Landsberg-*erecta*, cDNA banks from mature, sterile seedlings and from aerial tissue (no roots), and from flowers. We also distribute the 96 cosmid clones which form the basis of the Goodman Laboratory RFLP map.

In the current reporting period, we have filled the following requests for our materials:

Phage RFLP marker	4 x (2 to BRIDGE participants)
Cosmid RFLP marker	5 x (2 to BRIDGE participants)
Genomic libraries	12 x (3 to BRIDGE participants)
cDNA libraries	14 x (2 to BRIDGE participants)
YAC libraries	4 x (None to BRIDGE participants)

PHYSICAL MAPPING

C. DEAN, R. SCHMIDT and J. WEST, John Innes Centre, Norwich, UK (participant No. 9)

OBJECTIVES

1. Maintain data flow to international database.
2. Augmentation of YAC contigs by Goodman's cosmid library
3. Explore relationship between physical and genetic distances and gross architecture of the genome.
4. Look at telomere and centromere structures.
5. Transfer YAC collections to gene resource centre.

RESULTS

In order to join the YAC contigs identified by hybridization of the RFLP markers, as described in last years report, we have continued chromosome walking experiments, focusing on a 15cM region of chromosome 4. To date this region is covered by four large YAC contigs (involving more than 100 clones) covering >3Mb (Fig. 1). Rather than continue with global chromosome walking experiments, we are co-ordinating the mapping of a large number of new markers.

This objective was made much easier because of the completion of 300 recombinant inbred lines derived from a cross between Landsberg *erecta* and Columbia. The inbreds were selfed to F8, and F10 bulked seed is now available (and is housed at the Nottingham stock centre). We mapped 63 markers (31 and 32 from the Meyerowitz and Goodman RFLP maps respectively) onto 100 of these lines (which we are using as our basic mapping population). The RI lines are being distributed to as many laboratories as possible and new segregation data is being compiled on a central RI database in Norwich. All new markers mapping on chromosome 4 (we have now extended our initial objective and are doing the whole of chr4 rather than just the top half) and the top half of chromosome 5 have been hybridized to the YAC libraries. The collaborations which should add a large number of new markers onto the RI map in the coming year are with: Dr. Bob Whittier (Mitsui Corp., Japan) who has mapped 120 random Pst genomic clones; Dr. Joe Ecker (Penn. State) who is mapping microsatellite markers; Dr. Michel Caboche and colleagues of the CNRS sequencing initiative who are mapping 140 sequenced cDNA clones; Dr. Marc Zabeau (our Physical Mapping collaborator at Keygene) who are mapping a large number of AFLP markers (amplified fragment length polymorphism); and alterations closely linked to T-DNA was analyzed in detail. A recessive mutation in this line causes a leaky fusca phenotype that is associated with loss of osmotic tolerance, increased synthesis of anthocyanins and reduction of the rate of root elongation. The mutant locus was shown to contain a trimeric T-DNA insert, and preliminary data indicate that the tagged locus encodes a protein that shows sequence homology with the ß-subunit of trimeric G-proteins.

COLLABORATIONS

Over 25 laboratories screened so far the T-DNA tagged material in Köln. A joint EMBO-EEC training course was organized in 1992 on Advanced *Arabidopsis* Genetics in Köln with teachers from USA and Europe, and students from 10 European countries.

HIGHLIGHTS/MILESTONES

Characterized material of T-DNA tagged lines is provided for distribution to the Resource Centre. This material is being increased by the third year and beyond, as the genetic analysis proceeds.

CHARACTERIZATION OF AN ACTIVATOR/ DISSOCIATION (AC/DS) TRANSPOSABLE ELEMENT SYSTEM IN *ARABIDOPSIS THALIANA*

R. MASTERSON, Max-Planck-Institut für Züchtungsforschung, Köln, D (participant No. 23)

OBJECTIVES SET FOR THE REPORTING PERIOD

Establish conditions for the detection of germinal transposition events using plant resistance markers that allow the simultaneous detection of *DsDHFR* elements that have excised (NPTII; kanamycin resistance) and reinserted (DHFR; methotrexate resistance). Carry out large scale screening for *Ds*-related mutants.

RESULTS

We have previously shown that the over-expression of full length (pB-Ac101; ATG-1) and truncated (pB-Ac102; ATG-3) CaMV-Ac transposase fusions resulted in a minimal germinal transposition frequency of 27%. The use of a further truncated CaMV-Ac fusion (pB-Ac103; ATG-10) produced similar results. Approximately the same germinal frequency was obtained with a marked *Ds* element, *DsDHFR*, which contains a CaMV-DHFR gene fusion, and an unmarked *Ds* element termed *Ds35S-1*. This is a novel element that contains a CaMV 35S promoter located near the 5' end of the element, such that upon insertion of the *Ds35S-1* element into, or nearby, transcribed regions it is capable of resulting in over and anti-sense expression. Large scale screening of F2 and F3 generation progeny of *Ac/DsDHFR* and *Ac/Ds35S* crosses have led to the identification of several mutants, but a *Ds35S* insertion has yet to be identified that cosegregates with a mutant phenotype.

TRANSPOSON TAGGING IN *ARABIDOPSIS THALIANA*

M. van MONTAGU, Universiteit Gent, Gent, B (participant No. 36)

OBJECTIVES SET FOR THE REPORTING PERIOD

1. To analyze the distribution of reinserted elements.
2. Use pollen specific promoters to express transposons.

RESULTS

Around one hundred transgenic lines containing one or more *Ds* elements have been generated and partially analyzed. The *Ds* element contains the phosphinotricin acetyltransferase gene and was cloned in the 5'leader sequence of a chimeric NptII gene as an excision marker. *Ac* cDNA constructs under the control of the strong *Agrobacterium* Tr2' promoter were transformed into *Ds* containing lines. For fifteen of these we tested *Ds* transactivation by *Ac* in *in vitro* assays; large amounts of calli were screened for kanamycin resistance. At present we are mapping both *Ac* and *Ds* in eight of these double transformants. We are mapping a large number of *Ds* transformants with the final goal to obtain a set of lines that have *Ds* inserts spread out throughout the genome. We have developed a number of transformants that harbour an autonomous *Ac* element, with which we might be able to increase the efficiency of transposon tagging.

HIGHLIGHTS / MILESTONES

The transposon mutagenesis system which we started to develop last year has been optimised further. We now have both autonomous *Ac* and two-element lines that show a high frequency of excision and integration events. With these we hope to be able to select for insertion mutation events which will lead to the isolation of genes of interest in the near future.

ENHANCER DETECTION WITH TEN EN-I TRANSPOSABLE ELEMENT SYSTEM

A. PEREIRA and M. AARTS, Centre for Plant Breeding and Reproduction Research, Wageningen, NL (participant No. 42)

RESULTS

In the reported year we wanted to assess our 'two element *En-I* tagging system' for its utility in *Arabidopsis*. Each T_3 plant contains several *I* element copies with one or more *I* elements at new positions as compared to plants from the same as well as from different related progenies. Both reduction (infrequently) and multiplication (more often) of *I* elements was found. Therefore this system has a high frequency of transposition, with at least one newly transposed *I* element in every progeny plant, inevitably leading to transposon induced mutations. Among the progeny of a slightly leaky male sterile mutant a chimaeric plant was found displaying a fully fertile side branch on an otherwise male sterile plant, a phenomenon characteristic of transposon action. We found a correlating *I* element insert in F_2 outcrosses and cloned transposon flanking DNA by inverted PCR and determined the complete sequence of the cloned *Male Sterility 2 (MS2)* gene (see Fig. 2). Our objective for next year is to screen for mutants on a much larger scale and to position a number of transposed *I* elements on the genetic map. The enhancer trap system consists of 'three elements' on two T-DNA constructs. The *transposon* constructs contain, inserted in the leader of an SPT gene, a mobilizable *I* element with a minimal 35S promoter-GUS fusion. The *transposase* construct contains the *tnpA* and *tnpD* transposase genes under control of TR 2' and chiB (tapetum specific) promoter. Crosses were made between plants homozygous for *transposon* and *transposase* constructs. F_2's from these crosses did not give any streptomycin resistant progeny and also with blot hybridization no excision could be detected. The *transposase* constructs do not seem as effective in activating *I* elements as observed with the 'two element' system, but a wings-clipped *Enhancer* transposase source can now be crossed to *transposon* plants to activate the enhancer trap *I* elements.

GENE REPLACEMENT

GENE REPLACEMENT USING GENE RECONSTRUCTION

L. WILLMITZER and P.C. MORRIS, Institut für Genbiologische Forschung, Berlin, D (participant No. 38)

OBJECTIVES SET FOR THE REPORTING PERIOD

Isolation of genomic fragment bearing a repaired target hpt gene together with long flanking plant DNA sequences in order to enhance the length of homology in gene targeting experiments.

MAJOR PROBLEMS ENCOUNTERED

Instability of lambda and cosmid clones. False identification of a gene targeting event.

RESULTS

In our previous report we described how a model system for gene targeting studies was established by introducing a non-functional *hpt* gene between the CaMV 35S

promoter and the ocs polyadenylation signal into *Arabidopsis* by *Agrobacterium*-mediated transformation. Transformants containing a candidate active HPT gene were further analyzed by PCR for linkage of the CaMV 35S promoter and the sequence spanned by the deletion in the target *hpt* gene, and by Southern blot, to establish whether gene targeting had occurred. A targeting frequency of 3×10^{-4} was achieved.

In order to enhance the length of homology between target and targeting DNA from the 1 kb used in the previous experiments, the strategy envisaged was to clone repaired *hpt* genes from successful gene targeting experiments together with the largest possible length of flanking plant DNA, and after suitable alteration of the *hpt* gene so as to render it non-functional and thus reduce background from random integration (eg a 3' deletion of the *hpt* coding region), to use this long stretch of homologous DNA for further targeting experiments. One line (R15) contained a single target *hpt* gene on an 11 kb HindIII fragment, and thus this line was chosen for the recloning. However despite three attempts using two different vectors no stable clones could be isolated from this library. The same locus from the parental line (P23) prior to targeting was also cloned, but it also showed exactly the same aberrant restriction pattern and rearrangements as the clones from R15. Furthur analysis showed that the target *hpt* gene had not in fact been repaired in R15, but the targeting DNA had integrated some 6 kb 5' to the target on the same HindIII fragment. Our previous attribution of the hygromycin resistance of plant R15 to the result of a gene targeting event is therefore wrong.

HIGHLIGHTS/MILESTONES

The recombinant line R15 is not hygromycin resistant due to a gene targeting event, and the target site itself contains endogenous repetitive and high copy sequences. Thus further recloning attempts using this line in order to use the flanking sequences for enhanced homology are probably futile. Also, the analytical approach we used for detection of gene targeting is prone to error, and proof of targeting must be more rigorous to exclude such events.

WIDER CONSIDERATIONS

The frequency of homologous recombination achieved in these experiments is, with the removal of one of the candidate lines, even lower than previously estimated. This makes it even more important to define those conditions under which enhanced targeting frequencies can be achieved.

COOPERATIVE ACTIVITIES

Gene targeting experiments are being attempted by a number of labs in the Bridge programm using the *Arabidopsis* target lines described here. These workers have been updated with the nature of the difficulties we have faced using line P23.

SITE DIRECTED MUTAGENESIS OF ENDOGENOUS GENES IN *ARABIDOPSIS THALIANA*

P. van den ELZEN, S. OHL, Mogen International NV, Leiden, NL (participant No. 35).

OBJECTIVES SET FOR THE REPORTING PERIOD

1. Develop selection for α-amanitin resistance
2. Carry out gene targeting experiments

MAJOR PROBLEMS ENCOUNTERED

Selection for the targeted correction of the RNA polymerase II gene proved to be unexpectedly difficult.

RESULTS

Constructs were made that allow to select for the targeted modification of the gene AtrpII (encoding the large subunit of RNA polymerase II) to an allele conferring resistance to amanitin on the cell. However, *Arabidopsis* cells turned out to be a factor 100 less sensitive to the toxin than mammalian cells, although *in vitro* sensitivity of plant and mammalian RNA polymerase II is in the same range. Therefore, we decided to abandon the idea of using amanitin-selection in targeting experiments. However, initial targeting experiments were done using the modified AtrpII construct but screening via PCR has not lead to the identification of any targeting events. We will continue with targeting experiments and use PCR screening and will also start making constructs derived from the AtrpII gene in which a selective marker (kanamycin resistance) is present that will be used as replacement vectors in targeting experiments.

SITE DIRECTED MUTAGENESIS IN *ARABIDOPSIS THALIANA* USING AGROBACTERIUM AS A GENE DELIVERY SYSTEM

A.J.J. HOOYKAAS and A.C. VERGUNST, Institute of Molecular Plant Sciences, Leiden, NL (participant No. 14).

OBJECTIVES SET FOR THE REPORTING PERIOD

1. Construct Agrobacterium strains with defective *hpt*-gene.
2. Start transformation work using protoplasts.
3. Establish variables and frequencies of gene repair using PCR etc..

MAJOR PROBLEMS

Due to the difficulties of establishing a highly efficient transformation protocol of protoplasts with Agrobacterium as DNA donor we shifted to root transformation of *Arabidopsis*. Furthermore, due to problems with hygromycin selection attached to the HPT defective target lines we decided to continue with another model system to detect gene replacement.

RESULTS

Established protocols for *Arabidopsis* regeneration and naked DNA transformation use a gelling agent named alginate to immobilize protoplasts directly after isolation were used. Early embedding of protoplasts, necessary for good regeneration, appeared to hinder attachment of agrobacteria to protoplasts. *Arabidopsis* leaf mesophyll protoplasts turned out to be recalcitrant in liquid culture. The efforts to set up a liquid cultured using either a different hormone regime or the cellulose biosynthesis inhibitor 2,6-dichlorobenzonitril were continued, but resulted only in division at non-reproducible and low frequencies. Improvements in the regenera-

tion protocol of *Arabidopsis* protoplasts according to J. Masson had no elevating effect on division frequencies of protoplasts in liquid culture. It seemed not feasible to establish a highly efficient cocultivation protocol for *Arabidopsis* protoplasts with *Agrobacterium* that could be used in gene targeting experiments within the time schedule of this programme. Therefore, we changed our objective and optimized root transformation. Different parameters were tested for their influence on transformation frequencies. Hygromycin selection was used for optimization, because this resistance marker is also used in gene targeting experiments with HPT-defective target lines (see below). Due to problems concerning hygromycin selection we now use kanamycin resistance. Current transformation experiments yield 1 to 2 kanamycin resistant calli per root explant (one ≈ 0.5 cm root piece). To be able to detect homologous recombination events the model system developed in Willmitzers' group was used, but a specific primer combination, indicative for homologous recombination, did not yield the expected fragment in 20 resistant calli.

COOPERATIVE ACTIVITIES

There was collaboration with the group of part. no. 35, 38, 28, and 20. In April 1992 a Subgroup meeting was held in Leiden for the participants of the gene replacement section, and representatives of Subgroup members met recently at the symposium on gene targeting in plants organized by the Genetical Society.

GENE TARGETING VIA YACs

P. MEYER, Max-Delbrück-Laboratorium, Köln, D (participant No. 24).

RESULTS

One major objective was to obtain a transgenic *Arabidopsis* C24 plant containing a single copy of a negative selectable marker gene that is constitutively expressed. A binary vector, pPCV1'2'TKG2, has been constructed and transferred into C24. The plasmid contains an NPT II gene as a positive selection marker and the two negative selectable marker genes *tms 2* from *Agrobacterium tumefaciens* and *TK* from *Herpes simplex*. Both genes are driven by the bidirectional 1'2'promotor. The *tms2* gene was tested on medium containing 3μM NAM, and transgenic seedlings showed abnormal root development with reduced length growth and adventitious root formation while on wild type seedlings no obvious effect was visible. This confirmed that the *tms2* gene is active and can be used for negative selection at least on the callus and shoot level. Transgenic and wild type protoplasts have been selected with Gancyclovir concentrations of 10^{-3} — 10^{-7}M. No visible effect on neither transgenic nor wild type protoplasts has been observed at the concentrations tested. It seems that the *TK* gene cannot be efficiently used as a negative selectable marker in protoplasts. A new negative selectable marker for plants, the bacterial cytosine deaminase gene, will be tested for its applicability at the protoplast level. In *E. coli* this gene converts non-toxic 5-Fluorocytosine into toxic 5-Fluorouracil. This marker has been shown to be selectable on the callus and seedling level in tobacco but neither in *Arabidopsis* nor on a protoplast level. Another objective was the reisolation of the transgene together with a larger area of its chromosomal integration region. Southern blot analysis of high molecular weight DNA from A1/5-5 revealed a 400 kb NotI fragment suitable for cloning into a YAC vector. For recloning of such a large fragment an efficient yeast transformation system, with transformation frequencies of 1-2 x 10^6 transformants per ug

DNA, was established. To transfer YACs from yeast into protoplasts, experiments for PEG-mediated fusion have been carried out using either yeast spheroplasts or isolated yeast nuclei, but protoplasts never developed further than microcallus stage. In order to monitor the YAC-vector more accurately, a GUS reporter gene has been cloned into pYAC45. Since this GUS gene contains a plant intron, it should only be active in protoplasts. Fusion experiments with this vector, pYAC45GUSINT, have been initiated. As an alternative to fusion techniques the transfer of isolated YAC-clones or F-plasmids by PEG-mediated transfer into protoplasts will also be initiated. As a general vector for gene displacement an 18kb vector, pYGTG, has been constructed.

STIMULATION OF RECOMBINATION BY REC A

B. REISS, M. KLEMM, H. KOSAK, Max-Planck-Institut für Züchtungsforschung, Köln, D (particpant No. 32).

OBJECTIVES SET FOR THE REPORTING PERIOD

Look for recombination complexes

MAJOR PROBLEMS

Complexes formed between single stranded DNA and recA proved to be toxic for plant cells when used in a standard PEG transformation protocol.

RESULTS

A dual strategy was proposed for improving the frequency of gene targeting by the use of the recombination promoting enzyme recA:

(a) Formation of recombination competent complexes of recA enzyme and DNA, transformation of these complexes into target lines, and determination of the frequency of gene targeting.

(b) Expression of recA enzyme under the control of plant expression signals and determination of its effect on the frequency of gene targeting. When applied in PEG mediated transformations RecA complexes lead to cell death. In a preliminary set of experiments in which concentrations of recA and ssDNA which were not lethal for protoplasts were used, no increase in gene targeting frequency was observed. Therefore we concentrated on the production of transgenic plants expressing recA protein. A new binary vector was constructed which employed a selectable marker gene not appearing in any of the target lines commonly used to study gene targeting. The recA genes described earlier, either fused to the nuclear targeting sequence or the authentic gene, both under the control of the CaMV 35S promoter, were inserted into this vector and transgenic plants regenerated. The recA protein fused to the nuclear targeting signal was found exclusively and in high amounts in the nucleus. Because this fusion protein has been shown to be active in all assayable aspects relevant for promoting recombination, these transgenic plants contain high amounts of an active, prokaryotic recombination enzyme.

HIGHLIGHTS/MILESTONES

Transgenic plants have been generated which express bacterial recA protein to high levels. A fusion protein of recA and a nuclear localisation signal is very efficiently transported to the nucleus.

COOPERATIVE ACTIVITIES

Material, ideas, and valuable information relevant for the project was obtained on various occasions from the laboratories of P.J.J. Hooykaas (Participant 14), L. Willmitzer (Participant 38), and J. Paszkowski (Participant 28).

FLORAL INDUCTION

ISOLATION OF THE CO LOCUS AND IDENTIFICATION OF OTHER MUTATIONS AFFECTING FLOWERING TIME IN *ARABIDOPSIS*

G. COUPLAND, J. PUTTERILL, F. ROBSON, K. LEE and S. DASH, John Innes Centre, Norwich, UK (participant No. 5).

OBJECTIVES SET FOR THE REPORTING PERIOD

1. Identify cosmid clone containing the *CO* gene.
2. Attempt to identify the *CO* open reading frame by transcript analysis and examination of DNA of *co* mutants.

RESULTS

In our previous report we described a 1700 kb YAC contig which we made in the region of chromosome 5 known to contain co, which delays flowering time under long days, and we have now located the gene in a 350 kb segment within this contig. Two approaches have been taken to define the position of the gene, detailed RFLP mapping and complementation experiments. RFLP analysis located the gene on a 30-35 kb region contained within 4 overlapping cosmids. Complementation of *co* plants to normal flowering time confirmed the position of the gene and located it more accurately to within the 6kb overlap between *co*142 and *co*129, and this region is now being sequenced to try to identify the *CO* open reading frame. Overlapping transcripts of approximately 0.6 and 1.0 kb are located in this 6kb region, and sequencing of these is underway.

Within this project another class of flowering-time mutation which causes early flowering under short days is also being analyzed, and three mutants were identified. In addition two other mutants were provided by Maarten Koornneef. Allelism tests to determine whether any of these mutations are at the same locus are underway, as is the construction of double mutants carrying mutations for early and late flowering.

HIGHLIGHTS/MILESTONES

The *CO* gene was located on a short region of 6 kb permitting the analysis of the DNA sequence of the gene. 5 early flowering mutants were identified and their characterisation initiated.

WIDER CONSIDERATIONS

CO will be among the first genes determining flowering time to be cloned, and should contribute to our understanding of this important character.

COOPERATIVE ACTIVITIES

Participation in the EMBO workshop on *Arabidopsis* in Köln in April 1992, the BRIDGE meeting held in Gent in September 1992, and in the Flowering workshop held in Prague in March 1993. Interacted with BRIDGE collaborators José Martinez-Zapater, Maarten Koornneef and Caroline Dean to write a review on flowering time of *Arabidopsis*.

ISOLATION AND CHARACTERISATION OF THE FCA GENE AND ITS INVOLVEMENT IN THEIR VERNALISATION RESPONSE

C. DEAN and I. BANCROFT, John Innes Centre, Norwich, UK (participant No. 10).

OBJECTIVES SET FOR THE REPORTING PERIOD

1. Make high resolution map of markers surrounding the *FCA* locus.
2. Proceed to isolation of *FCA* possibly by using complementation and sequencing.

MAJOR PROBLEMS

Chimaeric YAC clones and the presence of segregating modifiers of flowering time in the *Arabidopsis* lines used in the RFLP mapping experiments made it difficult to walk across the fca locus. It took 65 YAC clones to walk over the 3cM interval between the two flanking RFLP markers.

RESULTS

The *fca* locus has been localised to an 80kb region within YAC clones EW20B3 and ABI10C10. The ara mutant and *agamous* were used as markers We also sub-cloned Sau3A fragments from YACs to provide small, very closely linked probes. Detailed restriction maps covering the FCA locus have been constructed and used to position the small subclones. Cosmid clones covering this region from a Landsberg *erecta* library constructed in our *Agrobacterium* binary vector are now being identified. We further demonstrated that large DNA technology could aid RFLP analysis to maximize the efficiency of localization of recombination points relative to chromosome walks.

HIGHLIGHTS AND MILESTONES

1. Completing high resolution map surrounding the *FCA* locus.
2. Building a Landsberg *erecta* library in an Agrobacterium binary vector for use in complementation experiments.
3. Localize the FCA locus to an 80kb region.

COOPERATIVE ACTIVITIES

Participation in the BRIDGE meeting held in Gent in September 1992. Laboratory participation in the Flowering workshop held in Prague in March 1993.

FLORAL INDUCTION IN *ARABIDOPSIS*

M. KOORNEEF and A.J.M. PEETERS, Agricultural University, Wageningen, NL (participant No. 21).

RESULTS

Double mutants between 10 late flowering mutants have been selected and are being checked for their genotype. Double and triple mutants between the late flowering genes co, gi, fwa and fca with the phytochrome mutants hy2 and hy3 have been obtained and grown in short day (SD) and long day (LD) conditions and in conditions with different light quality. The results indicate that the earliness in phytochrome deficient mutants is determined by different PHY species and not only by PHYB. The additivity of the flowering time effects of the hy and late flowering mutants indicate that the defects in the late flowering mutants are not mediated by the phytochrome system. The study of the ld and Fmc mutants indicate that these extreme late mutants which were not found in Ler are due to two gene differences with Ler, probably due to the interaction of a so called modifier in the ecotypes (Columbia and Estland respectively) not present in Ler. The respective genes and modifiers have been located on chromosomes 4 and 5. In collaboration with Dr. Jeff Leung in Dr. Giraudat's group the fwa locus was shown to be located on either one of two cosmids of 30 kbp. At the moment 8 recombinants with cross-overs between the ends of this two cosmid contig are available for a further refinement of the location of the fwa locus. The search for mRNA related to the cosmids is underway and DNA sequences homologous to the two cosmids will be isolated from the (dominant) mutant to allow complementation analysis and sequences of putative mutant alleles.

FLORAL INDUCTION: IDENTIFICATION AND CHARACTERIZATION OF GENES INVOLVED IN FLORAL INDUCTION

J.M. MARTINEZ-ZAPATER, CIT-INIA, Madrid, E (participant No. 22).

OBJECTIVES

1. Make high resolution map surrounding *fve*
2. Proceed to isolation of *fve* by complementation and sequencing

RESULTS

We have integrated two *Arabidopsis* RFLP maps in the region surrounding the *fve* locus. Due to the segregation of Niedersenz and Ler alleles at different loci affecting flowering time, we have had unexpected difficulties in the recognition of the Fve late flowering phenotype. 53 additional putative recombinants between *fve* and *cp2* in the cross between the Fve marker line and Ndz and 154 putative recombinants between the same markers have been identified. The resolution of the cointegrated map around the *fve* locus is still low, but it will be improved when the analysis of these recombinants is finished. The *fve* locus lies between RFLPs CITd84 and PhyB, a region where we have 3 recombinants. Three alternative approaches approaches to isolate *fve* are being taken. These are to label molecularly the *fve* locus (we are screening for deletion alleles of the locus, and for intragenic recombinants in F2 progenies derived from crosses between *fve* alleles in Ler and in Col), and to avoid the problem of the modifier loci by con-

structing a line carrying the *fve* mutant allele and surrounding markers. Based on the estimation of the location of the *fve* locus with respect to RFLPs, we have isolated three YAC contigs of 520, 450 and 160 kbp from RFLPs CITd84 on the left side and from RFLPs PhyB and 216 on the right side of fve, and these are being extended in both directions. The results of morphological characterisations indicate that the *fve* locus plays a role all along the development of the plant, from the first stages of the rosette till the inflorescence.

HIGHLIGHTS/MILESTONES

We have cointegrated the two RFLP maps of *Arabidopsis* in the region of chromosome 2 containing the *fve* locus.

Physiological and morphological analysis of two independent *fve* mutants indicate the *fve* locus plays a role along all the developmental phases of the plant under any set of environmental conditions.

COOPERATIVE ACTIVITIES

The floral induction subgroup met twice along the year: The first time in Amsterdam and Wageningen with the occasion of the International Workshop on Flower Development and Plant Reproduction and the second time in Ghent at the plenary meeting of the *Arabidopsis* T-project.

The floral induction subgroup has written a chapter on The Transition to Flowering in *Arabidopsis* for a book on the biology of this species. This has been a specially interesting experience that has allowed to unify criteria and hypothesis about this developmental process and to draw a general model on the genetic control of the transition to flower.

MOLECULAR CHARACTERIZATION OF APETALA2

M. van MONTAGU, Universiteit Gent, Gent, B (participant No. 37).

OBJECTIVES SET FOR THE REPORTING PERIOD

1. Continuation of expression analyses of the APETALA2 gene at the transcription level by the in situ hybridization technique and at the protein level by immunolocalization studies.
2. DNA sequence analyses af the cloned mutant alleles ap2-3, ap2-6, ap2-7, ap2-8 and ap2-9 as well as related genes of other species to determine functionally important domains in the protein.
3. AP2/GUS chimeric gene fusions will be introduced into wild-type and mutant backgrounds. The temporal and spatial GUS expression pattern will be analyzed histochemically.

RESULTS

In situ hybridization experiments and RNA gel blot studies indicate that in wild-type plants the AP2 gene is expressed in all four floral organs as well as vegetative tissue. These results show that AP2 is unique among the floral homeotic genes. AP2/GUS chimeric gene constructs have been made, introduced into wild-type *Arabidopsis* plants and subsequently crossed into ap1-1, ap2-1, ap3-1, pi-1, ag-1 and lfy-6 mutant backgrounds to determine whether any of these gene products regulates AP2 at the transcription level. To identify regions within the AP2 protein

that are essential for its function we planned to isolate and sequence all 10 published AP2 alleles. So far the ap2-1, ap2-5 and ap2-7 alleles have been sequenced. By comparison with wild-type gene sequences point mutations that are likely to be responsible for the observed mutant phenotypes have been identified. Genomic libraries of two additional extreme alleles, the strongest allele ap2-2 and the slightly less extreme allele ap2-9, have been constructed and sequencing is in progress. AP2-like cDNA clones have been isolated from Petunia and Antirrhinum respectively and sequenced. Analysis of the AP2 mutant genes and the Petunia and Antirrhinum AP2-like genes allowed us to define a 160 amino acid region, the AP2-domain, which is crucial for AP2 function in *Arabidopsis* and highly conserved in Petunia and Antirrhinum. Scanning electron microscope analysis indicates that AP2 plays a critical role in the normal formation of the seed coat.

HIGHLIGHTS/MILESTONES

APETALA2 is a unique, novel regulatory protein, unlike other floral homeotic proteins. Its sequence is conserved which suggests that its a component of an evolutionary conserved floral control mechanism.

SEED DEVELOPMENT

ISOLATION AND CHARACTERIZATION OF *ARABIDOPSIS* GENES INVOLVED IN ABSCISIC ACTIONS

J. GIRAUDAT, CNRS, Gif sur Yvette, F (participant No. ...).

OBJECTIVES SET FOR THE REPORTING PERIOD

1. Continue isolation and characterization of genes conferring aba-insensitivity.
2. Analyse expression and potential modes of action of genes involved in drought resistance.

RESULTS

Objective No. 1:

We have undertaken to clone the abil and abi3 loci which, on the basis of the mutant phenotypes, are likely to encode elements of two partially overlapping ABA-signal transduction pathways. The *abil* locus is located at position 44.7 on chromosome 4, and a refined RFLP map in the vicinity of this locus has been made, and an approximately 500kb chromosome walk using YAC clones across this region has also been made. We have now established by RFLP mapping that the abil locus has indeed been crossed during this walk, and further refined its location to an approx. 150kb region. A set of overlapping cosmid clones representing 200 kb of contiguous wild-type sequence in this area has been assembled, and a λphage contig of DNA from the homozygous Abil/Abil mutant covering the 150kb region which contains the ABI1 locus has been assembled by taking advantage of the above YAC and cosmid contigs. Since the Abil mutant allele is dominant over the wild-type allele, the inserts of these phage clones are being assessed for their ability to create the abil mutant phenotypes when used to transform wild-type *Arabidopsis* plants. The *abi3* locus is located at position 23.5 on chromosome 3. We reported last year the isolation of an 11kb genomic fragment (wild-type) which achieved functional complementation of the (recessive) abi3 mutation in transgenic plants. This year, the abi3 gene and a corresponding cDNA clone have been iden-

tified and characterized. Several lines of evidence support the identification of the abi3 gene. It co-segregates with the abi3 locus upon fine RFLP mapping. The complementing 11kb fragment does not appear to contain any additional genes as judged from nucleotide sequence analysis and Northern analysis. Finally, this gene is expressed in developing siliques, as would be expected for the abi3 gene based on the mutant phenotypes, and carries a mutation that leads to a truncation of the encoded protein in the most severe abi3 mutant allele. The ABI3 protein is predicted to contain 720 amino acids, with a theoretical 79500 Da molecular weight. Its sequence contains a putative nuclear localization signal and several regions with features previously described in activation domains of transcription regulators. Furthermore, the ABI3 protein displays three discrete domains of high sequence similarity to the maize VP1 protein. Like the VP1 protein, the ABI3 protein thus seems to participate in abscisic acid-regulated gene expression during seed development. The expression pattern and physiological role of the abi3 gene, as well as its functional relationship to the maize vp1 gene, are now being analyzed further.

Objective No. 2:

Arabidopsis plants develop over weeks an adaptive response which includes new root morphogenesis when subjected to progressive drought. These characteristic short tuberized roots are able to survive in desiccated soil and to rapidly resume growth upon rehydration. Two cDNA libraries have been constructed starting from the root systems of control well-watered or drought adapted *Arabidopsis* plants respectively. Differential screening has identified at least four different transcripts. The abundance of two of these transcripts is decreased in response to progressive drought stress, wherease the level of the other two increases. Determination of the nucleotide sequence of full length cDNA clones representative from each class is being completed. Also, in order to dissect the regulatory pathways involved in this adaptive response, we are comparing the expression patterns of these genes in wild-type and in abi mutants, both in plants submitted to progressive drought and in seedlings treated with exogenous ABA.

HIGHLIGHTS/MILESTONES

Isolation of the abi3 gene demonstrates the feasibility of map-based cloning in *Arabidopsis.*

COOPERATIVE ACTIVITIES

With participant No. 21 which is engaged in cloning the fwa locus located nearby abi1. We benefitted from their independent RFLP data, and in turn provided them with the above described molecular tools.

With participant No. 20 who provided us with new published abi3 mutant alleles.

ACQUISITION OF DESICCATION TOLERANCE IN DEVELOPING SEEDS OF *ARABIDOPSIS THALIANA*

C.M. KARSSEN, Agricultural University, Wageningen, NL (participant No 16).

OBJECTIVES SET FOR THE REPORTING PERIOD

Extent mutant analysis to include studies of membrane configuration and composition and the role of disaccharides.

MAJOR PROBLEMS

Because seeds of the double mutant are desiccation intolerant, they lose viability, when harvested and stored. A scheme was developed in which the double mutants are grown directly from almost ripe seeds taken out of the siliques and immediately put to germinate in water.

RESULTS

This year two new ABA-insensitive mutants, abi3-4 and *abi3-5* became available. These were physiologically analyzed and compared with the *abi3-1* mutant and the ABA-deficient and -insensitive double mutant *aba-1,abi3-1*. Seeds of *abi3-4* and abi3-5 mutant plants remain green throughout seeds development, are severely insensitive for ABA, do not develop dormancy and acquire a transient desiccation tolerance. Carbohydrate analysis of wild type and these mutant seeds during development showed that carbohydrates were not sufficient to establish desiccation tolerance. However, a correlation exists between seed longevity and a low ratio between mono-/oligosaccharides in the seeds. The presence of proteins which cross react with antibodies raised against ABA or desiccation induced proteins ('pcC6-19') in *Craterostigma plantagineum*, was tested in seeds of different genotypes. Cross reaction was found in all genotypes except the desiccation-intolerant double mutant. However, cross-reacting proteins were only found in nearly mature seeds, whereas desiccation tolerance is acquired already at 12-13 days after pollination. Probably, this protein is associated with general seed maturation processes rather than correlated with desiccation tolerance. There is an optimum stage of seed development around 13 days after pollination for induction of desiccation tolerance. We analyzed the effect of *in vitro* induction of desiccation tolerance on carbohydrate composition and synthesis of proteins cross-reacting with the 'pcC6-19' antibody. Both treatments induced desiccation tolerance, but had no effect on carbohydrate accumulation. *In vitro* treatment leads to synthesis of proteins cross-reacting the 'pcC6-19' antibody.

SEED DEVELOPMENT AND ABSCISIC ACID

M. KOORNEEF, K.M. LÉON-KLOOSTERZIEL, E.P. van LOENEN-MARTINET and M.H.C. BLANKESTIJN-de VRIES, Agricultural University,Wageningen, NL (participant No. 20).

OBJECTIVES

The isolation and characterization of mutants affecting abscisic acid (ABA) metabolism and seed development.

RESULTS

The new extreme abi3-4 allele was shown by Giraudat to be due to a stop codon in the cloned ABI3 gene and thereby provided additional prove that the real ABI3 gene had been isolated. The characterization of these mutants together with the group of Karssen resulted in arguments for the role of carbohydrates in the development of desiccation tolerance in seeds. Thus far three non-allelic mutants without increased ABA resistance but with reduced seed dormancy have been isolated and are currently being analysed with respect to their genetics and interaction with ABA deficient and resistance mutants. A search for similar mutants in T-DNA tagged lines obtained from the Nottingham Stock Center has been started. A set of recombinant inbred lines provided by Drs Lister and Dean via the Stock Center is being used to map the seed dormancy difference between the lab ecotypes Ler and Col of which the latter is less dormant than Ler. Scanning and light microscopic analysis of the maternally inherited abberant testa shape *(ats)* mutant, located in the middle of chromosome 5, indicated that rather specific layers of the testa are affected. This results in a less elongated shape of the seed which develops within the first three days after pollination when the embryo proper occupies only a small fraction of developing seed (see Fig. 3). To our knowledge this is the first time that the importance of the testa for the ultimate seed shape has been established experimentally. In addition it was shown that this mutation results in a maternally inherited reduced dormancy. Most combinations of EM2 promotor-GUS construct (obtained from Dr. Delseny) with the various ABA related mutants have been made and will be analysed in collaboration with Dr. Delseny's group. The RAB17 construct is being combined with the extreme *abi3* alleles and the *aba/abi3-1* double mutant for further analysis of the expression of this promotor in collaboration with Dr. Pages.

EXPRESSION OF THE ABA-RESPONSE GENES IN *ARABIDOPSIS* SEEDS

M. PAGÈS, C. ARENAS and E. CARRERA, CID. CSIC, Barcelona, E (participant No. 27).

OBJECTIVES SET FOR THE REPORTING PERIOD

1. Continue study of expression of ABA-regulated genes in mutants
2. Initiate studies aimed to characterise ABA-induced promoters.

RESULTS

Previous results suggest that alternative pathways involving different molecular mechanisms may be responsible for seed specific expression and ABA-osmotic stress induction of the *rab* gene. We obtained from Dr. Michel Delseny the clone rlea2, a *rab* homolog, and we have studied the developmental and hormonal regulation of the endogenous gene in comparison with that of the maize promoter in the rab17/GUS transgenic *Arabidopsis* plants. ABA and water stress induce the *Arabidopsis rab* homolog, indicating that distinct cis-elements and/or protein factors are involved in the expression of *rab* genes in embryo and vegetative tissues in monocot and dicot plants. Therefore we have concentrated our efforts on the preparation of different constructs for the detailed study of the *rab* promoter regulation. Transient assays using these constructs are being performed. Mutants impaired in the regulation of the *rab* promoter will be isolated by by selecting for

an altered expression of the reporter gene. Integration of the construct 35S/MA16 in transgenic *Arabidopsis* was confirmed by Southern analysis, and expression of the mRNA and protein by Northern and western blot respectively. The constitutive expression of the MA16 protein provides a tool to further characterize the function of this protein. Screening of *Arabidopsis* libraries with maize *rab* genes originated some positive clones. One genomic clone showing strong hybridization with the rlea2 was sequenced, but it is not the corresponding genomic DNA.

COOPERATIVE ACTIVITIES

We have collaborated with Michel Delseny, Jose Miguel Martinez Zapater and Maarteen Koornneef groups. Participation of the group in the BRIDGE meeting held at Gent, and in the 'Signal Transduction in Microorganisms and Plants' meeting held at Lunteren in September 1992. One Spanish scientist and one French scientist visited the Perpignan and Barcelona lab for one week to discuss and perform experiments.

GENES INDUCED BY ABA DURING DEVELOPMENT

M. DELSENY, University of Perpignan, Perpignan, F (participant No. 11).

RESULTS

Fusions of two Em promoters and deletion derivatives have been made with a GUS or a luciferase reporter gene and have been transferred into *Arabidopsis*. The results indicate that the two full length promoters are specifically expressed in the embryo and not in the endosperm. Expression of At Em 1-GUS fusions coincides with the *in situ* hybridization patterns, being more intense in the provascular tissues. No expression was observed in adult leaves or roots, whether the plant are water stressed or not. An enhancer element has been located in the -250 -300 region. Preliminary experiments using cloned transcription factors (TAF 1 which should bind the Em box, and the TATA- binding factor) in gel shift assays are being carried out. The Em-GUS constructions have been given to Marteen Koornneef and have now been transferred in *aba* and *abi 1,2,3* backgrounds. Work which is now planned will consist in using the transgenic plants to deregulate Em expression and to find new regulatory mutants. Two cDNA clones coding for *Arabidopsis* LEA proteins have been selected and genomic clones are now being sequenced. A new RAB 17 cDNA clone has been characterized. These clones are characterized in collaboration with Montserrat Pages in Barcelona.

EMBRYOGENESIS

T-DNA INSERTIONAL MUTAGENESIS TO ISOLATE GENES REGULATING EMBRYOGENESIS IN *ARABIDOPSIS THALIANA*

P. GALLOIS and M. DEVIC, Université de Perpignan, Perpignan, F (participant No. 44).

OBJECTIVES SET FOR THE REPORTING YEAR

1. Continue to screen for GUS activity.
2. Make F1 progeny of lines and look for mutations affecting embryogenesis.

3. Make seed populations available to resource centre.
4. Isolate interesting flanking sequences.

RESULTS

During the first phase of the project a population of 750 transformed lines of *Arabidopsis* has been made in Perpignan. The construct transferred via *Agrobacterium* contains a promoterless GUS gene and a kanamycin resistance gene located between the left border and the right border of the T-DNA. The transformants are screened for both GUS activity and/or embryo lethal mutations.

Up to now 400 lines have been screened for GUS expression in the seed and siliques. Six lines have been selected for further analysis in which the expression is limited to the embryo or to the embryo and a few other organs. In addition, four lines have been selected which show a segregation of a lethal mutation with a 3:1 ratio of normal/aborted seeds in the siliques. 265 lines containing GUS inserts have been bulked up and will soon be sent to the Resource Center. An IPCR protocol has been developed which allows the amplification of the plant DNA adjacent to the left border or the right border of the T-DNA inserted in the plant genome. Using line 276-1, 500 bp of genomic DNA upstream of the GUS gene have been amplified by IPCR. This fragment has been sequenced and will be used as a probe to isolate corresponding wild type DNA. Next year will see the characterisation of DNA directing different patterns of expression in the plant embryo. These sequences will be compared with sequences obtained independently in Leicester which direct similar patterns of GUS gene expression. This will yield interesting information on the organisation of promoters of genes expressed in the plant embryo.

GENE EXPRESSION DURING EMBRYOGENESIS STUDIED BY *IN SITU* HYBRIDISATION

F.T. OKKELS, K. HENRIKSEN, B. LINDBERG, S. GULDAGER PETERSEN and J.E. NIELSEN, DANISCO A/S, Copenhagen, DK (participant No. 45).

OBJECTIVES SET FOR THE REPORTING PERIOD

Development of an in situ hybridization (ISH) method for detection GUS mRNA in GUS transgenic *Arabidopsis* embryos.

MAJOR PROBLEMS ENCOUNTERED

To achieve enough sensitivity to hybridize to a rare mRNA like GUS in sections.

RESULTS

ISH experiments with biotinylated oligoes and biotinylated, dioxigenated or Sulphur-35 labelled hydrolized riboprobes on sections of *Arabidopsis* embryos and various plant tissues gave good results with mRNA's occurring in high amounts in the plant material (poly-A tails, BNYVV virus and Histon IV). Various procedures were investigated with different fixations, probe concentrations, proteinase concentrations, hybridization and washing stringency, all these experiments led to the conclusion that the GUS mRNA was rare and near the detection limit with the methods used. Sulphur-35 labelling method is used with NPT and GUS probes at the moment. Positive results with GUS and NPT probes on RNAse protection assay have been achieved.

HIGHLIGHTS/MILESTONES

1. GUS and NPT riboprobes work in RNAse protection assays.
2. The potato rRNA probe works in ISH.

INSERTIONAL MUTAGENESIS TO ISOLATE GENES CONTROLLING EMBRYOGENESIS IN *ARABIDOPSIS*

K. LINDSEY, University of Leicester, Leicester, UK (participant No. 44).

OBJECTIVES SET FOR THE REPORTING PERIOD

1. To continue screening transgenic lines for GUS fusion expression in zygotic embryos;
2. To generate F1 (T2) progeny and screen for mutants;
3. To make F1 populations available to the Seed Centre;
4. To start isolation of T-DNA-flanking sequences from lines of interest.

RESULTS

Three lines that exhibit GUS fusion activity in seeds of transformants containing a GUS enhancer trap have been identified for more detailed analysis: these are designated AtEM-101; AtEM-201; and AtEN-101.
GUS activity in AtEM-101 in the T2 seed is restricted to the embryo. As embryogenesis continues, GUS activity in the developing root region increases, but is restricted to this part of the embryo. Following germination, GUS activity is found strongly in the root tip and more diffusely in the cotyledons, but not in the true leaves. In more mature plants, activity is restricted principally to the root tips and the seeds, with low levels of activity in the silique wall. Southern analysis showed that this line contains a single *gus*A insertion event. Restriction analysis and sequencing has confirmed that an IPCR product derived from the T-DNA junction region has been cloned. This was used to probe a wild-type *Arabidopsis* genomic library and an homologous genomic fragment has been isolated.

AtEM-201
GUS activity is restricted in the seed to the embryo, and is detectable in early heart-stage embryos. As embryogenesis continues, activity becomes restricted to the basal part of the embryo. In seedlings (up to 7d. post germination), activity is found in root tips and also in cotyledons and hypocotyl, but in the mature plant is restricted to root tips, particularly in young lateral roots; no activity has been found in leaves or flowers. This line contains a single T-DNA insert. T-DNA 5' flanking sequences have been amplified by IPCR, and a 6 kb fragment is ready to be further sub-cloned for sequencing.

AtEN-101
GUS activity is restricted to the endosperm. In the seedling, activity is found in the cotyledons and roots, but in mature plants is restricted to the roots and the tapetum of the anther. Sequences flanking the single T-DNA insert have been amplified by IPCR, and larger wild-type genomic fragments isolated. Under greenhouse conditions, all three transgenic lines show no detectable mutant phenotype, but we will be investigating environmental conditions that may reveal developmental aberrations in AtEM-101, 201 and AtEN-101 plants homozygous for T-DNAs.

HIGHLIGHTS/MILESTONES

1. The conditions for IPCR have been optimised for our transgenic lines.
2. A number of genomic fragments, expected to contain sequences that direct gene expression in embryos and endosperm, have been isolated.
3. Progress is being made on the identification and characterization of phenotypic mutants.

COOPERATIVE ACTIVITIES

Gent, September 1992, 4 days: BRIDGE plenary meeting and discussions of joint work plan with Leicester, Copenhagen and Perpignan participants.

T-PROJECT

“LIPASES”

LIPASES

COORDINATOR:
R. VERGER, CNRS, Marseille, F

A GENERAL OVERVIEW
(see also specific reports on the following pages)

OBJECTIVES

Characterization of Lipases for Industrial Applications.
Three-Dimensional Structure and Catalytic Mechanism.

HIGHLIGHTS / MILESTONES

1. Complete ^{1}H-NMR assignment of porcine pancreatic colipase B as well as procolipase. Analysis of the sequential and medium range NOEs, backbone J-couplings and the identification of slowly exchanging amide protons have indicated the presence of a large number of short β-sheet regions at 15-17, 27-30, 37-40, 47-50, 68-70 and 84-88. Some NOEs and slow exchanging amide protons are missing from these sections, which suggests the occurrence of deviations from a regular β-sheet structure. The location of these stretches as well as the irregular β-sheet fold is in agreement with the X-ray structure of procolipase. A more exact definition of these regions was achieved based on the complete folding of the protein. The analysis also showed the existence of three definite turns at residues 13-14, 45-46 and 65-66.

The published X-ray structure of procolipase (resolution 3Å) has a similar fold to that of the NMR structure. However, the average rmsd of backbone atoms with respect to the solution structures is 1.9Å, suggesting the existence of small but significant differences between the two structures. The poorly defined regions in the NMR structure are similar to those in the X-ray structure. A detailed analysis of the differences between the X-ray and NMR structures, including the orientation of the side chains, is in progress and will include an X-ray structure with higher resolution. The disulfide bridges observed in the X-ray structure analysis were used for the NMR structure determination of procolipase. A separate DG calculation without disulfide bridges yielded a similar fold to that observed with disulfide bridges and this fold is incompatible with the disulfide bridge between residues 17 and 87 which was previously thought to exist on the basis of chemical studies. The final set of constraints will be compared with possible alternative sets of disulfide bridges.

Procolipase has 5 more residues at the N-terminus than to colipase. In the X-ray study of procolipase, the N-terminus was not observed and was assumed to be of no importance for the structure of colipase or procolipase. In the NMR study, no long-range NOEs have been observed as yet from the N-terminus to the remainder of the protein. Comparisons between the backbone ^{1}H chemical shifts of colipase and procolipase indicate however that the differences are not confined to the N-terminal residues, but are equally significant among the residues in the C-terminal half of the sequence, while the backbone ^{1}H chemical shifts occurring in residues 16 to 45 are relatively unperturbed. This finding together with differences between

colipase and procolipase as regards the linewidth in the less well defined regions suggests that procolipase and colipase may differ more in their dynamics than in their structure. This aspect will be included in the further NMR study on colipase and procolipase.

2. Interfacial activation of the lipase-procolipase complex by mixed micelles detected by X-ray crystallography.

3. A structural domain (the lid) present in pancreatic lipases is absent from the guinea pig (phospho)lipase.

4. Cutinase is inhibited by classical fluorophosphate inhibitors (E 600 and DFP). Inhibited cutinase has a 3 D structure which is almost identical to that of the wild type protein and no loop movement is observed. The oxyanion hole is preformed.

5. The first crystallization experiments with the highly purified *Bacillus* lipase already gave promising results in that an initial diffraction data set was recorded at a resolution of 3.5 Å.

6. Structural elucidation of several *Pseudomonas* lipases is in progress, and that of two *Geotrichum* lipases is feasible.

7. The reserved micellar system (AOT in isooctane) induces cutinase unfolding which exposes some tyrosine residues. Up to 97 % of the cutinase can be extracted into reversed micelles and 30 % back-extracted into a new aqueous phase.

8. The solid-gas bioreactor as well as NMR spectroscopy are appropriate techniques with which to study the role of water in lipase catalyzed reactions. The optimal activity and stability of cutinase was observed when one or more hydration layers are present around the enzyme.

9. A lipase from *Staphylococcus hyicus* was cloned and expressed in large amounts in *E.coli*. Surprisingly, α phospholipids are very poor substrates when spread at the air-water interface, contrary to what was deduced from the bulk kinetics.

10. The surface pressure was found to control the lipase stereo selectivity.

WIDER CONSIDERATIONS

During the closing session of the B.A.P. held at Troia (Portugal) in November 1989, not a single lipase structure was known. By now, this obvious gap has been partly filled thanks to the efforts of the teams participating in the BRIDGE T-lipase project. Our Lipase network in Europe has solved 5 to 7 structures: Mucor lipase in its open form; cutinase native and inhibited; pancreatic lipase/colipase; pancreatic lipase/colipase/mixed micelles complex; colipase in solution (NMR); *Pseudomonas, Geotrichum* and *Rhizopus* lipases.

What knowledge is at present lacking about heterogeneous lipase biocatalysis? Several participants have suggested the following topics: Lipase/lipid interactions — Lipase adsorption phenomena. Plant lipases — Lipase engineering — Effects of water on lipase catalysis in organic media — Thermophilic lipases and bioreactors based on modified lipases.

COOPERATIVE ACTIVITIES

Rome, May 19th (1992). Meeting with E. Cernia, L. Tranchino, B. Nieuwenhuis and R. Verger to organize the annual meeting of the lipase T-project in Capri (CEC/CTB workshop, 1992).

Capri, October 1-3 (1992). Co-organization with E. Cernia, L. Alberghina, R.D. Schmid and R. Verger of a CEC/CTB workshop of the Lipase T-project on 'Lipase: Mechanism, Structure and Genetic Engineering'. Monitoring Unit and coordinators meetings with B. Nieuwenhuis, A. Albert, L. Thim, H. Lenting, M. Egmond, F. Spener and R. Verger. Scientific and critical evaluation of the Lipase T-project after presentation of the research activity reports of each participating group on October 3rd, 1992.

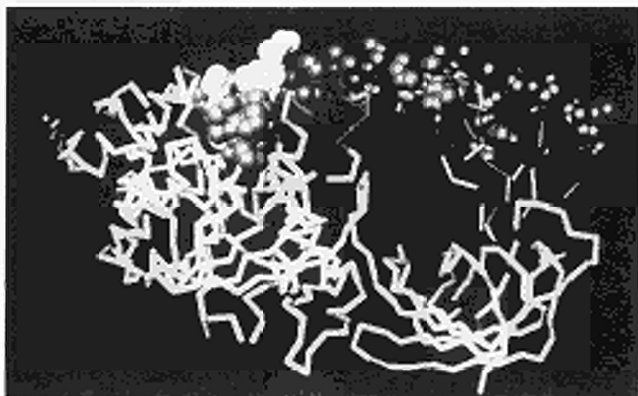
Brussels, February 22nd (1993). Monitoring Unit and coordinators meeting with P. de Taxis du Poët, L. Thim, R.D. Schmid, M. Egmond, O. Misset and R. Verger. Suggestions and recommendations about possible modifications/re-orientations of the project in the light of the 3rd call for proposals.

Copenhagen, March 15th (1993). Scientific discussions with the group headed by L. Thim. Selection of the invited speakers for the Elsinore meeting (October 1993).

EUROPEAN DIMENSION

The enthusiasm and the professionalism of Dr. B. Nieuwenhuis acted like an active catalyst in the launching of our project, and all the participants and coordinators gratefully acknowledge his stimulating example. The aim of our programme is to promote a free flow of information among the partners of the BRIDGE T-lipase project. This challenging team-work has been carried out in a spirit of collaboration, trust and openness. Four issues of the Lipase Newsletters have been co-edited by B. Nieuwenhuis and R. Verger. More than twenty purified lipases have been sent to Marseilles for kinetic analysis. Several staff members and students have commuted between various laboratories during the reporting period:
Long term (> 1 year): Ransac, S. (Marseilles → Groningen); Martinez, C. (Marseilles → Vlaardingen); Carrière, F. (Marseilles → Copenhagen); Longhi, S. (Milan → Marseilles).
Short term (< 1 year): Deveer, A.M.T.J. (Utrecht → Marseilles); Ladefoged, C. (Copenhagen → Marseilles); Melo, E. (Lisbon → Marseilles); Ransac, S. (Groningen → Bochum); Tilkorn, A.C. (Münster → Marseilles); Taipa, A. (Lisbon → Bochum).

While the project has been running it can be observed that collaboration among the project teams has been increasing and that the interactions among researchers in Europe have become more frequent due to the existence of our stimulating BRIDGE-LIPASE project.



Triacylglycerol lipases and colipases of the digestive tract and exocellular fungal lipases: Structure, interfacial binding and catalysis (BIOT CT-910274)

COORDINATOR:
R. VERGER, CNRS, Marseille, F
PARTICIPANTS:
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J.M.S. CABRAL, Instituto Supérior Técnico, Lisboa, P
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R. VERGER, CNRS, Marseille, F

OBJECTIVES

For many years, lipases have been attracting the attention of scientists in academic and industrial circles. One of the most intriguing and unique features of lipases is the way in which they are 'activated' by interfaces. The aim of this proposal is to elucidate of

(i) the structure;
(ii) the interfacial binding;
(iii) the catalytic mechanism of triacylglycerol lipases and colipases of the digestive tract and exocellular fungal lipase (PGS-lipase).

PGS-lipase was chosen because of its potential industrial applications.

There is indirect evidence that colipase activates the pancreatic lipase-catalyzed interfacial hydrolysis by anchoring lipase to its substrate in the presence of bile salts. The colipase/lipase/interface is a challenging system of lipolytic regulation via protein/lipid and protein/protein interactions.

RESULTS

See list of publications and general overview.

HIGHLIGHTS / MILESTONES

1) The 3 D-structure of the pancreatic lipase/procolipase complex and the interfacial activation of this complex by mixed phospholipid/bile salts micelles were both revealed by x-ray crystallography.

2) Preparation and characterization of human pancreatic colipase; Epitope mapping using 11 monoclonal antibodies.

3) Immunochemical identification of the participation of the N-terminal region of colipase to the formation of the stable active complex at a lipid-water interface.

4) Extension of the 3 D-resolution of the native cutinase in steps to 1.25 Å and 1.1 Å resolution.

5) Active site, oxyanion hole and lipid binding site mutants of cutinase crystallized in the same space group as the native protein and diffract to maximal resolutions comprised between 1.6 and 2.1 Å.

6) Cutinase was inhibited by diethyl p-nitrophenyl-phosphate, di-isopropylfluorophosphate and n-hexyl phosphonate ethyl ester. The 3 D-structure of inhibited cutinase is almost identical to that of the native enzyme.

7) The reserved micellar system (AOT in isooctane) induces cutinase unfolding which exposes some tyrosine residues. Up to 97% of cutinase can be extracted into reversed micelles and 30% back-extrated into a new aqueous phase.

8) The solid-gas bioreactor as well as NMR spectroscopy are well suited techniques to study the role of water in lipase catalyzed reactions. The optimal activity and stability of cutinase was observed when one or more hydration layers are present around the enzyme.

9) A lipase from *Staphylococcus hyicus* was cloned and expressed in large amounts in *E. coli*. Surprisingly, α phospholipids are very poor substrates when spread at the air-water interface in contrast to what observed from bulk kineties.

10) It was shown that surface pressure controls lipase stereo selectivity.

11) Epitope mapping and immunoinactivation of human gastric lipase were achieved using bulk and monomolecular film techniques.

COOPERATIVE ACTIVITIES

Compiègne, April 26th (1992). Scientific discussions between J. Cabral and M.D. Legoy.

Marseilles, June 23nd (1992). Invitation to professor F. Spener (Münster) who presented a seminar entitled: 'Multifunctional FABP from fatty acids binding to growth inhibition'.

Utrecht, December 5th (1992). Scientific discussions with G.H. de Haas, R. Kaptein, S. Ransac and R. Verger.

Exchange of staff and students

Long term (> 1 year): Ransac, S. (Marseilles → Groningen; Martinez, C. (Marseilles → Vlaardingen); Carrière, F. (Marseilles → Copenhagen); Longhi, S. (Milano → Marseilles).

Short term (< 1 year): Deveer, A.M.T.J. (Utrecht → Marseilles); Ladefoged, C. (Copenhagen → Marseilles); Melo, E. (Lisbon → Marseilles); Ransac, S. (Groningen → Bochum).

EUROPEAN DIMENSION

See comments in the general overview.

JOINT PUBLICATIONS / PATENTS WITH TRANS-NATIONAL AUTHORSHIP

Ransac S., Deveer A.M.T.J., Rivière C., Slotboom A., Gancet C., Verger R. & de Haas G.H. (1992). Competitive inhibition of lipolytic enzymes. V. A monolayer study using enantiomeric acylamino analogues of phospholipids as potent competitive inhibitors of porcine pancreatic phospholipase A^2. Biochim. Biophys. Acta 1123: 92-100.

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Hjorth A., Wöldike H.F., Thim L., Gormsen E. & Verger R. Mammalian pancreatic lipase and variant thereof. Int. Patent Appln. No. 91610056.3.

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Moreau H., Abergel C., Carrière F., Ferrato F., Fontecilla-Camps J.C., Cambillau C. & Verger R. (1992). Isoform purification of gastric lipases: towards crystallization. J. Mol. Biol. 225: 147-153.
Van Tilbeurgh H., Gargouri Y., Dezan C., Egloff M.P., Nésa M.P., Rugani N., Sarda L., Verger R. & Cambillau C. (1993). Crystallization of pancreatic procolipase and of its complex with pancreatic lipase. J. Mol. Biol. 229: 552-554.
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3-Dimensional structure and catalytic mechanism of 2-3 selected lipases of industrial relevance (BIOT CT-900181)

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OBJECTIVES

The project will include the study of 3 lipases:
A. Guinea Pig Pancreatic Lipase (GPL) and mutants thereof
B. Human Pancreatic Lipase (HPL) and mutants thereof
C. Mucor Miehei Lipase (MML)
As previously reported, our study of GPL has shown that the 3D structure around the active site (Asp, His, Ser) and the lid region is extremely important in the understanding of the mechanism of action of lipases in general. As a consequence of these results, it has been decided not to work on human bite salt activated lipase (BSAL), but to include HPL and mutants thereof in the programme instead.

RESULTS

A. Guinea Pig Pancreatic Lipase (GPL)

The isolation, sequencing, cloning, expression and enzymatic characterization of GPL has been published (ref. 1). Crystallographic studies are currently in progress on GPL, and single crystals have been grown (the largest was 0.3 x 0.2 x 0.1 mm). Unfortunately, these crystals were extremely fragile, and a suitable stabilizing solution could not be found. Several crystals were mounted directly from the crystallization drops and exposed to X-rays. An attempt to collect data on the largest crystal using the Xentronics area detector gave very weak diffraction to about 8Å resolution. These data indexed reasonably well in a large rhombohedral cell with parameters $a = b = c = 225$Å, $\alpha = \beta = \gamma = 81°$ (this can be re-indexed into a hexagonal cell 302x302x429.2Å). Data reduction was less than satisfactory giving a merging R-factor of 28.5% for 87% of the data to 8Å resolution. Subsequent analysis of the hk0 reciprocal lattice plane suggested that the space group was R32. In order to calculate a realistic solvent content in the range 40-60%, there would need to be at least 12 molecules per asymmetric unit (possibly as many as 18!). Therefore, because of the fragility of the crystals, their poor diffraction and large number of molecules per asymmetric unit, this crystal form was not analyzed further.

In more recent experiments, a new crystal form has been obtained in the presence of phospholipid/bile salt mixed micelles using a recipe previously used to grow crystals of human pancreatic lipase (HPL) in the activated conformation (Verger and van Tilbergh, personal communication). These crystals were of octahedral morphology, suggesting at least orthorhombic, if not tetragonal symmetry. Similar crystals were grown using DNPP-inhibited protein. The largest of these grew to 0.3x0.2x0.2 mm, but as with the previous form showed poor X-ray diffraction. Spots were not visible much beyond 10Å resolution and there was insufficient data available for successful indexing using both Xentronics and R-AXIS systems. This second crystal form remains uncharacterized. Experiments are continuing towards

obtaining new crystal forms for X-ray diffraction work using both active and inhibited protein.

It is anticipated that a mutant rGPL will be available shortly where the glycosylation site Asn334 has been mutated to Gln. Although not heavily glycosylated, this site lies in the 'hinge' region between the N and C domains (by homology modelling with HPL). It is conceivable that random glycosylation, at this point, may result in conformational heterogeneity by affecting the angle between the two domains. Removal of the glycosylation may eliminate this heterogeneity and thereby aid successful crystallization.

The amino acid sequence of GPL is highly homologous to other known pancreatic lipase sequences, with the exception that it carries a large deletion in the lid domain that mediates access to the active centres of the other lipases. It is proposed that this deletion is directly responsible for the anomalous behaviour of this enzyme. In the absence of useful X-ray data, GPL modelling has been based on the HPL structure (Winckler *et al*, Nature *343* (1990), 771-774) and sequence comparison between GPL and HPL. The GPL model was built using the Turbo-FRODO program for residue replacement, deletions (18-residues in the lid domain) and insertions (Thr 54b, Asp 350b, Gly 424b). This model was subsequently subjected to molecular dynamics using the X-PLOR program.

From comparison of GPL modelling and HPL 3D structure, the core of the N-terminal domain appeared to be conserved overall, with the exception of the lid domain. Furthermore, within a 10Å radius sphere of the active serine (Ser 152 Oγ), there are no insertions/deletions and only four minor residue changes pointing away from the active site (Ile 78 (HPL) $\rightarrow$ Thr (GPL)) or buried ((Ala 157 (HPL) $\rightarrow$ Thr (GPL); Leu 264 (HPL) $\rightarrow$ His (GPL); Tyr 267 (HPL) $\rightarrow$ Ile (GPL)). The recent 3D structure of HPL in its active conformation (van Tilbeurgh *et al*, Nature *362* (1993), 814-820) showed how the active site, hidden under two surface loops, opens up like the petals of a flower. The conformational transitions in the lid and ß5-loop are characterized by a profound change in secondary and tertiary structure. In view of these results, GPL was also modelled based on this new HPL structure, the major difference with the previous model being the conformational change in the β5-loop thus creating the oxyanin hole.

The enzymatic characterization of native as well as recombinant GPL has been published in details elsewhere (ref. 1). The main conclusions from these results are that GPL differs from other pancreatic lipases in that: (1) it is not interfacially activated, (2) its activity is unaffected by the presence of bile salts and/or colipase using tributyrin as substrate, and (3) it exhibits equally phospholipase A_1 and lipase activities. The amino acid sequence of GPL is highly homologous to that of other known pancreatic lipases, with the exception of deletion in the so-called lid domain that regulates access to the active centres of the other lipases. We propose that this deletion is directly responsible for the anomalous behaviour of this enzyme. Thus GPL challenges the classical distinction between lipases, esterases and phospholipases.

B. Human Pancreatic Lipase (HPL)

A cDNA clone encoding the sequence of human pancreatic lipase (HPL) was subcloned into the baculovirus transfer vector pVL1392 and used in co-transfection of *Spodoptera frugiperda* (Sf9) insect cells with wild-type *Autographa californica* nuclear polyhedrosis virus (AcNPV) DNA. A single recombinant protein (50 kDa) secreted by Sf9 cells was detectable in the culture medium 24 hours post infection

using both anti-HPL polyclonal antibodies and potentiometric measurements of lipolytic activity. The expression level reached 40 mg/l of enzyme at 6 days. A single cationic exchange chromatography was sufficient to obtain a highly pure recombinant HPL as demonstrated by N-terminal sequencing, amino acid composition and carbohydrate analysis as well as by mass spectrometry. These analyses revealed the production of mature protein with the correct processing of signal peptide and a homogenous glycosylation pattern. The kinetic properties of recombinant and native HPL were compared. Both enzymes showed similar profiles of interfacial activation, inhibition by bile salts and re-activation by colipase.

C. Mucor Miehei Lipase (MML)

Two new crystal forms of the MML enzyme have been obtained. The first was using protein inhibited by a phosphonate ester with a 12-carbon acyl chain (referred to as C12). X-ray data were collected on these crystals to a maximum resolution of 2.6Å. The structure was solved by the molecular replacement technique using the native structure as a search model. As expected, there was a trimer in the asymmetric unit, and the C12 inhibitor was clearly visible in the electron density at the active site, with the lid in the open position. The second crystal form was produced by growing native enzyme crystals in the presence of phospholipid mixed micelles. The structure was solved by MR.

HIGHLIGHTS/MILESTONES

1. Kinetic characterization of native and recombinant GPL
2. Modelling of GPL based on HPL 3D structures
3. Development of a Baculovirus expression system suitable for lipases
4. Development of a one-step purification procedure for lipases produced in the Baculovirus expression system.
5. Expression and purification of HPL and mutants thereof in the Baculovirus system

WIDER CONSIDERATIONS

The Baculovirus expression system is extremely efficient for production of small amounts of lipase mutants for studies of structure/function relationship.

COOPERATIVE ACTIVITIES

Capri, October 1-3, 1992. BRIDGE Lipase Meeting
Marseilles, August, 1992. Working visit by Dr. Claus Ladefoged, Novo Nordisk, to CNRS Marseilles in order to transfer the oil drop tensiometer know-how.
Copenhagen, March 15, 1993. Visit by Robert Verger to Novo Nordisk. Scientific discussions.
Marseilles, April 30, 1993. Visit by Frédéric Carrière to CNRS Marseilles. Seminar on the Baculovirus expression system.
Delft, April 7, 1992. Meeting of coordinators
Brussels, February 22, 1993. Meeting of coordinators
Copenhagen, March 1, 1993. Planning of the next BRIDGE-Lipase meeting (Elsinore, October 10-13, 1992).

EUROPEAN DIMENSION

As a result of the agreement between the EFTA countries and the EC, it has been possible to arrange the next BRIDGE-Lipase meeting as a combined meeting

between the EC BRIDGE lipase project and the Nordic Industrial Foundation protein engineering programme.

LIST OF JOINT PUBLICATIONS/PATENTS WITH TRANS-NATIONAL AUTHORSHIP

Hjorth, A., Carrière, F., Cudrey, C., Wöldike, H., Boel, E., Lawson, D.M., Ferrato, F., Cambillau, C., Dodson, G.G., Thim, L. and Verger, R. A structural domain (the lid) found in pancreatic lipases is absent in the guinea pig (phospho)lipase. Biochemistry (1993), **32**, 4702-4707.
Thirstrup, K., Carrière, F., Hjorth, S., Rasmussen, P.B., Wöldike, H., Nielsen, P.F. and Thim, L. One-step purification and characterization of human pancreatic lipase expressed in insect cells. FEBS Lett. (1993) **327**, 79-84.

Characterization of lipases for industrial application (BIOT CT-900194)

COORDINATOR:
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R. VERGER, CBBM / CNRS, Marseille, F

OBJECTIVES

- Obtain structural information of lipases from prokaryotic species, in particular Pseudomonas species.
- Gather information concerning mechanism of action, substrate specificity and inhibitory compounds.
- Generate tailor-made lipases for application in e.g. Detergent industry using understanding of structure-function relationships

MAJOR PROBLEMS ENCOUNTERED

- Delays in crystallographic analyses.
- Lacking structural information for *Pseudomonas glumae* lipase prevented studies to correlate functional properties investigated with structural data.

Due to these delays encountered it was decided to include also other lipases in the programme aimed at elucidating structure-function relationships using specific (pseudo)glyceride substrates. In particular those lipases were considered of which a high resolution structure is already available, either through the Bridge-T project or from the protein databank.

RESULTS

1. Progress of crystallographic studies on *Pseudomonas glumae lipase*
(A. Cleasby until Autumn 1992 and M.E.M. Noble from January 1993)

A solvent flattened MIR map for *P. glumae* lipase has been the focus of the crystallographic studies carried out by Dr Anne Cleasby during this review period. Particularly helical regions in the protein structure were identified with confidence, however the extensive beta sheet structure forming the core of the protein has been more difficult to interpret. Using non-crystallographic symmetry various attempts were made by Dr Cleasby to optimize the interpretation of the map. Her initial attempts to relate the various molecules through symmetry transformations using the interpreted regions of the protein proved to be unsuccessful, however.

Dr Martin Noble has tried initially to produce an optimal MIR map. Various approaches to heavy atom refinement were tried, without producing a high figure of merit beyond 4Å resolution. Also, a number of different solvent flattening strategies have been used to improve the experimental phases, including application of histogramming techniques according to the program SQUASH. In addition, several new techniques have been used to identify and locate the non-crystallographic symmetry elements within the crystal with highly promising results.

Apart from the work focussed on interpretation of the MIR map, a broader search has been initiated for alternative crystal forms. This work will be continued for the *Pseudomonas glumae* enzyme as such and in the presence of inhibitors.

2. Progress of synthetic effort

The synthesis and purification has been completed for all six enantiomeric triglyceride analogues containing an O-methyl ether-, a decanoyl ester-, and a decanoyl amide function, respectively, at either position of 1-aminoglycerol and 2-aminoglycerol. The amounts produced should be sufficient to not only screen the activity of *Pseudomonas glumae* lipase, but also of the various lipases being investigated in the Bridge-T lipase project. A comparison of the effects being obtained for different lipases, together with their respective structural information will provide us with detailed information concerning the ways positional specificity may be determined for these lipases. In addition to these studies it was considered important to investigate the enzyme-substrate interactions in more detail requiring further synthetic substrates. A summary of the set of properties of these compounds is:

(i) a glycerol backbone having only one hydrolysable bond (primary ester function)
(ii) chain length variation at chiral centre (2-position)
(iii) chain length variation at site where hydrolysis occurs
(iv) chain length variation at site where no hydrolysis occurs

So far two compounds have been synthesized in the (S)-configuration having C9 chains at the chiral centre and the non-hydrolysable bond, respectively, together with either a C3 or C9-chain at the site where hydrolysis takes place. The synthesis of the corresponding (R)-isomers is near completion.

3. Progress of mechanistic studies on lipases

Activities concerned with the kinetic aspects of *Pseudomonas glumae* lipase have been postponed until all synthetic efforts have been completed and the crystal structure of the enzyme has been obtained.

HIGHLIGHTS / MILESTONES

Although crystallographic analysis of *Pseudomonas glumae* is taking somewhat longer than expected, attempts to complete this work will continue. Studies aiming at understanding e.g. stereo- and positional preference of lipases will be carried out using other lipases and the specific (pseudo)-glycerides mentioned above.

WIDER CONSIDERATIONS

Now that the Bridge T-project provides us with molecular details of lipases from various sources it will allow us to gain insight in the ways water-soluble enzymes are able to attack water-insoluble substrates. The picture that emerges indicates that true lipase action requires a (considerable) conformational change of the enzyme before effective interaction with the lipid substrates can take place. This indicates that further studies (e.g. molecular dynamics) will be needed to guide us in understanding the details of lipolytic action. The relatively high symmetry of triglyceride molecules is a complicating factor, when one wants to investigate to what extent (stereo)specific interactions between lipases and single substrates are coupled to the recognition of organised lipid-water interfaces and vice-versa. Most

likely future studies will require modification of both the enzyme (by protein engineering) and substrates to proceed in this area.

COOPERATIVE ACTIVITIES

April 7 1992 Coordinators meeting at Gist Brocades, Delft, The Netherlands
April 21 internal meeting Bridge Lipase T-project
May 18 -20th Visit Dr C.Cambillau, CNRS Marseille to Unilever Research Vlaardingen
June 19th Visit Dr H.M. Verhey and prof.dr G.H. de Haas to Unilever Research Vlaardingen
July 28th Visit prof.dr G.H. de Haas to Unilever Research Vlaardingen
October 1st-3rd Bridge Congress Meeting Capri, Italy
October 22nd Internal meeting Bridge Lipase T-project
November 17 (partial) team Meeting at Utrecht
February 22nd, Coordinators meeting Brussels
Staff exchanges: Dr C.Martinez from CNRS Marseille to Unilever Research Vlaardingen (post-doc position for 1 year)

EUROPEAN DIMENSION

The T-project on lipases takes advantage of the fact that research on lipase structure and function is focussed particularly in Europe. While the project is running it can be observed that collaboration across the different project teams is increasing. Thus interactions among research teams in Europe have become more frequent due to this project.

JOINT PUBLICATIONS

Peters, A.R., Dekker, N., van den Berg, L., Boelens, R., Kaptein, R., Slotboom, A.J. and de Haas, G.H. (1992), Biochemistry 31, 10024-10030
Deveer, A.M.T.J., Franken, P.A., Dijkman, R., Meeldijk, J., Egmond, M.R., Verheij, H.M., Verger, R., and de Haas, G.H. (1992) Biochim. Biophys. Acta 1125, 73-81
Deveer, A.M.T.J., den Ouden, A.T., Vincent, M., Gallay, J., Verger, R., Egmond, M.R., Verhey, H.M., and de Haas, G.H. (1992) Biochim. Biophys. Acta 1126, 95-104
de Haas, G.H., Dijkman, R., Lugtigheid, R.B., Dekker, N., van den Berg, L., Egmond, M.R. and Verhey, H.M. (1993) Biochim. Biophys. Acta, in press
Deveer, A.M.T.J. (1992), thesis University of Utrecht, 'Mechanism of activation of lipolytic enzymes'
Frenken,L.G.J. (1993), thesis University of Utrecht, '*Pseudomonas glumae* lipase: Characterization, biogenesis and protein engineering'

Exploring the structure-function relationship of *pseudomonas* and *bacillus* lipase (BIOT CT-910272)

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U. WINKLER, RUB, Bochum, D
B.W. DIJKSTRA, RUG, Groningen, NL

OBJECTIVES

The second reporting period (01/04/1992 — 31/03/1993), together with the first reporting period, comprises 20 months of the total project. According to the Working Programme (Annex I of the EC-contract and Annex I of the Consortium Agreement), in this period the activities should be directed towards purification of enough lipase for subsequent crystallization experiments, 3D-structure determination, site directed mutagenesis and enzymatic characterization of the lipases.

MAJOR PROBLEMS ENCOUNTERED

The purification and crystallization of the *Pseudomonas* lipase was severely hampered by problems encountered during the fermentation of said microorganism. The initial level of lipase in the fermentation broth (30-60 mg/L) could not be reproduced, instead, only 10 mg/L was repeatedly found. This posed tremendeous problems to the Bochum group in purifying enough lipase of good quality for the crystallization trials. A recent study of the fermentation process revealed that the majority of the (very hydrophobic) lipase ($>90\%$) was bound to the cells while these were removed by centrifugation. Treatment of the cells with Triton X-100 solubilized the enzyme; now some 100 mg/L lipase can be recovered.

RESULTS

1. Pseudomonas aeruginosa lipase

1.a Purification of Pseudomonas lipase (RUB)

From the first fermentation broth containing 30-60 mg/L, three batches of purified lipase were prepared containing in total 10 mgs of pure enzyme. This material was used by the Groningen group for crystallization experiments (see below). Although the lipase was pure in the sense of protein content, the samples still contained lipopolysaccharides (LPS), a cell wall component of Pseudomonas species, which seems very difficult to get rid of.

The culture supernatants containing low levels of lipase (10-20 mg/L) were concentrated by ultrafiltration which, however, was accompanied by an enormous increase in viscosity. After ultracentrifugation of this mixture, the supernatant was extracted with heptane which was effective in reducing the viscosity without affecting the lipase activity.

Purification of lipase from extracted cells (see above) is currently underway.

1.b Enzymatic characterization of Pseudomonas aeruginosa lipase (RUB)

- The presence of an intact disulfide bridge connecting Cys183 and Cys235 was demonstrated by a shift of the electrophoretic mobility after treatment of purified lipase with dithiotreïtol. Additionally, it could be shown that the disulfide bond is susceptible to reduction only after the protein was pretreated with detergent (e.g. 0.025% SDS).
- Digestion of lipase with protease V8 yielded 2 lipase fragments of 25 and 26 kDa respectively without affecting the enzyme activity. Experiments using the different buffer systems favouring cleavage either behind Asp-residues or Asp's and Glu's indicated that Asp38 is more accessible than Glu46 as predicted from the 3D-model.
- Digestion of lipase with subtilisin resulted in a complete loss of enzyme activity. Analysis on SDS-PAGE showed that the 29 kDa lipase protein band completely disappeared. At present, experiments are performed to work out conditions allowing limited proteolysis and detection of peptide fragments based on predictions derived from subtilisin digestion of *Pseudomonas glumea* lipase.
- Three different peptides were identified which are surface-exposed according to the 3D-model and should be accessible for recognition by antibodies. These peptides were chemically synthesized, conjugated with a high molecular weight carrier molecule (KLH) and used to immunize rabbits. Antibodies should allow to identify surface-exposed domains of the *Pseudomonas aeruginosa* lipase.

1.c Molecular genetics of lipase (RUB)

- Strains are constructed which are isogenic to wild type *Pseudomonas aeruginosa* except for having an antibiotic resistance insertion in either the lipase structural gene lip A (tet^r) or the lipase helper gene lip H (cb^r) by using a newly developed system which allows to specificallly screen for *Pseudomonas aeruginosa* transformants containing such insertions. Both genes have been isolated, subcloned and resistance genes have been inserted. Subcloning into appropriate shuttle vectors and transformation of *Pseudomonas aeruginosa* wild type strains are currently done.
- The lipase structural gene lip A was amplified by PCR, subcloned into pT7-7 and transformed into *Escherichia coli* BL21/DE3. Upon induction with IPTG only low lipase activity (2% of the amount produced by *Pseudomonas aeruginosa* PAO1) was detected indicating that a functionally active *lip H* protein was necessary to obtain enzymatically active recombinant protein.
- The lipase operon consisting of genes *lip A* and *H* was subcloned into vectors pBlue-scriptII SK and pT7-7 and transformed into *Escherichia coli* JM109 and BL21/DE3. Upon induction with IPTG both strains produced enzymatically active lipase at a level comparable to wild type *Pseudomonas aeruginosa* PAO1. Analysis of whole cell lysates by SDS-PAGE and Western-blotting indicated that a significant amount of lipase protein was present intracellularly as an inactive 32 kDa precursor.
- *Pseudomonas aeruginosa* wild type strain ADD1976 containing a chromosomal insertion of the T7 RNA-polymerase gene was transformed by electroporation with plasmid pEB12 containing the lipase operon *lip AH*. After induction with IPTG this strain produced 200 µg/L of enzymatically active lipase which is about 6 times the amount produced by wild type strain *Pseudomonas aeruginosa* PAO1.

— Genes *lip A* and *H* were both amplified by PCR, subcloned into vector pUC18 and transformed into *Escherichia coli* JM109. Determination of the DNA-sequence from both 3'-OH and 5'-P-ends yielded a correction of the published DNA-sequence coding for the C-terminal part of protein *lip H* which now perfectly fits sequences of other known H-analogous proteins. Additionally, a protein of 37 kDa was obviously overexpressed as judged from SDS-PAGE analysis of whole cell lysates. Presently is examined whether this overexpressed protein crossreacts with antibodies against the *lip B* protein from *Pseudomonas glumae*.

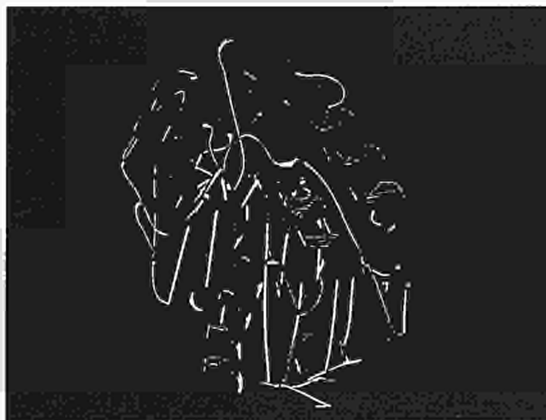
1.d Crystallization of Pseudomonas lipase (RUG)

Until now, no crystals of sufficient size and quality of the *Pseudomonas* lipase have been obtained. The 10 mgs of pure enzyme, which have been obtained, were subjected to a great variety of crystallization conditions and only in the case of the precipitants 2-methyl-2,4-pentanediol and ethanol (at 4°C and in the presence of n-octyl-β-D-glucoside) some tiny crystals and clusters of crystalline plates were obtained, however, not suited for X-ray diffraction.

Conditions in which other *Pseudomonas* lipases (from *P. cepacia, P. glumae, P. putida* and *P. fluorescens*) gave crystals, were not successful with the lipase from *Pseudomonas aeruginosa*. This can be explained by the extreme hydrophobic nature of this lipase and/or by the presence of contaminating lipopolysaccharides which are present in the purified enzyme preparation (see above). The latter — which usually form a heterogeneous molecule population — may strongly interfere with the crystal growth. Currently, there are attempts to remove the LPS via specific purification protocols.

1.e Model building of Pseudomonas lipase (RUG)

In the present reporting period, the 3D-model of the *Pseudomonas aeruginosa* lipase which was built as explained in the previous progress report, was further refined and brought in alignment with experimental results such as the presence of a disulfide bridge, the absence of interfacial activation (no flap) and proteolytic cleavage sites. Figure 1 shows a ribbon plot structure structure.



2. *Bacillus subtilis lipase*

2.a *Analysis of the protein sequence (Gb)*

The cleavage position of the signal sequence of the precursor protein was confirmed experimentally by amino acid sequencing of the N-terminus of the mature, purified lipase: H_2N-AlaGluHisAsnProValValMetVal., etc.

2.b *Expression and overexpression of the Bacillus lipase (UCL)*

— The signals for secretion and for translation initiation in *B. subtilis* are very good as well as the translation and transcription arrests, suggesting that the poor yield of the enzyme in *B. subtilis* resulted from poor transcription and/or low copy number of the gene.

— Physical deletion of the whole *lip* gene and somc of its surrounding DNA was necessary in order to be able to construct a stable strain with the *lip* gene on a multicopy plasmid while avoiding recombination with homologous chromosomal DNA. The appropriate deletion was constructed *in vitro* and a K_N^R::lip disruption on the chromosome, constructed previously (V. Dartois, PhD-thesis, 1992), was replaced by the deletion by screening for K_N-sensitive transformants.

— A *lip* overproducing plasmid was constructed by amplifying the whole lip gene by PCR, flanked with appropriate restriction sequences and inserted into plasmid pMA5. In a further step, the plasmid was deleted *in vitro* from its *E. coli* moiety and transformed into *B. subtilis*. The final construction (BCL1051) is the current strain for overproduction of *B. subtilis* lipase.

2.c *Purification of Bacillus lipase (UCL)*

The overproducing strain gave rise to expression levels in the culture supernatant which were 40-100 fold higher as for the wild type strain. From 30 L supernatant, a total of 135 mg highly purified lipase was obtained (overall yield only 8-10%). This amount is more than enough for the crystallographic experiments to be carried out in this project.

2.d *Enzymatic characterization of Bacillus lipase (UCL)*

— From chromatofocussing experiments an **isoelectric point** of 9.9 was deduced, which corresponds nicely with the calculated value of 9.79.

— *Bacillus subtilis* lipase shows 1,3-positional **specificity**. This was deduced from hydrolysis experiments with triolein: Only 1,2-diolein is formed and 2-mono-olein is accumulating; 1-mono-olein is not detetcted.

— The temperature optimum for **activity** is 35°C

— The pH-optimum for **activity** is pH = 10 with 50% values at pH 6 and 11.5

— The enzyme is **stable** up to temperatures of 40°C (100% activity after 30 min. at 40°C) and the stability gradually decreases at higher temperatures (0% activity after 30 min at 55°C).

— The enzyme is stable in a pH range of 4-13, with the highest **stability** at the alkaline side (100% activity recovery after 24 hrs at pH = 12, still 75% after 24 hrs at pH = 13). Below pH 4, a rapid inactivation is observed.

— The **stability** of the enzyme in organic solvents is as follows: stable for 24 hrs in 30-60% DMSO, 30% DMF and 30% methanol, less stable in 60% DMF and

60% methanol, but very unstable in isopropanol, ethanol, pyridine and acetone (all at 30 and 60 % v/v and room temperature).

2.e Crystallization of Bacillus lipase (RUG)

The large amount of this enzyme which was made available allowed for a great variety of crystallization experiments at high concentrations of lipase (10 mg/mL). So far, some crystals of reasonable quality were obtained with PEG-4000 (31-36%) as a precipitant, in combination with Li_2SO_4, octylβ-D-glucoside, alkaline pH (around 9.0) and at 22°C.
Two of the crystals were mounted and analyzed with X-rays. In one case, a 3.5 Å data-set was obtained which is complete up to 4.5 Å resolution.
Using the model of the *Bacillus* lipase, which was built analogeous to the *Pseudomonas* lipase model, it was tried to find the orientation of the lipase molecule in the diffraction dataset by a cross rotation function. Further analysis is in progress.

2.f Model building of the Bacillus lipase (RUG)

Since there exists some homology with *Pseudomonas* lipases, the model for the *Bacillus* lipase was built using the model of the former as a template. With the exception of the active site serine, the other active site residues are still hypothetical.

HIGHLIGHTS/MILESTONES

1. Thanks to the creation of an overexpressing strain, some 130 mgs of highly purified *Bacillus* lipase could be obtained.
2. The first crystallization experiments with the highly purified *Bacillus* lipase gave already promising results in the sense that a first diffraction data set at 3.5 Å resolution could be recorded.
3. The model for the *Pseudomonas* lipase has been refined and brought in alignment with experimental data, thus increasing its realibility.

COOPERATIVE ACTIVITIES

Meetings — The project team had the following project meetings in the reporting period: 30 June 1992 (Groningen), 13 November 1992 (Bochum), 5 March 1993 (Delft). The next meeting is scheduled for 2 July 1993 in Louvain-la-Neuve.
On 12 January 1993, there was a special meeting between Jäger and Gist-brocades scientists discussing the problems with the expression of the *Pseudomonas* lipase and the experimental set-up which finally led to the solution of the problem.

Materials and staff exchanged — Bochum has obtained cell-free supernatants of several *Pseudomonas aeruginosa* fermentations, as well as from detergent treated cell pellets.
Groningen has received in total 10 mgs of purified lipase from *Pseudomonas aeruginosa* (RUB) and 60 mgs purified lipase from *Bacillus subtilis* (UCL).
M. Angela Taipa from the Instituto Superior Tecnico Secça de Biotechnologia (Lisboa, Portugal) worked in Bochum from 8 March-3 April, 1993. She did biochemical purification and characterization of the lipase from *Chromobacterium viscosum*.

Lectures — During the last project meeting in Delft, Dijkstra (RUG), Colson (UCL) and Jäger (RUB) gave lectures for the R&D-organization of Gist-brocades about the lipase research.

PUBLICATIONS

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Molecular structure and specificity relationship of microbial triacylglycerol lipases (BIOT CT-910258)

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OBJECTIVES

Purification and crystallization of selected microbial lipases. X-ray structure elucidation of the lipases from Geotrichum candidum and from Pseudomonas strains. Cloning, sequencing and expression of lipases from Rhizopus oryzae, Candida cylindracea and Geotrichum candidum. Kinetic and inhibition studies aiming at the understanding of their mechanism of action.

RESULTS

The project is focussed on the structure elucidation, protein engineering and mechanistic investigation of various fungal and bacterial lipases, with emphasis on the Geotrichum/Candida, the Rhizomucor/Rhizopus and the Pseudomonas lipase family.

1. Lipases of the Geotrichum/Candida family.

Investigations were carried out on 3 lipases from this organism, namely G. candidum Amano (GCM) (Münster and GBF), G. candidum CMICC 335426 (GCL-A and GCL-B) (Unilever). Whereas GCL-B is unique with respect to its high specificity for cis delta-9 unsaturated FA, GCM is only moderately specific, but hydrolyzes phospholipids as well (Marseille). The structure of G. candidum ATCC 34614, a homologue of GCM, was published by M. Cygler et al., Montréal.

Lipase GCM: a purification protocol is available (Münster), and crystallization has been achieved (GBF) resulting in a x-ray data set for the native enzyme (resol. about 2.5 Å). Microheterogeneity was checked by MALDI-MS (Münster).

Lipase GCL-B and -A (Unilever): both genes have been fully sequenced. Comparative peptide mapping gave indication of some heterogeneity in the amino acid sequences, as confirmed by amino acid sequencing of the pertinent peptides, and one O-linked site and two potential N-linked sites have been shown to be glycosylated by comparing results before and after endoglycosidase treatment. Carbohydrate analysis revealed the major sugar component to be mannose. Galactose, N-acetylglucosamine and glucose are also present. GCL-B could not yet be expressed in E. coli, but expression in S. cerevisiae was proven by Western blot analysis. However, the intracellular enzyme is inactive. After removal, by using aqueous two-phase systems, of the Triton-X100 used in GCL-B isolation, the

enzyme could be crystallized (GBF) and preliminary x-ray analysis showed diffraction down to 4.5 Å.

Next steps: optimization of expression of active GCL-B (Unilever). Optimization of crystallization protocol of GCL-B (GBF). X-ray studies of GCL-B and GCM (with M. Cycler, Montreal). Mechanistic studies (Münster, GBF, Unilever, Marseille, Graz).

Candida cylindracea

All investigations were carried out on the lipases of Candida cylindracea ATCC 14830 (CCL-LIP1-LIP5) (Milano). In the reporting period, a fifth gene coding for lipase was cloned and sequenced, thus characterizing a multigene family composed of five structure genes. All genes are likely to be expressed, since they are preceeded by cononical CAAT and TATAA boxes. Suitable expression vectors for S. cerevisia have been constructed for high-level expression and regulation by the C-source in the medium. Since Candida lipase has been shown to use codon CUG for serine, whereas CUG in yeast (and in all other organisms studied so far) codes for leucine, a rational approach for mutagenesis of the 16 — 19 serine residues in the Candida lipase multigene family has been planned, based on a 3D-structure model. Mutagenesis of the first two codons has been performed by PCR and results are being evaluated.

Next steps: expression of multigene family in yeast. Mutagenesis of serine residues. Comparison of (unpublished) Candida x-ray structure (M. Cygler, Montreal) with multigene family sequences.

2. Lipases of the Rhizopus/Rhizomucor family

Investigations were carried out on the lipase from Rhizomucor miehei (RML), and on three Rhizopus lipases, namely the enzyme from Rhizopus niveus (RNL), from Rhizopus arrhizus (RAL) and from Rhizopus oryzae (ROL).

Rhizomucor miehei (GBF): the structure of the enzyme was solved at a resolution of 2.3 Å, based on the data of the C-alpha-trace published by Derewenda et al., using synchrotron radiation.

Rhizopus niveus (GBF): based on the RML coordinates and the published sequence of RNL, which is 63% homologous to RML, a model of the RNL was prepared.

Rhizopus oryzae: based on joint activities at the GBF and Münster, the lipase was cloned at the GBF. Only one gene could be found, implying post-translational modification to account for the two different phenotypes of this enzyme. Homology to the published sequences of RNL and R. delemar is 99% (2 amino acid replacements out of 269). Overexpression in E. coli led to inclusion bodies which were located in the periplasmic space. A refolding protocol was elaborated providing high yields of a purified lipase with a spec. act. of 8600 U/mg, identical to the native enzyme. The enzyme was modeled on basis of the RML structure and the RNL model, and appropriate sites for specific mutagenesis were identified.

Rhizopus arrhizus: the enzyme was purified from a commercial sample (Münster) and sent to Marseille for crystallization, possibly for x-ray analysis.

Next steps: elucidation of mechanism of action of ROL by site-directed mutagenesis (GBF, Münster). Modelling of lipase-substrate interactions and proof by site-directed mutagenesis (GBF, Münster).

3. Lipases of the Pseudomonas family

There might be several families of P. lipases. Investigations were carried out on 3 lipases, namely on the lipase from Pseudomonas spec. ATCC 21808 (PSL), from Pseudomonas cepacia (Amano)(PCL) and from Chromobacterium viscosum (Asahi Chemicals)(CVL).

Pseudomonas spec PSL: a native data set was obtained and awaits structure analysis by molecular replacement (GBF). The sequence of the enzyme is available through patent information.

Pseudomonas cepacia PCL: a purification protocol was established (GBF), and crystals were obtained which diffract to < 2.5 Å. The N-terminal sequence is identical to 22 residues with PSL and CVL.

Chromobacterium viscosum CVL: a purification protocol from a commercial sample (Asahi Chemicals, formerly Toyo Jozo) was established and the enzyme was characterized, with special focus on microheterogeneity (Münster). Isoforms were isolated using preparative IEF. Inhibition studies revealed little susceptibility towards serine inhibitors. Complete inhibition was observed with tetrahydrolipstatine in isopropanol (Münster), and attempts to crystallize the complex are underway (GBF). Enantioselectivity of CVL lipase was investigated with 2-O-hexadecylglycerol obtained from Graz.
CVL was crystallized in an orthorhombic form (GBF), and five heavy atom derivatives could be obtained. The electron density map is resolved at a R-factor of 6.2%. To date, about 40% of the amino acids have been fitted.

Next steps: completion of the CVL structure, and joint publication of the CVL and Pseudomonas glumae structures with the Münster/Unilever/Oxford groups. Solution of PSL and PCL structures by isomorphous replacement. Studies on mechanism of action by site-directed mutagenesis based on the gene from P. glumae.

4. Other microbial lipases

A thermophilic and a psychrophilic lipase were screened and partially characterized (GBF).
The 16 kDa thermophilic lipase originates from a strain of *Bacillus thermocatenulatus*. A purification protocol has been established. N-terminal sequencing revealed 15 hydrophobic residues among 29 amino acids, and no homology to other lipases, including a patented thermophilic lipase from a Bacillus stearothermophilus. The 19 kDa psychrophilic lipase originates from an unclassified gram-negative strain isolated from antarctic sediments. A purification protocol has been established.

5. Kinetic and mechanistic studies

A range of triglyceride analogues were synthesized (Graz) and provided to Münster for kinetic studies. Lipid ether analogues were supplied to the GBF for attempted co-crystallization.

HIGHLIGHTS/MILESTONES

Purification protocols available for most lipases under study. Diffracting crystals obtained for most lipases under study. Structure elucidation of several Pseudomonas lipases in progress, of two Geotrichum lipases feasible. Candida and Rhizopus lipases cloned and studies on site-directed mutagenesis initiated.

Mechanistic studies initiated via genetic engineering/computer modelling and via kinetic studies of substrate analogues.

WIDER CONSIDERATIONS

The project proceeds smoothly, but competition is strong, in particular from Canada and the USA. Efforts are being made to enhance competitiveness by joining forces with other BRIDGE lipase groups, and by collaboration with the group in Montreal, Canada.

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G. Wohlfahrt, H. D. Beer, M. Hirai, U. Menge, D. Schomburg and R. D. Schmid: Protein design of lipases from Rhizopus species. Poster prestented at CEC International Workshop 'Lipase: Structure, Mechanism and Genetic Engineering', Capri, Oct. 1992

T-PROJECT

"LACTIC ACID BACTERIA"

Improvement and exploitation of lactic acid bacteria for biotechnology purposes (BIOT CT-910263)

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ORGANISATION OF THE PROJECT

The project is organised on a CORE and LEAF structure that facilitates coordination and provides maximum integration of participating groups whose research is targeted to specific objectives. CORE groups emphasise developments in genetic analysis of Lactic Acid Bacteria (LAB) and link to the LEAF groups that focus on key sectors of research needed to underpin a more scientific and controlled use of these bacteria.

CORE TECHNOLOGIES

OBJECTIVES

1. Gene analysis and the study of gene expression and regulation in LAB including the characterization of chromosomal loci in *Lactococcus*.
2. Development of genetic tools, particularly food-grade integration/stabilization/expression systems.
3. Study of conjugation mechanisms in *Lactococcus* and *Lactobacillus*.
4. Analysis of recombination systems in *Lactococcus*.
5. Characterization of plasmid replication, stability and incompatibility functions in *Lactococcus*.

RESULTS

1. Gene expression and regulation

(a) Studies relating to control of expression of the lactose operon provided clear *in vivo* evidence for autoregulation of the repressor gene, lactose gene control by glucose repression and the terminator that follows the *lac*R gene. The versatility of the lactose promoter in providing variable levels of heterologous gene expression was demonstrated. Advanced molecular analysis of the *lac*R repressor has located the recognition helix and provided mutants with an improved operator building capacity. The role of tagatose-6-phosphate as the physiological inducer of the *lac* operon has also been established.

(b) Efforts to study genes whose expression responds to environmental stimuli focused on the isolation of heat shock genes in lactococci. Chromosomal regions harbouring *dnaK* and *groEL* were identified and the former was characterized down to the DNA sequence level. This region was shown to consist of four ORFs, three of which are co-transcribed. ORF1 corresponded to the *orf* 9 and *orf* 39 of *C. acetobutylicum* and *B. subtilis*, respectively. ORF2 exhibited homology with *grp*E found in many bacterial species and ORF3 represented the DnaK protein.

(c) Several of the regulatory mechanisms involved in the control of the *trp, his* and *leu-ilv* operons have been identified and all three pathways were shown to be controlled at the level of transcription initiation and transcription elongation. The inability of dairy strains of lactococci to synthesise histidine and branched chain amino acids, even though they harbour the relevant operons, was shown to be due to multiple lesions which included frameshifts, stop and missense codons. Phenotypic analysis indicated that different mutations are present in *his* and *ilv* operons in different dairy strains. Systematic sequencing of small fragments of the *his* operon of *cremoris* and *lactis* subspecies of *L. lactis* has shown that they are on average approximately 20% divergent whereas strains of the same subspecies are less than 3% divergent.

(d) The gene encoding the *S* layer of *Lactobacillus helveticus* CNRZ 892 has been cloned and partially sequenced and homology between it and a gene encoding the *S* layer of another *Lb. helveticus* strain was observed. The promoter of the *S* layer gene is being characterized as a prelude to expression studies.

2. Development of genetic tools

(a) A family of food-grade integration vectors for *Lactococcus* species was constructed. These are based on the broad host range plasmid pWV01 from which

the *rep*A gene has been deleted. While these can be maintained in hosts harbouring *rep*A *in trans*, they are unable to replicate in other Rep^- hosts. However, when they harbour a segment of lactococcal chromosome, they integrate by Campbell-type recombination into the homologous region of the genome. The vectors also contain sucrose genes, which constitute a food-grade selectable marker. This system has been used successfully to introduce genes in single or multiple copies in the target lactococcal chromosome. Further successful applications include the delivery of mutated genes into the chromosome, its use as a random mutagen and the construction of chromosomal transcriptional fusions.

(b) The chromosomally-integrated lactose operon in *L. lactis* MG5270 was exploited for the stabilized, controlled expression of heterologous genes. Integration in the *lac*G gene provided a useful strategy for food-grade controlled and stabilized expression of cloned genes and the effectiveness of the system was successfully demonstrated using the *Listeria* bacteriophage lysin.

(c) The *Vibrio* luceriferase gene has been effectively exploited as a rapid, sensitive reporter system to quantify levels of gene expression in LAB and it is intended to use this system for the isolation of physiologically controlled promoters. Another family of vectors based on a thermosensitive plasmid and incorporating the transposable element, ISS1, was developed to analyze genes involved in growth control, the stress response and the transition to stationary phase.

3. Conjugation

(a) The sex factor element which is responsible for conjugal transfer of chromosomal and plasmid DNA in *L. lactis* 712 is being subjected to molecular characterization. The gene encoding the aggregation protein which is responsible for cell to cell adhesion during conjugation was identified. A 30 kDa surface protein was isolated from lactococci harbouring the plasmid encoded fertility factor, *clu*, which is responsible for high efficiency mating in lactococci. The sugar cellobiose was shown to induce the Clu phenotype in *clu* strains. The gene encoding the Aggregation Promoting Factor (APF) in *Lactobacillus plantarum* 4B2 has been cloned and is being sequenced and the gene and its product are being compared with the surface adhesion of *Enterococcus faecalis.*

(b) A region on the lactococcal phage resistance plasmid required for its conjugative mobilization was localized and sequenced. Two ORFs were identified, one of whose products exhibited homology with mobilization proteins from two *Staphylococcus aureus* plasmids.

4. Recombination systems in *Lactococcus*

A *rec*A mutant of *L. lactis* MG1363 was constructed using the temperature sensitive delivery vector pG^+ host. The mutant was extremely sensitive to UV light and to DNA-damaging agents and was at least 10^4-fold less able to perform homologous recombination. This strain will prove valuable in advancing the genetic analysis of lactococci and will be used in a range of studies such as those concerning plasmid recombination events and prophage repression.

5. Plasmid replication, stability and incompatibility

The minus origin of the rolling circle replication (RCR) plasmid, pWV01, has been characterized. This was found to contain two inverted repeats which, when deleted, sharply reduced the copy number of the plasmid. The replication protein of

pCI305, which is likely to replicate by a theta mode, is being isolated and purified to allow for *in vitro* analysis.

The small theta replicating plasmid, pWV02, is being used as the basis for the construction of a highly stable food-grade vector in which sucrose genes will constitute the selective marker.

The region directing the replication of pCI528 was localized and sequenced. Significant homology between the Rep protein and the equivalent proteins of other theta replicating plasmids was observed. In addition, a non-coding AT-rich region upstream of *repB* contained several structural motifs usually present in replication origins.

HIGHLIGHTS / MILESTONES

— Protein engineering of the LacR repressor.
— Identification of factors mediating aggregation and clumping involved in conjugation in *L. lactis* 712.
— Identification of a locus involved in conjugative mobilization of lactococcal plasmid pCI528.
— Development of food-grade integration vectors.
— Identification and analysis of silent genes in *Lactococcus*.
— Cloning and characterization of a gene cluster encoding the heat shock gene *dnaK*.

PROTEOLYSIS LEAF

OBJECTIVES

1. Molecular characterization of proteinase enzymes of LAB and analysis of factors controlling proteinase gene expression.
2. Biochemical and genetic analysis of peptidase enzymes in *Lactococcus* and *Lactobacillus*.
3. Analysis of the amino acid and peptide transport systems in *Lactococcus* and *Lactobacillus*.
4. Cheese manufacturing trials using lactococcal strains with a manipulated proteolytic system.

RESULTS

1. Proteinases

It has been demonstrated that proteinase production by *Lactococcus lactis* is controlled at the transcriptional level. In this control a chromosomally located gene is involved. Successful attempts to engineer the stability and specificity of *L. lactis* SK11 proteinase have been reported. Furthermore, a new serine proteinase was discovered which is membrane-bound. This proteinase (nisP) is coded by one of the genes of the nis operon. The enzyme splits a leader sequence from the pre-pro-nisin.

A serine proteinase of *Lactobacillus bulgaricus* ACADC235 has been studied. The chromosomally located gene encoding the proteinase was cloned in *E. coli* and partially sequenced. It was also observed that casein is phosphorylated and can be dephosphorylated by extracellular acid phosphatases of lactobacilli and lactococci. The dephosphorylated casein seems to be more readily attacked by proteinase.

2. Peptidases

An endopeptidase in *L. lactis* that can cleave peptides with 6 to 24 amino acids has been discovered. The substrate specificity and the nucleotide sequence indicate that it is a new endopeptidase, although some of the biochemical properties are similar to previously described enzymes.

The cloning and sequencing of an endopeptidase (Pep 0) and a tripeptidase (Pep T) have been reported. The Pep T gene is homologous to the tripeptidase of *Salmonella typhimurium*. None of the peptidase genes sequenced so far indicate the presence of an N-terminal signal peptide. Inactivated *pep X* and *pep 0* genes had no effect on growth in milk. However, inactivation of both *pep X* and *pep N* severely impaired growth in milk.

Restriction site polymorphism has been observed predominantly in *pep N Communautégenes and also in pep X* genes in a number of lactococci. In *L. lactis* ssp. *cremoris* Wg2 90% of the lysyl-aminopeptidase activity is due to Pep N whereas in other strains this activity is mainly due to other enzymes (Pep C). The genetics of lactococcal general aminopeptidases (GAP) have been characterised and studies initiated on the sequencing of a lactococcal dipeptidase gene.

There has been considerable progress in the analyses of the proteolytic system of *Lb. delbrueckii* spp. *lactis* strain DSM 7290. By screening a low copy number plasmid library of the lactobacillus genome on *E. coli* strains lacking the respective enzyme activities eight peptidase genes were cloned. The *pep P* gene has been overexpressed in *E. coli* and the product was found to be a serine proteinase. A dipeptidase (*pep D*), an amino peptidase (*pep L*), a cysteinpeptidase (*pep C*), and a prolidase (*pep Q*) are or have been overexpressed and the enzymes are currently being biochemically characterised. An amino peptidase from *Lb. delbrueckii* ssp. *bulgaricus* with substrate specificities different from Pep N and Pep C has been purified. The protein has a mol. mass of 32 kD. A second amino peptidase (Pep C-like) and an endopeptidase are currently being biochemically characterised.

Some of the protein degradation products formed by the extracellular proteinase(s) can be taken up by the peptide and amino acid uptake systems, while others will be degraded further by extracellular peptidases. So far no solid evidence for the presence of extracellular peptidases has been presented. By using mono- and/or polyclonal antibodies some peptidases have been found to be located at the cytoplasmic membrane. This aspect will receive further attention by several participants in the next period.

3. Transport Systems

A branched chain amino acid transport system in *Lb. delbrueckii* ssp. *lactis* WS87 which was homologous to amino acid-transport systems of other bacteria has been cloned and sequenced. In a study of the peptide transport system of *L. lactis*, two systems were found: a di-/tripeptide transport system that is driven by the proton motive force and an oligopeptide transport system that is driven by ATP (primary transport system). The gene for the di/tri-peptidase transport system and the genes encoding the oligopeptide transport system have also been cloned and sequenced.

4. The effects of proteinases and peptidases on cheese-ripening

The effect of recombinant strains of lactococci in which the proteolytic system had been manipulated on cheese ripening was studied. Strains were used with an elevated level of proteinase activity; harbouring a neutral proteinase from

B. subtilis and overexpressing aminopeptidase Pep N activity. Only with strains harbouring the neutral proteinase were enhanced rates of cheese ripening observed. Very promising results have been obtained with strains that express the neutral proteinase mixed with the original strains.

HIGHLIGHTS / MILESTONES

- Demonstration that the DtpT polypeptide represents a novel secondary transport protein.
- Improved assay to facilitate the study of the transport of non-radiolabelled (oligo) peptides.
- Identification of an oligopeptidase (Pep O) important in the hydrolysis of β-casein
- cloning and sequencing of genes encoding a tripeptidase (Pep D).
- Cloning and partial sequencing of a gene encoding a new lactococcal endopeptidase.
- Demonstration that the peptidolytic genes determine the peptide profile in hydrolysed casein.
- Excellent progress in the use of *E. coli* based genetic strategies to clone protease/peptidase genes for lactobacilli.
- Characterisation of a cell-wall serine proteinase and an endopeptidase of *Lb. delbruckii* spp. *bulgaricus*.

PHAGE LEAF

OBJECTIVES

1. Characterization and molecular analysis of phages isolated from members of the LAB, i.e. *Lactococcus* and *Lactobacillus* species and *Streptococcus thermophilus*.
2. The study of phage/host interactions in LAB, particularly phage resistance mechanisms, lysogeny and cell surface bacteriophage receptors.

RESULTS

1. Molecular characterization of bacteriophage

Two different lactococcal phages are being analyzed at the DNA sequence level. Phage r1-t, lysogenic for *L. lactis* subsp. *cremoris* R1, possesses a linear double stranded DNA genome of 34 kb with cohesive ends. 95% of the genome has been sequenced and most of the ORFs which have been identified are in the same orientation. In addition, it has been found that the phage genome integrates at a unique site in the R1 chromosome.

Phage Tuc2009 from *L. lactis* subsp. *cremoris* UC509 possesses a 40 kb genome which is circularly permuted with terminal redundancy. Approximately 80% of the genome has been sequenced. The attachment site (*att*P) and ORFs responsible for two of the major structural proteins of the phage have been identified. ORFs exhibiting homology to other characterized lysin and integrase genes have also been located.

The temperate *S. thermophilus* phage TP-J34 is also being characterized at a molecular level. This phage can be induced by exposure of the host to mitomycin C or UV-light treatment or by a 40-45°C temperature shift. Prophage-cured derivatives of the host have been isolated and were found to form aggregates

which readily sedimented. Interestingly, rare non-aggregating cured derivatives were shown to harbour a small segment of TP-J34 DNA within their chromosomes. Lytic growth of the phage on cured derivatives produced progeny with a 2.8 kb deletion in their genome. Subcloning and mapping studies have localized the site of this deletion as well as the *att*P and *pac* sites. In addition, the region likely to encode the phage structural proteins has been identified.

A temperate *Lb. casei* phage was shown to possess a 47 kb genome with cohesive ends. The *att*P has been localized and oligonucleotide probes (generated on the basis of N-terminal sequence analysis) are being used to identify the gene encoding the most abundant of the 11 proteins constituting the phage capsid. Detailed deletion analysis has shown that a region of at least 4 kb of the phage genome is dispensable for lytic development.

Significant progress has also been made in the molecular characterization of the *Lb. delbrueckii* temperate phage mv4. The endolysin structural gene (*lys*A) has been sequenced. A second ORF (*lys*B), located upstream of *lys*A, has been identified and this may play a role in translocation of LysA across the membrane. Both genes are part of an operon structure. The 132 bp *att*P site of mv4 has been characterized and sequence analysis of the surrounding region has identified a 3272 bp segment encoding six closely spaced or overlapping ORFs, including a 1277 bp ORF which could encode a putative integrase of 427 amino acids. The genetic organisation of the region encoding the 34 kDa major capsid protein identified five ORFs - one encoding the g34 structural protein, a second encoding a 20 kDa immunoreactive protein and three other ORFs of unknown function.

Comparison of the regions of mv4 encoding the structural proteins and the lysis functions with the corresponding areas of the LL-H virulent phage indicates that the former are highly conserved while considerable divergence is observed in the latter.

2. Bacteriophage resistance

Three distinct lactococcal R/M systems are being characterized. The pFW094 plasmid-encoded *Lla* A1 system has been cloned and sequenced and one ORF was found to encode the *Lla* A1 endonuclease while two were required for the *Lla* A1 adenine methylase. Significant homology between this and the *Dpn*1 R/M system was observed, particularly between the genes encoding the respective methylases. The endonuclease involved in the pJW563 encoded *Lla*B1 R/M systems has been purified and its recognition sequence has been determined. In addition, the entire system has been cloned and sequenced, allowing the identification of the ORF for *Lla*B1 endonuclease.

The second methylase (*mscr*F1B) associated with the chromosomally encoded *Scr*F1 R/M system of *L. lactis* subsp. *cremoris* UC503 has been sequenced. The coding region was 1080 nucleotides in length, sufficient for a peptide product with a predicted molecular mass of 41,847 Da. While the deduced amino acid sequence indicated that the gene product displays 10 motifs with an alignment and architecture characteristic of all 5 methylcytosine methylases, significant differences between it and the product of the other *Scr*F1 — linked methylase (i.e. *mscr*F1A) were observed.

The plasmid which encodes the R/M systems conferring phage resistance in *S. thermophilus* TO39 has been transferred to other hosts by conjugal mobilization and it has also been cloned in *E. coli* as a prelude to more in depth molecular analysis.

The interaction between the plasmid-encoded abortive infection determinants (*abi*-416, *abi*-420 and *abi*-105) and their target phages is being analyzed. The respective target sites have been localized within genomic regions of 8.3 kb on phage bIL41, 5 kb on bIL170 and 2.7 kb on bIL66. The *abi*-416 target on phage bIL41 corresponded to an operon coding for phage structural proteins. Comparison of this region in *abi*-416 sensitive and resistant phage is being pursued to determine how the *abi* gene product interferes with phage multiplication. Similar analysis is being performed on the *abi*-105 target on bIL66. This corresponds to a gene of unknown function which is expressed early in the phage multiplication cycle and which, in the presence of *abi*-105, is overexpressed. In the course of this work protein and DNA homologies between isometric-and prolate-headed phages, which were previously considered to be unrelated, were observed. This may explain how some Abi systems (e.g. *abi*-105) are active on both types of phages.

The Abi system encoded by plasmid p1149-3 of *L. lactis* subsp. *cremoris* was shown to mediate resistance to phage p416 by blocking phage-specific transcription.

The gene encoding the receptor for phage CNRZ 832, lytic for *Lb. helveticus*, has been cloned and sequence analysis is in progress.

HIGHLIGHTS / MILESTONES

— The sequence analysis of the entire genomes of two distinct temperate lactococcal phages is a significant achievement. This will allow very exact comparison as well as providing information on their genomic structure and organisation. It will also help elucidate the regulatory mechanisms and the sequence of gene expression involved in mediating the lysogenic and lytic cycles of phage development in lactococci.

— The continuing investigation of bacteriophage resistance systems, including the mechanisms involved in abortive infection, will contribute enormously to the ultimate goal of generating lactic acid bacteria with enhanced phage resistance traits for use in modern, intensive milk fermentation processes.

ANTIMICROBIALS LEAF

OBJECTIVES

1. Isolation of novel bacteriocins and the development and application of a standard purification protocol.
2. Molecular characterization of selected bacteriocins.
3. Analysis of mode of action of bacteriocins and the cognate immunity mechanisms.
4. Isolation and characterization of the genetic determinants responsible for production activation and secretion of bacteriocins.
5. Application of bacteriocins to food systems.

RESULTS

1. Additional screening resulted in a number of novel bacteriocins different from those studied previously. Various bacteriocins of *Lactobacillus sake* have been identified by groups in UK, Spain, Germany and Norway and a bacteriocin of *Lactobacillus acidophilus* isolated in Italy was shown to be different to a Dutch acidocin B, based on hybridization experiments.

Within the reporting period most groups were involved in the purification of bacteriocins. Plantaricin F, plantaricin S, sakacin S and M, acidocin B, and other bacteriocins of *Lactobacillus plantarum* and *Pedicoccus* sp 347 were completely purified. A general procedure for the purification of the bacteriocins was established.

2. The amino acid sequence and composition of the purified bacteriocins revealed that although some bacteriocins were identical, at least 20 different compounds are studied within the LEAF. All bacteriocins are small peptides consisting of 25-50 amino acids, usually a high percentage of apolar amino acids is present. Some bacteriocins (lactacin 481 and lactocin S) belong to the lantibiotics since they contain unusual amino acids like lanthionines. This has resulted in the classification of bacteriocins based on physico-chemical structure and antimicrobial spectrum.

3. Research on the mode of action of bacteriocins has also made good progress. It is now generally accepted that bacteriocins dissipate the trans-membrane proton gradient in sensitive cells. Incubation of sensitive cells with pediocin PA1 resulted in leakage of cytoplasmic components: the size of these components increased when higher concentrations of this bacteriocin were used.

The mechanisms involved in immunity were studied with lactococcin A. Membrance association of the immunity protein was demonstrated. Exposing sensitive cells to an excess of the immunity protein *in vitro* did not affect the bacteriocin killing of the cells, indicating that the immunity protein does not simply protect the cells by binding to the bacteriocin or to externally exposed domains on the cell surface. Specific antibodies raised against this immunity protein have become available. This will facilitate the study of mechanisms of immunity in more detail.

4. The structural gene for acidocin B production has been identified. In addition, transfer of the genes responsible for production and immunity of acidocin B and pediocin PA1 to other lactic acid bacteria has been achieved. For a number of other bacteriocins the localization of the genes and the molecular characterization is in progress. This was facilitated by the possibility to generate DNA probes based on the amino acid sequences derived from the purified bacteriocin. The genes for the processing and excretion of lactococcins and pediocin were also characterized. The genes involved in processing and secretion of the bacteriocin showed homology with the haemolysin A secretion system.

5. Only a few studies have been performed concerning the application of bacteriocin producing lactic acid bacteria or bacteriocins in foods. In a sausage fermentation pediocin production by *P. acidilactici* P-2 could be detected. The activity of pediocin of strain P-2 and added pediocin inhibited *Listeria spp* completely. However, the active concentration of bacteriocin decreased by 20-30% within 13 days.

Application of acidocin B in milk products showed that the activity in milk was 4 times lower than in buffer. Fractionation experiments supported the idea that acidocin B is bound to casein without inactivation.

Cheese making experiments showed that the development of clostridia was delayed but a shift in the clostridium population towards insensitive strains was observed.

HIGHLIGHTS / MILESTONES

— Based on a standard method for determining the inhibitory spectrum of the bacteriocins approximately 20 novel bacteriocins were recognized within the LEAF, of which 10 substances were subjected to further characterization.

— A general procedure for the purification of bacteriocins has been developed.
— Bacteriocins were classified into classes based on antimicrobial spectrum and amino acid sequence.
— Mode of action studies of the bacteriocins showed dissipation of trans-membrane potential of sensitive cells.
— Some genes responsible for bacteriocin production and immunity have been successfully introduced in other lactic acid bacteria. In addition, overproduction of bacteriocins has been established in one case by cloning the genes on a high copy number vector.
— Research was initiated on immunity and resistance mechanisms.

METABOLISM LEAF

OBJECTIVES

1. Biochemical analysis of fermentation pathways and their products in LAB.
2. Cloning and characterization of selected genes relevant to sugar and citrate metabolism in LAB.

RESULTS

1. Biochemical analysis of fermentation pathways and products thereof in LAB

For *Leuconostoc oenos* isolated from Portuguese wines the pH was the most important factor affecting growth and substrate utilization. *Lactobacillus* strains isolated from meat and vegetable fermentations were found to differ in their potential to compete under environmental conditions in fermenting sausage.

A new *Lactobacillus* that constitutes up to 80% of the microbial flora of some sour dough fermentations was identified by using special isolation techniques. By a combination of biochemical and molecular taxonomic methods this new isolate has been identified as a new species, named *Lactobacillus pontis* after the BRIDGE project.

During maltose utilization by *Lb. sanfransisco* significant amounts of glucose are being released; this may have important ecological consequences during sour dough fermentation.

The pathway for the production of erythritol by *Leuconostoc oenos* as detected by ^{13}C NMR was established by an enzymatic analysis that included the purification of a phosphoketolase with activity for fructose-6-phosphate and xylulose-6-phosphate. Malolactic fermentation generated a proton motive force in *Leuconostoc oenos* at pH 3 that was dependent on the external concentration of malic acid.

In vivo NMR studies showed that the stimulation of lactate dehydrogenase by the intermediate fructose diphosphate explains the pattern of end-products formed during the cometabolism of glucose and citrate in *Lactococcus lactis*. In the cometabolism of dairy *Leuconostoc* strains, α-acetolactate and acetoin were found to be produced only when the sugar was exhausted. This is likely to be caused by the inhibition of α-acetolactate synthase by intermediates of sugar metabolism in this organism.

2. Cloning and characterization of selected key genes relevant to sugar and citrate metabolism

Biochemical, physiological and *in vivo* NMR studies established that diacetyl is formed from α-acetolactate in *L. lactis*. Key enzymes in this pathway are α-acetolactate decarboxylase, diacetyl reductase, and α-acetolactate synthase. These enzymes have been purified and biochemically characterized. The genes for acetolactate synthase (*ilvBN*) and acetolactate decarboxylase (*aldB*) have been obtained and are used in genetic constructions to engineer diacetyl production. This also applies to the *ldh* gene that has been cloned and sequenced from two sources, citrate-utilizing and non-citrate utilizing *L. lactis* strains.

The *lac* genes of *Lb. helveticus* appeared to have an operon organisation and were found to be located in close proximity to the *gal* operon. The *galKT* genes from *Lb. helveticus* were cloned in *S. thermophilus* and found to partially complement the galactose-deficiency of this host.

HIGHLIGHTS / MILESTONES

- Protein engineering of the LacR repressor and identification of tagatose-6-P as the physiological inducer of the *lac* operon in *L. lactis.*
- Identification and phylogenetic positioning of a new important sourdough starter, *Lactobacillus pontis*, named after this BRIDGE programme.
- Determination of the pathway leading to diacetyl formation in *L. lactis.*
- Malolactic fermentation at low pH in *Leuconostoc oenos* generates a proton motive force.

SCREENING LEAF

OBJECTIVES

1. The application of SDS-PAGE fingerprinting for the classification of LAB.
2. Identification of LAB producing exopolysaccharides.

RESULTS

1. From different participants a total of 450 lactic acid bacterial (LAB) strains were obtained which were isolated from a variety of fermented food products. The strains were lyophilized and stored in liquid nitrogen. All strains have been submitted to the SDS-PAGE fingerprinting technique for identification. Identification to species level was successful for over 90% of the strains. The protein patterns serve as new reference for further identification and classification of LAB strains. In the complex *Lactobacillus casei* group, e.g. the strains selected by SDS-PAGE fingerprinting were essential to develop species-specific rRNA targeted oligonucleotide probes for *Lb. paracasei* and group-specific probes for *Lb. casei/Lb. rhamnosus, Lb.casei/Lb. paracasei/Lb. rhamnosus* and *Lb. buchneri/Lb.parabuchneri/Lb. kefir.* New species-specific oligonucleotide probes for the differentiation of several lactobacilli such as *Lb. brevis, Lb. hilgardii* and *Lb. lindneri* were developed. In collaboration, a new species, important in sourdough preparations, was detected and described. In traditional Greek cheeses a new group of electrophoretically different *Streptococcus thermophilus* strains was detected. In Portuguese table wines, Port wines and musts, *Lb. paracasei* and *Leu. oenos* were detected.

Using the native protein extracts of the strains mentioned above, a large screening program was started for esterase and amino- and dipeptidylamino-peptidase

activities of LAB strains. Representative species of all genera of LAB have been included. Considerable enzymatic differences were observed between the two *Streptococcus thermophilus* groups.

2. More than 600 strains isolated from traditional European food products and processes were screened for exopolysaccharide (EPS) production. Most the EPS+ strains belong to *Lb. paracasei* and *Lb. sake*. Physical and chemical analysis of the EPSes was started. Preliminary results show that (i) the viscosities are substantially different, and (ii) the sugar composition of most EPSes is also substantially different, although all of the EPSes analyzed were shown to be non-charged heteropolymers. One particular EPS was found to be twice as viscous as xantan gum with similar shear thinning properties. It contained glucose and rhamnose only (ratio 2:1). Using a synthetic growth medium, the EPS production could be enhanced to almost sevenfold initial production on standard medium. Because of its potential interest, the localization and organization of the genes involved in the production are being studied.

HIGHLIGHTS / MILESTONES

- Detection and description of a new *Lactobacillus* species that plays a major role in sourdough preparation.
- Development of new rRNA targeted oligonucleotide probes for reliable identification of various *Lactobacillus* species.
- Creation of an SDS-PAGE protein pattern database of more than 1800 LAB strains, suitable for reliable identification of most LAB species
- Isolation and characterization of an exopolysaccharide produced by a *Lb. sake* strain and enhancement of its production by a factor of seven.

WIDER CONSIDERATIONS

Consumers continue to set high standards for food quality and safety. The wide range of fermented foods in which Lactic Acid Bacteria (LAB) are essential are in special demand. Thus it is important that the roles of the diverse LAB in foods such as fermented dairy products (cheeses yoghurts etc.) fermented meats, fermented vegetables, fermented breads and wine are properly understood and maximised in production processes that range from large commercial scale to those in small units that are often special features of specific regions in Europe.

The T-Project on Biotechnology of Lactic Acid Bacteria harnesses the research expertise of 34 groups that share interests in a range of LAB. The organisation of the Project is such that both the development of new technologies and the application of these technologies to key traits of LAB are facilitated.

Fundamental genetic studies are providing improved understanding of LAB that were previously ill-defined and, therefore, unpredictable in food systems. Detailed analysis of plasmids, and, more recently, of the chromosomes of LAB offer potential for improved stability by vector strategies that are genetically safe for in-food use.

The study of the proteolytic systems present in LAB is of particular importance for those bacteria that participate in dairy fermentations. A complex battery of enzymes is responsible for the ability of LAB to grow in milk and is also involved in the ripening of cheeses to provide the desired flavour and texture. The T-Project has provided an integrated study of the biochemistry and genetics of the proteinases and peptidases and of the peptide transport systems present in lactococci. This knowledge will have important application in scientific selection of

cheesemaking strains and controlled, and possibly, accelerated ripening of cheeses. In addition to the excellent progress on understanding the proteolytic system of lactococci, the T-Project has added considerably to the knowledge available on lactobacilli that are important in a wide range of food fermentations.

Bacteriophage (virus) attack of LAB is a significant problem, especially in dairy fermentations where it can lead to downgrading of product and consequent commercial losses. T-Project research has already provided strains with enhanced phage resistance. Continued studies on the phages themselves and on the mechanisms of phage resistance encoded by either plasmid or chromosomal DNA is likely to provide commercially useful strains that may combine a multiplicity of mechanisms and provide maximum resistance against phage attack.

The T-Project on Biotechnology of LAB has focused considerable effort on the improved understanding of antimicrobials produced by these bacteria. Several new antimicrobial compounds, some of which possess considerable activity against the pathogen *Listeria monocytogenes*, have been discovered within the framework of the project. The well coordinated research effort is aimed at helping the understanding of molecular structure/function relationships of the antimicrobials as well as at the provision of bacterial strains with enhanced production characteristics which can provide a cost effective natural preservation of foods.

New insights have been obtained into the metabolism of lactose, maltose, citrate and malate which are vital to the industrial use of LAB in the dairy, bread and wine industries. Metabolic engineering of sugar metabolism was achieved, demonstrating the potential of genetic engineering to control metabolism for biotechnological applications. The T-Project takes cognisance of the importance of isolating and identifying LAB from a wide range of food and feed fermentations. New techniques and strategies for the identification and classification of LAB have important roles in ensuring that a wide range of strains are available for study and for commercial use. The collaborative efforts that resulted in the isolation, characterisation and identification of a new *Lactobacillus* strain from sour dough fermentation emphasise the benefits of the BRIDGE T-Project. It is fitting that the new isolate has been designated *Lactobacillus pontis.*

COOPERATIVE ACTIVITIES

A plenary meeting of all 34 groups participating in the project was hosted by Professor Charles Daly at Cork, Ireland, in May 1992. In addition to the T-Project meeting at which 7 keynote lectures and 99 research posters were presented, there was a special session attended by 32 members of the Lactic Acid Bacteria Industry Platform (LABIP). These arrangements allowed for a very useful exchange of views between the project scientists and representatives of the key industries that use LAB.

Meetings of the CORE and LEAF subgroups were hosted by the AFRC, Norwich, UK; Cranfield, UK and U.e. de I. De Biotecnologia de Alimentos, Sevilla, ES and Universität Kaiserlautern, D and Universitat Hohenheim, D in the period November to December 1992.

The very extensive collaboration between the participating laboratories continued and was even increased in the second year. This was highlighted by extensive sharing of materials such as DNA and RNA probes, cloned genes, antibodies and vectors. Close collaboration between research groups ensured a minimum of duplication of research effort. Sharing of methodologies ensured rapid progress. Staff exchanges included 12 extended visits totalling 64 man-months and an additional

25 short visits of up to three weeks duration. In many cases the short visits were used to provide techniques and methodologies that were not available in the home laboratory.

EUROPEAN DIMENSION

An internationally-coordinated research programme is essential in view of the wide range of commercially important traits possessed by a diversity of LAB. The collaborations within the project have brought European research on LAB to a leading role worldwide. This has been achieved by a meaningful crossfertilisation involving a multidisciplinary scientific approach and transfer of knowledge across several genera of LAB. Some of these e.g. *Lactobacillus* species, have proved quite difficult to study and progress based on individual laboratory inputs would have been difficult. International collaboration has built up a genuine team approach to research on LAB. This has ensured that progress is maximised through avoidance of duplication and the availability of a critical mass of resources to tackle difficult tasks. While research on LAB important in dairy fermentations has been taken to new heights by the European collaboration within the T-Project, there has also been major achievements within LAB from meat, bread, vegetables and wines. In the past a somewhat fragmented approach to LAB research hindered progress and lacked the benefits of knowledge and skills transfer between participating scientists. The frequent meetings of participants in the T-Project have built up a unique team effort that facilitates meaningful collaborations, stimulates early exchange of information and accelerates the progression of the overall objectives of the project. An exciting development has been the many examples of short scientific visits to use specialised facilities available in host laboratories.

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T-PROJECT

“PLANT REGENERATION”

Factors regulating growth and differentiation of plant cells. A basis for the understanding of plant regeneration (BIOT CT-900206)

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REPORT OF THE GENERAL COORDINATION OF THE T-PROJECT

For those approaching for the first time the activities of this T-programme, it can be useful to recall that the T-Project has been built on five contracts corresponding to five working groups focusing respectively on the *Molecular Analysis of Auxin-specific Signal Transduction in Plant Cell Communication, the Perception, Interactions and Responses to Plant Growth Regulators, on rol Genes as privileged Tools to study Plant Morphogenesis, on the Molecular Analysis of Higher Plant Embryogenesis and on the Regulation of the Inductive Phase of Microspore Embryogenesis.*

The internal coherence of the T-Project is based on common concerns for elucidating the mechanisms of action of a variety of signals regulating growth and differentiation of plant cells and understanding the regulation of the initial steps of key morphogenetic events at the origin of the regeneration of plants from organ fragments or tissue culture, namely cell division, somatic embryogenesis from cell cultures, microspore embryogenesis and rhizogenesis.

The second year of activity of the T-Group has seen major successes in terms of scientific results and in the production of many different tools which will be of utmost interest for future developments. A detailed description of the progress achieved in the programmes of the five groups involved in the T-Project can be found in the reports elaborated by these groups.

The second general meeting of the T-Group has been held in Copenhagen in November 1992. It was hosted by Maribo seed, a very active industrial partner of the T-Project. The report of the research activities of the different teams offered an excellent opportunity to appreciate the progress made during the last year. External experts were invited and actively participated to the presentations and discussions. Several sectorial meetings have also been organized during 1992. The third and last general meeting is planned to be held in Wageningen in December 1993 and will be organized by S. de Vries.

The assistance of the EC staff and particularly the unlimited efforts of Etienne Magnien must be acknowledged for their decisive contribution to the evolution of the T-Group.

HIGHLIGHTS / MILESTONES

Only a few flashes on some of the exciting results obtained are highlighted here.

The purification and partial or complete sequencing of several very important proteins such as new auxin-binding proteins (ABPs), ethylene-binding protein

(EBPs) and fusicoccin-binding proteins (FCBPs) have been achieved. Synthetic peptides corresponding to the presumed auxin-binding site of ABPs have been prepared and the corresponding antibodies raised and characterized in terms of activity. The structure of a new hydroxyproline-poor extracellular AGP secreted by carrot cells has been resolved. Several chitinases secreted by carrot cells have been produced in significant amounts and characterized. Several *Arabidopsis* genes with high homology with cell cycle regulation genes such as two *cdc2* genes and three cyclin genes have been cloned and sequenced as well as a gene encoding a small GTP-binding protein *Rha1*.

In terms of regulation of the cell cycle in plants, the availability of new tools corresponding to *cdc2* and cyclin genes permitted to study for the first time the influence of auxins and cytokinins on their expression, in relation with the foci of cell division in plants. Interestingly, mutants *cdc2* genes overexpressed in *Arabidopsis* and tobacco strongly modify cell division in plants providing new perspectives to alter plant development.

The mechanisms by which *rol* genes of the T-DNA of *Agrobacterium rhizogenes* trigger morphogenetic changes are actively investigated with stimulating new results. For example, various evidence suggest that some of these genes and especially *rolC* might influence hormone biosynthesis and/or activation. On another hand, the *rolB* promoter has been dissected and the control of the gene expression studied in details leading to new interesting views on the role of the rolB gene product in the induction of totipotent meristems. In terms of cell differentiation the collection of monoclonal antibodies raised against cell wall or cell surface epitopes has been a fantastic tool to reveal cell and tissue specific differentiation steps in *Arabidopsis* roots.

Really exciting results have been obtained this year concerning the regulation of somatic embryo development in carrot. The isolation and characterization of extracellular glycoproteins involved in the regulation of somatic embryogenesis and more specifically in the rescue of the temperature sensitive mutant *ts11* have led to the characterization of the role of a specific extracellular 32 kD endochitinase. Even more exciting is the discovery that Nod-factors mimic the 32 kD endochitinase in rescuing *ts11*. This opens the possibility that regulators more or less similar to bacterial Nod factors might be produced by plant cells.

The isolation and analysis of cDNA clones and specific proteins associated with the induction of microspore embryogenesis are in progress. The organisation of microtubules and microfilaments as well as DNA synthesis and protein phosphorylation have been investigated under embryogenic and non-embryogenic culture conditions.

New methodologies have also been built which promise important developments in the near future. They concern for example procedures for internal sequencing of proteins or video recording to follow the evolution of individual cells in culture to identify the development of embryos from single embryogenic cells.

WIDER CONSIDERATIONS

Significant progress has been made by the five subgroups involved in the T-Project. This has already markedly extended our level of knowledge in different domains such as growth factors perception and transduction mechanisms, regulation of cell division, induction and development of somatic embryos, microspore embryogenesis.

Powerful tools have been built such as a variety of nucleic acid probes, mutated genes, gene constructs, transgenic plants, antibodies, oligosaccharides, new cold and labelled ligands, cell lines, etc. The power of these tools is well illustrated by the fact that their use immediately allowed new discoveries in terms of regulation of cell division and differentiation in plants. This "production" is one of the best reflect of the impact of the T-Project. Many of these tools have been circulated inside the T-Group, but also outside, illustrating the very positive "contaminating" aspects of the T-Group activities.

The discovery of new genes involved in several critical and complex aspects of plant differentiation provides unique possibilities to manipulate plant growth and development. This will undoubtedly have important practical implications in the production of transgenic material having improved performance. New and exciting avenues for the future are thus clearly opened.

COOPERATIVE ACTIVITIES

This year has also seen a reinforcement of the cooperation between the various subgroups and teams. This cooperation has been consolidated throughout frequent discussions, sectorial meetings, considerable exchange of tools and personnel and the most important by common experiments and joint publications. Interestingly, "mixed" workshops occurred, where members of different groups gathered and interacted to build fruitful new substates of the initial network.

More structured contacts have been established during the last year with industries interested in the activities of both our T-Project and the *Arabidopsis* T-Project. A Plant Industrial Platform (PIP) for both Projects has been created. Operational links are now built with mutual exchanges and diffusion of information and attendance to meetings.

T-News, our internal link, is successfully continuing to diffuse our activities to selected scientists and decision makers, outside the T-Group. Nine issues have now been circulated.

EUROPEAN DIMENSION

Many of the subprojects investigated need the cooperation of different disciplines of Plant Sciences, cooperation which can only be found through transnational efforts.

Obviously, the multidisciplinary character of this EEC programme is one of its major strength, providing the adequate range of expertise, instruments and facilities as well as the possibility to use the large variety of tools built up by the group.

Furthermore, special efforts have been devoted by the team of coordinators to facilitate as much as possible the circulation of young scientists attached to the T-Project (attendance to sectorial or general meetings, visits of other european laboratories, etc.). This has been considered as specially important in order to give them access to the transnational network of research in the domain as well as to the "european" spirit of our activities.

Molecular analysis of auxin-specific signal transduction in plant cell communication (BIOT CT-900178)

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P.A. MILLNER, University of Leeds, Leeds, UK (ULeeds)
F. OKKELS, Danisco/AS, Copenhagen, DK.

OBJECTIVES

Molecular, structural and functional analysis of ER located auxin binding proteins (MPI); Production of antibodies to auxin binding site or other important epitopes, epitope mapping (HRI); Development of an assay system for auxin induced membrane potential changes (ISV); Cloning and partial analysis of cell division control genes and genes encoding small GTP-binding proteins (RUG); Identification and study of functional and physiological properties of plant G-proteins (ULeeds); Identification and study of novel auxin binding proteins (MPI, Danisco).

RESULTS

Since the last report a special effort has been made to characterize the maize endoplasmic reticulum located auxin-binding protein (i.e. ZmERabp1) as well as auxin-modulated ion channels from tobacco by the patch-clamp technique. It was found that auxin stimulates an outwardly directed H^+-current and that ZmERabp1 plays an important role in this process. Antibodies directed against the ZmERabp1 were able to block the H^+-current; conversely, antipeptide antibodies (D16), which were raised against a peptide from the presumed auxin binding site and shown to have auxin agonist activity on tobacco protoplast membrane potential, were able to stimulate this current in the absence of externally applied auxin. Together with a time resolved analysis of the tobacco hyperpolarisation response (in collaboration with B. Van Duijn and K. Libbenga, ULeiden) these observations suggest that the D16 antibody recognises part of the auxin-binding site of ERabps. Protoplasts derived from transgenic tobacco plants overexpressing ZmERabp1 showed an increased sensitivity to auxin in the hyperpolarisation assay. This response was blocked by application of ZmERabp1-specific antibodies indicating that despite of a C-terminally located ER retention signal a small fraction of the ZmERabp1 protein is apparently able to escape the ER retrieval mechanism (ISV, HRI, MPI). Future studies will aim to elucidate the intracellular trafficking of this protein. Patch clamp analysis revealed the presence of an auxin-modulated anion channel in tobacco protoplasts. This anion channel showed a fast activation and deactivation kinetics and a slow inactivation upon prolonged stimulation. 1-NAA, a synthetic auxin analogue, caused a time and concentration dependent shift of the peak current leading to the activation of the anion channel at more negative potentials. Future work will be devoted to the analysis of the auxin modulation of this channel, in particular whether this channel is directly modulated by auxin or whether abps are involved in its modulation.

To study the auxin perception chain and the putative target proteins for ZmERapl, peptides were synthesized corresponding to domains predicted to be localised at the outer surface of this protein (ULeeds). These peptides were tested for their effects on ion channels in *Vicia faba* (in collaboration with M. Blatt, Wye, UK). It was found that one of the peptides was a potent and reversible inhibitor of the inward Ca^{2+}-potassium rectifier with a concentration of half maximum effect below 10 μM. This peptide is currently used to search for a putative docking protein for ZmERabpl. Auxins and cytokinins play a major role in the control of plant growth and development, partly by affecting cell divison. As a first step to study the regulation of cell division in plants, several genes were cloned from *Arabidopsis thaliana* encoding proteins homologous to cell cycle controlling proteins from other eukaryotes. It was found that, for example, expression of a cdc2 gene was indeed affected by auxins and cytokinins (RUG). In addition GTP-binding proteins were studied to unravel their role in phytohormonal signalling chains (ISV, RUG, ULeeds).

HIGHLIGHTS / MILESTONES

— whole cell patch clamp analysis has confirmed a role for ZmERabpl in the control of ion fluxes;

— the calcium-dependent ATPase of maize shoot ER has been thoroughly characterized;

— an auxin-modulated anion channel was identified in tobacco protoplasts;

— mutant *cdc2* genes influence cell division in plants and provide perspectives to alter plant development;

— the availability of two *cdc2* genes and five cyclin genes allow to study the influence of phytohormones, in particular auxins and cytokinins, in cell division;

— the cell-specific expression pattern of an *Arabidopsis* gene encoding a small GTP-binding protein is a major step forward to understand its cellular function.

COOPERATIVE ACTIVITIES

— T-project meeting, Copenhagen.

— Transgenic tobacco plants provided to subgroup partner Barbier-Brygoo (CNRS), M. Venis (HRI); antibodies from HRI and MPI to various partners, further samples to K. Libbcnga (Lciden) and H. Felle (Giessen).

— Exchange of materials with DANISCO/AS.

WIDER CONSIDERATIONS

In-depth characterization of basic cellular processes should be carried out on the easiest model systems, e.g. *Arabidopsis* for plants. Fundamental research should not be coupled with short-time commercial consideration.

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Plant growth regulators: Perception, interaction and response (BIOT CT-900158)

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OBJECTIVES

The objectives set involved progress in the following:

1. Isolation and characterisation of gene(s) for auxin binding proteins (ABP).
2. Isolation and characterisation of gene(s) for ethylene binding proteins (EBP).
3. Isolation and characterisation of gene(s) for fusicoccin binding proteins (FCBP).
4. Separation of components of transduction chains.
5. Development of protoplast systems to test functionality of components.
6. Development of *in vitro* reconstitution systems to test functionality of components.
7. Isolation of genes for components of transduction chains.
8. Transformation of plant cells with gene constructs.'

MAJOR PROBLEMS ENCOUNTERED

Changes in conformation and activity of membrane-derived proteins as a result of solubilisation.

RESULTS

In Leiden further investigation of auxin-inducible genes from tobacco has revealed that many share similarities with glutathione-S-transferases, enzymes which are involved in the biosynthesis of various signal compounds in mammalian cells. These genes can also be induced by salicylic acid and yeast extract. The D16 antibodies (from Professor M.A. Venis) inhibit auxin-induced gene expression but not the effects of salicylic acid or yeast extract. Further progress has also been made on the role of ion fluxes in auxin signal transduction.

In Bonn specific binding of [125I]ABP to plasmamembrane has been demonstrated and results indicate that reduction of a disulphide bridge leads to disturbance of the ABP-membrane interaction. One of three antibodies raised to 12 oligopeptides from ABP promotes hyper-polarisation in protoplasts but all three increase auxin binding to ABP.

In Rome extensive internal sequence information on the 30 kDa band of the FCBP has been obtained in collaboration with Mogen; sequencing of the 90 kDa band is proceeding. Experiments using a photolabile radioactive derivative of FC indicate that the 90 kDa protein is the functional FC receptor. The endogenous compound inhibiting in vivo FC-stimulated proton extrusion has been purified and preliminary data indicate that it is a highly hydroxylated aliphatic substance.

Recent work suggests that a phospholipid associated with the receptor may modulate binding.

In Aberystwyth two EBPs from *Phaseolus* (28 and 26 kDa) have been purified and antibodies raised to each. This work indicates that considerable homology exists between the two proteins and homologous proteins exist in other species. In collaboration with Mogen, extensive internal sequence information has been obtained for the 28 kDa band; there is no significant homology with any known proteins. cDNA libraries are currently being screened with oligonucleotide probes derived from the sequences. Further progress has been made on the role of protein kinases in the transduction of the ethylene response.

At Mogen, in addition to the work in EBP and FCBP noted above, a new method for obtaining internal sequence information has been developed with a sensitivity down to 100 pmol of protein.

HIGHLIGHTS / MILESTONES

1. Extensive internal sequences of EBP.
2. Extensive internal sequences of 30 kDa FCBP and progress in sequencing 90 kDa component.
3. New method developed for internal sequencing of proteins.
4. Demonstration of hydrophobic interaction of ABP with plasmamembrane.
5. Demonstration that a number of auxin-inducible genes share marked similarities with glutathione-S-transferases.

WIDER CONSIDERATIONS

The progress made over the period of the contract has already considerably extended our knowledge of hormone perception and transduction mechanisms and has revealed new and exciting avenues for the future. The discovery of new genes involved in these processes will undoubtedly have important commercial implications both for plant regeneration and for the production of transgenic material having improved performance and requiring lower inputs.

COOPERATIVE ACTIVITIES

A) Within the group: collaboration between Aberystwyth, Rome and Mogen in protein purification and sequencing; anti-ABP oligopeptide antibodies from Bonn to Leiden.

B) With other groups in the T-project:
Anti-ABP oligopeptide antibodies from Bonn to Gif-sur-Yvette (Prof. J. Guern), anti-ABP antibodies from HRI (Prof. M.A. Venis), to Leiden and Aberystwyth.
Dr N Harpham (Aberystwyth) has spent the past year working at Mogen and at the Institute of Molecular Plant Sciences on protein sequencing and screening cDNA libraries.
Dr M-R Fullone (Rome) has likewise spent a period at Mogen screening maize cDNA libraries for the FCBP gene.
Meeting of whole T-group in Copenhagen (November 1992).
A number of other visits have been made between the laboratories and to other groups in the T-project particularly that coordinated by K. Palme.

EUROPEAN DIMENSION

Unquestionably much of the success of the project is attributable to the transnational nature of the group, providing as it does a wide range of expertise and instrumentation as well as the exchange of various tools. Additionally, the opportunities provided to young people in the project to attend meetings and to exchange ideas with their peers in other European laboratories is of great value, academically and otherwise.

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The *rol* genes as privileged tool to study plant morphogenesis (BIOT CT-900179)

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OBJECTIVES

Isolation of genes encoding *rolB* regulatory proteins (DGBM). Identification of genes coregulated with *rolB* (DGBM). Intracellular localization of *rolB* (DGBM, ISV, CEMV). Titration of RolB in connection with hormonal sensitivity (DGBM, ISV, CEMV).
Analysis of phenotypes, development and hormonal balance of rol-transgenic plants (MPI, UIA, CEMV). Analysis of rol-transgenic roses (LVMH).

MAJOR PROBLEMS ENCOUNTERED

No major problem has been encountered in any of the subgroup's laboratory. Some of the objectives for the previous year proved harder than expected. Some of the projects, though providing interesting results, are perhaps somewhat behind schedule. A few parallel lines of research stimulated by these results have been undertaken.

RESULTS

The research projects of our subgroup as a whole concern various aspects and levels of the morphogenesis triggered by the T-DNA genes of *Agrobacterium rhizogenes* in plants. In DGBM the study of the regulation of *rolB* has led to the identification of three regulatory domains (one of which around the TATA box) in the 341 nucleotides proximal to the translational start codon and the conclusion that auxin responsiveness and expression in the plant meristems of *rolB* are controlled by the same promoter domains. Developmental regulation of both *rolB* (DGBM) and *rolC* (CEMV) has been shown and, in the case of *rolB*, linked to hormonal regulation (DGBM). Analysis of the effects of *rolB* in flower and root neoformation programmes *in vitro* has led to the unexpected and important conclusion that this gene, rather than promoting specifically root morphogenesis induces formation of meristems, whose developmental fate depends upon the local (hormonal?) conditions of the tissue (DGBM). At DGBM, in collaboration with ISV, the morphogenetic capability and the regulation of the homologous of *rolB* on the cucumopine Ri plasmid (rolß) is also being studied. The gene product of *rolB* has been immunologically localized in the cytosol of transformed cells (MPI), and its enzymatic activity has been characterized as a glucosidase capable of hydrolysing indoxyl-glucose and -galactose but not IAA-glucose as previously suggested (MPI

and UIA). Anthers transgenic for *rolB* under the control of a tapetum-specific promoter contain higher levels of free IAA and a concomitant decrease in indoxyl-glucose (UIA and MPI). Despite these evidences, the actual biochemical role of *rolB* in triggering morphogenesis is still unclear. Concerning other Ri T-DNA genes, expression in *E. coli* of *rolA* (MPI) and *rolD* (DGBM), purification of the respective proteins and production of polyclonal antibodies has been achieved in order to proceed with the localization and clarification of the biochemical role of the *RolA* (MPI) and *RolD* (DGBM) proteins and thus complete the work on *rol* oncogenes. Alterations in the nitrogen metabolism, including a decrease in total aminoacids in leaves and a lowering total polyamine levels has been observed in *rolA*-transgenic tobacco plants and linked to modification in growth kinetics (CEMV). Another interesting Ri T-DNA gene, ORF 13, is apparently responsible for the biosynthesis of a diffusible morphogenetic substance whose nature, despite the considerable efforts at ISV, is still elusive. Numerous observations suggest that this substance does not belong to one of the classes of known plant hormones and that it does not alter substantially hormone balance (ISV and UIA) and it either finely modulates cytokinin concentration or the sensitivity to this hormone of transformed cells (ISV). Analysis of the promoter of ORF 13 has revealed its wound inducibility and that the response is confined to a few cell layers next to the wound (ISV); a detailed cis analysis of the ORF 13 promoter is currently underway at ISV. On the more applicative side, *rolC* has been shown to induce a quantitatively interesting increase in the foliage mass in the agronomically important crop plant *Medicago sativa* (IREV). *rolC*-transgenic roses have been obtained for different cultivars, finally overcoming serious transformation/regeneration problems with this species (LVMH). Growth parameters of transgenic plants are currently being analysed at greenhouse level both at IREV and LVMH.

HIGHLIGHTS / MILESTONES

Hormonal, developmental and tissue-specific expression of *rolB* have been linked to the same promoter elements (DGBM). The overall effect of *rolB* has been shown to be the induction of totipotent meristems, not specifically root meristems (DGBM). Progress in the analysis of the biochemical role of *rolB* could lead to new insight in auxin biosynthesis and/or activation (MPI, UIA).

WIDER CONSIDERATIONS

The results obtained by our subgroup as a whole (DGBM, ISV, MPI, UIA, CEMV, LVMH, IREV) have more than confirmed that the study of the *rol* genes provides unique entry points on several critical and complex aspects of plant differentiation and provides unique tools to manipulate plant growth and development. Access to plant regulatory factors and genes involved in differentiation and possibly to plant cellular oncogenes will be possible through the *rol* gene system. Investigation on the biochemical role of the *rol* gene products has demonstrated that plant physiology and development can be modified by enzymatic systems capable to interfere with hormone biosynthesis and/or release from inactive conjugates. Basic aspects of plant physiology such as cell competence and meristem formation can be addressed through the *rol* genes. The *rol* genes are now being utilized as morphogenes for applicative purposes.

COOPERATIVE ACTIVITIES

Exchange of all types of tools (strains, constructs, transgenic plants, antibodies, protocols and techniques, early preprints) has taken place between all participating laboratories. Collaborative efforts within our subgroup (in particular DGBM with ISV and IREV; MPI with UIA, CEMV and LVMH) and with other laboratories

within the T-Project (e.g. DGBM with the laboratory of Mario Terzi) has led to several joint publications. Several joint meetings between partners in the subgroup have taken place. Leaders of the DGBM and ISV laboratories and of MPI, UIA, CEMV and LVMH have met several times. A general subgroup meeting was held in Gif (ISV) in May 1992 and a general T-Project meeting in Copenhagen in November 1992.

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The molecular analysis of higher plant embryogenesis (BIOT CT-900177)

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OBJECTIVES

The central themes in this project are a.) analysis at molecular and biochemical level of several secreted glycoproteins that influence somatic embryogenesis in carrot cultures and b.) analysis of molecular markers specific for different stages of embryogenic cell formation in carrot cultures. The second year has been characterized by a detailed analysis of the material prepared in the first year. (AUW/CPRO): Identification by automated cell tracking of single embryogenic cells, molecular, biochemical and functional analysis of 10, 32 and 47 kD secreted proteins. (UP): investigation of the events leading to the formation of single embryogenic cells. (JII): structural and molecular analysis of arabinogalactans (AGPs) from the culture medium. (M).: purification and identification of the 32 kD and other secreted chitinases, cloning of the corresponding cDNAs. (UE): search for putative cell wall substrates for the 32 kD chitinase, relationship between XET activity and somatic embryogenesis.

RESULTS

(AUW/CPRO): The fatty acid binding properties of the 10 kD extracellular lipid transfer protein were further analyzed. The biochemical data thus obtained support the proposed role of this protein in cutin synthesis. The impaired somatic embryogenesis of the carrot cell line ts11 at non-permissive temperature was shown to bc rcscued by lipo-oligosaccharides from *Rhizobium* (nod-factors). The rescue activity was comparable with that of the purified 32 kD endochitinase. The role of the 32 kD endochitinase was further investigated by supplementing the medium of wild type carrot embryo cultures with anti-32 kD endochitinase antibodies. Somatic embryogenesis was inhibited transiently when the antibodies were added at the start of the culture. Video recordings of immobilized suspension culture cells showed anti-32 kD induced arrest of globular embryo formation from proembryogenic masses, but not proembryogenic mass formation from single cells. The computerized object stage (cell scanner) for time-lapse analysis of embryo development of suspension cells has been optimized. The latest modifications were aimed at the registration of high sensitivity fluorescent images of living cells with a confocal scanning laser microscope (CSLM) attached to the cell scanner. For this purpose, a dedicated video registration system was developed, consisting of a character generator for position identification on the recorded video images, coupled on one side to the video output of the frame-grabber of the CSLM and on the other side the position code output of the cell scanner. This way, the

developmental fate of single cells under conditions leading to the formation of somatic embryos, over 30,000 single cells were analyzed, of which 155 cells formed somatic embryos. These cells could be classified into distinct morphologic classes and from each class the percentage of cells that indeed entered somatic embryogenesis were determined. It could be shown that the morphology of the intermediate proembryogenic stages differed between the different cell classes. Initial experiments to follow the developmental fate of single cells labeled with the monoclonal antibody JIM 8 are in progress and await analysis of the video recordings.

(UP): Starting from hypocotyl explants, the response of the various explant tissues to various combinations of auxin agonists and antagonists were investigated using optical and electron microscopy, time-lapse on different sub-populations of cells and immunological probes. The perception of auxin and its evolution in the early stages of development was measured in binding and in displacement experiments with various auxin analogues capable of discriminating different classes of auxin-binding proteins. Transient variations in the amount of DNA/cell were measured with flux cytometry and microdensitometry, while genetic segregations were followed using RFLP and RAPD markers. Using those approaches, we have: a. identified the tissue generating the somatic embryos; b. determined the time course of the activation of the cell cycle in the proliferating tissue; c. determined the time of appearance of the first totipotent cells; d. demonstrated that two types of cells present in the explant show different responses to auxin, viz. elongation (parenchymatic cell) and proliferation (phloematic cambial cells). The indications we obtained on acquisition of totipotency point to a sort of transition occurring in procambial cells that resembles some of the steps occurring *in vivo* when vegetative meristems become reproductive.

(JII): Using an anti-AGP monoclonal antibody (MAC 207), we have immunoaffinity-purified a major soluble AGP from the culture medium of suspension-cultured cells of carrot. Although the sugar analysis, β-glucosyl Yariv binding, and high alanine, serine and proline content of the protein are consistent with it being an AGP the amino acid composition unexpectedly revealed this molecule to contain no detectable hydroxyproline. This suggested that this glycoprotein is not a classical AGP, but represents the first example of a new class of hydroxyproline-poor AGPs. Deglycosylation of the protein revealed the presence of a single core protein. Direct visualisation showed putative AGP monomers, approximately 25nm by 15 nm that exhibited a strong tendency to self assemble into higher order structures. FTIR microspectroscopy of dessicated AGP demonstrated a higher order of structure present in the carbohydrate moiety than previously supposed. The deglycosylated AGP has been cleaved, and the N-terminus and internal regions of this protein are currently being amino acid sequenced. In addition, interactions between the native AGP and other components of the ECM are also currenty under investigation.

(M): Large scale purifications of the 32 kD chitinase from spent medium of carrot cultures have been carried out in order to raise specific antibodies and facilitate amino acid sequencing. Sequences obtained from peptides generated by digestion of the 32 kD chitinase with trypsin or endoproteinase Lys-C cover about 50% of the polypeptide chain and show significant similarities to class I and class IV chitinases. Furthermore, a 34 and a 29 kD chitinase having pIs at 4.0 and 3,9, respectiviely, have been purified from the carrot suspension culture medium. Based on their MW, amino acid composition, specific activity and serological relationships, the 29 kD chitinase probably is a class II chitinase whereas the 34 kD

chitinase appears an acidic class I chitinase. On the basis of the partial amino acid sequences we are in the process of cloning the 32 kD chitinase cDNA by PCR.

(UE): We are investigating whether some of the secreted glycoproteins that influence somatic embryogenesis act on cell wall components, either to release oligosaccharides that influence embryogenesis, or to cause direct physical changes to the cell wall. a. Xylglucan endotrans-glycosylase. Our studies of enzymic activities of carrot culture filtrates have found relatively low but detectable levels of cellulase activity which might loosen the wall by hydrolysing xyloglucans. However, we have also found considerable levels of xyloglucan endotransglycosylase (XET) activity which cleaves a xyloglucan chain, and then transfers the cut end onto a chemically similar molecule. Should this reaction occur between xyloglucan molecules attached to different cellulose microfibrils, it could loosen the wall. XET assays conducted during the course of embryogenesis revealed a decrease in intracellular specific activity for the first 4d which coincided with the formation of globular embryos from proembryogenic masses. During this phase of development it appears essential, for embryogenesis to proceed successfully, that proembryogenic mass cells do not elongate. Between 4 and 12d of embryogenesis, intracellular XET specific activity increased, and XET was secreted into the culture medium. This was associated with the appearance of heart-, torpedo-, and cotyledonary embryo stages, during which there is the requirement for localised cell expansion, which is presumably caused by wall loosening. In addition, a comparison of XET levels between different carrot cell suspensions indicated that on a per cell basis, highest rates of XET production were by cells undergoing elongation. Therefore, the data are compatible with a role for XET in wall loosening. It appears that the carrot XET may be different from those reported in literature, and hence worthwhile purifying. Interestingly, the radiolabelled oligosaccharide acceptors produced here for use in the XET assay, have also been shown to bind to membranes by co-workers of (UP). **b.** 32-kD endochitinase. In order to identify the putative substrates for this protein, we have radiolabelled carrot cell cultures with N-acetyl-*o*-[1-^{14}C]glucosamine in an attempt to produce a range of potential substrates for the endochitinase. After 24h, radiolabel was found to be incorporated into GlcN/GlcNAc residues of 80% acetone-precipitable material present in the culture medium, including glycoproteins, and also into GlcN/GlcNAc residues of unprecipitated oligomers. Radiolabel was also incorporated into GlcN/GlcNAc residues of cell wall polymers. These fractions were tested as potential substrates for the carrot 32-kD endochitinase and the sugarbeet chitinase Kit 4. So far, no evidence has emerged of any radiolabelled fraction acting as a substrate for either enzyme.

HIGHLIGHTS / MILESTONES

AUW/CPRO: Nod-factors mimic the 32 kD endochitinase in ts11 rescue. Effect of the anti-32 kD serum on wt embryogenesis. Identification of single embryogenic cells. **UP**: Identification of explant cells capable of transition into embryogenic cells. **JII**: Structure of AGP resolved. Peptides of the core obtained. **M**: Identification of secreted chitinases and cloning of very closely related members of the encoding multi-gene family. **UE**: Close correlation between XET activity and somatic embryo formation

WIDER CONSIDERATIONS

Significant progress has been made in all areas of the project and the objectives set for the second year have either been achieved or have exceeded expectations.

The established close collaborations have been further built upon and the high level of mutual interactions have resulted in numerous joint and complementary experiments.

COOPERATIVE ACTIVITIES

General activities: The second meeting of Group 4 was held in Padova in May 1992, a general meeting as well as the third Group 4 meeting were held in Copenhagen in November 1992. Specific activities: Visits of participants of the different subggroups in May 1992 and in March 1993. There have been numerous and regular exchanges of cell lines, cDNA libraries, crude and partially purified culture media, specific mono-and polyclonal antibodies, cDNA clones, purified secreted proteins, purified cell wall fragments as well as experimental protocols between all participants.

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Regulation of microspore embryogenesis (BIOT CT-900160)

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OBJECTIVES

Many plant species cannot be regenerated from single cells *in vitro*. We are investigating the mechanism controlling the induction of cell proliferation in plants using *Brassica napus* microspores as a model system. It is envisaged that the information obtained will be useful to elucidate not only how microspore embryogenesis but also plant cell proliferation in general is initiated. The research will help to establish why some agronomically important plant species cannot be regenerated from microspores *in vitro*. The production of haploid plants from microspores is of major commercial importance to plant breeding companies.

We wish to identify some of the genes required for the induction of microspore embryogenesis, structural and functional cellular components associated with the induction process and how the genes required for the induction of microspore embryogenesis are inherited in a plant population. A 32°C temperature treatment of freshly isolated late to early-binucleate microspores for 8hrs is sufficient to induce the embryogenic process.
The following main objectives were set for the second year of our study:

a) Isolation and analysis of cDNA clones associated with induction of microspore embryogenesis. Begin to isolate and sequence genomic clones and synthesize antibodies against gene products associated with induction of microspore embryogenesis.

b) Continuation of structural analysis of the inductive phase of microspore embryogenesis (microtubule and microfilament changes) and initiation of in situ hybridization studies with probes provided by MPI and others.

c) Analysis and characterization of protein changes during induction of microspore embryogenesis.

MAJOR PROBLEMS ENCOUNTERED

— The group of Dr Pechan moved to MPI fur Biochemie in Munchen during the second half of 1992. This caused some disruption to the ongoing research activities. The Munchen laboratory is now fully operational.

— As heat shock proteins appear to play a role in induction of cell proliferation, more emphasis has been placed on the analysis of these proteins than initially anticipated.

RESULTS

At MPI, cDNA libraries constructed from *B. napus* cv Topaz microspores have been differentially screened for clones associated with the induction of embryogenesis. Three categories of induced clones have been identified. One of the clones was identified to be associated with the electron transport in the mitochondria.

Sequence homologous to 5'-end of *S. cerevisiae* RAD6 has been cloned by PCR from the genomic DNA of *Brassica napus* cv Topaz to study the role of cell cycle regulated genes in the induction of microspore embryogenesis. However, no specific expression of RAD6 like sequences upon microspore treatment at 32°C for 8 h was detected.

Total proteins isolated from *Brassica napus* microspores, separated on SDS-gels, were analysed on Western blots with various antibodies. Heat shock proteins 68 and 70 were found to be constitutively expressed, whereas heat shock protein 17 was induced only in microspores cultured at 32°C for 8 h.

As the heat shock 17 is showing promising results, its corresponding gene is being isolated from the Brassica genomic library. The library is also used to isolate and sequence complete genes from 3 cDNA clones associated with induction of microspore embryogenesis. These results, and those obtained in the first year of the project, will be used to initiate the functional analysis of genes and their products in microspore embryogenesis.

At AUW, the influence of elevated temperature on the orientation of the microtubular spindles in metaphase microspores has been studied. The disappearance of certain microtubular elements leads to significant changes in position of nuclei resulting in new division patterns characteristic of embryogenic development. Microfilaments appeared to be of less importance in the change to the embryogenic pathway of development.

A non-radioactive and fast immuno-cytochemical technique was applied to trace the induction of DNA synthesis in nuclei of microspores and pollen grains by the use of pulse or continuous labelling with BrdU. It was found that elevated temperature induced nuclear DNA synthesis in the nuclei of vegetative cells (of bicellular pollen) which normally never enter S-phase. The reentering of S-phase and the induction of S-phase in microspores and pollen was analyzed and quantified (cooperation with the Institute of Experimental Botany in Olomouc, The Czech Republic).

The detection of newly and specifically expressed mRNAs by in situ hybridization techniques applied on sections of microspores and pollen that were induced to become embryogenic is being carried out with the digoxigenated probes provided by the MPI. cDNA probes hybridizing with actin mRNA and tubulin mRNA were already tested because the changes in the synthesis of cytoskeletal configurations might well be related to changes in the synthesis of cytoskeletal proteins and thus to changes in gene expression. First positive results were obtained concerning an actin probe and a probe specifically expressed during the induction phase of embryogenesis.

At IAPV, genetic analysis of parentals, F1, F2 and backcrosses from the Topas (high embryogenic) and Hanna (low embryogenic) cross has been carried out on field-grown plants.

Five parentals plants and two replicates per plant were studied. It was found, based on the analysis of 50 F2 plants, that the influence of environment on microspore embryogenesis masked any genetic effect. Some differences among F2 plants were observed. Nevertheless, the variation between plants was high.

The parentals and F2 plants have been studied using RAPDs. From a total of 120 different primers used, only 32 were informative primers. Some of them segregated in a 3:1 proportion in the F2 generation.

A reaction mixture using two primers increases the polymorphism. Consistently, the presence of a band in doubled haploid plants has been found which were absent in the parental plants.

At N, experiments have been carried out to characterise the highly embryogenic barley (*Hordeum vulgare*) microspore culture system in order to enable a better comparison with the already well characterised *Brassica napus* microspore culture system.

Embryogenic divisions were observed after 48-78 hours culture at 25°C (permissive temperature). Lower culture temperatures were tested in order to define a non-permissive temperature to parallel the *Brassica napus* system. However, high frequencies of embryogenesis were observed at 18°C and 12°C.

In common with *Brassica napus*, uninucleate microspores and binucleate microspores (pollen) may be induced to undergo embryogenesis. Spikes containing microspores at the mid-uninucleate stage were subjected to a cold treatement of 4°C for 28 days prior to mechanical isolation of the microspores. DAPI staining revealed that, following cold pretreatement, the majority of isolated microspores were binucleate. An alternative pretreatement involved culturing excised anthers on 0.3 M mannitol for 4 days. Microspores isolated from these anthers were still uninucleate which may have significance for transformation experiments. However, to date, isolations following mannitol treatement have been less embryogenic than those following cold treatement.

Three different types of cell could be distinguished in the initial stages of microspore culture. One type consisted of small, plasmolysed microspores which were apparently dead. A second type consisted of swollen microspores, rich in granular cytoplasm. A third type also consisted of swollen microspores, but possessing a large vacuole traversed by cytoplasmic strands. It is proposed that the swollen, granular type divide and grow while the swollen, vacuolar type die.

At CPRO-DLO, the work has been focused on the identification of proteins that might be involved in the induction of embryogenic development of *Brassica napus* microspores. Two dimentional (2-D) gel electrophoresis was used to analyse the [35S]-methionine incorporation in proteins during the first 8 hours under embryogenic conditions at 32°C. The obtained patterns were compared with those found in microspores cultured 8 hours under gametophytic conditions (18°C). Eight proteins (out of a total of approx. 700 protein spots) were synthesized exclusively at 32°C and not at 18°C. Another set of proteins was synthesized at a much higher rate at 32°C compared to 18°C. Some of these proteins were identified as heat shock proteins by immunoblotting with monoclonal antibodies against plant HSP70 or HSP17. Labeling experiments with 32P showed that the HSP70-like protein could be phosphorylated *in vivo* in the embryogenic cultures. This presumptive (phosphorylated) HSP70 was also recognized strongly by the MPM-2 monoclonal antibody that recognizes a phosphorylated epitope specific to a number of mitotic proteins. Immunolabeling of sectioned microspores with the MPM-2

antibody revealed that the presumptive phosphorylated HSP70 was located predominately within the nucleus (Agricultural University). The intensity of labeling varied between embryogenic (32°C) and gametophytic (18°C) microspore cultures. Apart from the protein analysis, experiments have been performed to investigate the cell cycle phase at which embryogenesis is initiated, and whether the organization of microtubules play a role in this process. Ploidy levels were examined in embryos derived from late-uninucleate (LU) and early-binucleate (EB) microspores. It was hypothesized that LU microspores which are in G2 should produce diploid embryos, whereas the EB microspores which are in G1 should produce haploid embryos. Results however, were identical for both microspore populations: approximately 85% of the embryos were haploid. From experiments with antimicrotubule drugs colchicine and oryzalin, it was concluded these drugs could effectively disturb normal cell division. Enhancement of the rate of microspore embryogenesis, as reported by other investigators, was not obtained.

Microspore embryogenesis was also attempted in *Arabidopsis*. The *Brassica napus* microspore culture system has proved to be a very good model to study embryogenesis, but as a disadvantage hardly any embryogenic mutants exist in the species. Microspore embryogenesis in *Arabidopsis* could fill that gap. First results are promising. Microspores with 5-8 vegetative-like nuclei developed in culture, but development into embryos had not yet been achieved.

HIGHLIGHTS / MILESTONES

- Expression of HSP 17 protein and mitochondrial 18s rRNA gene subsequent to induction of microspore embryogenesis
- *Brassica* RAD6 like sequences are not specifically expressed upon induction of microspore embryogenesis.
- Presence of simple repetitive sequences in clones associated with induction of embryogenesis (one being involved in electron transport in mitochondria).
- Successful transfer of *Brassica* microspore culture technology to all participating groups.
- The cytoskeletons of microspores and pollen were investigated *in planta* and under embryogenic and non-embryogenic culture conditions.
- The structural analysis of the inductive phase of microspore embryogenesis has been done by the analysis of the organisation of microtubules and microfilaments and the analysis of DNA synthesis and phosphorylation under embryogenic conditions.
- *In situ* hybridisation techniques have been successfully applied and first results are being obtained.
- A reproducible and sensitive system has been developed for comparative analysis of protein synthesis during the first 8 hours of microspore culture.
- *In vivo* phosphorylation of a HSP70-like protein was demonstrated.
- A promising start was made with development of a microspore embryogenesis system for *Arabidopsis*.

WIDER CONSIDERATIONS

The *Brassica napus* microspore culture system proved to be a very good model both as a vehicle for the study of plant cell proliferation and an important tool for

the plant breeder. In the normal process of pollen development, the pollen mother cell gives rise to haploid microspores which, after mitosis, mature into pollen grains capable of pollinating flowers. One of the two sperm nuclei fertilizes the egg resulting in a diploid embryo development and a new plant generation. However, by forcing the microspore itself to undergo embryo development, haploid or diploid plants are formed which are of great interest to plant breeders. A programme of research on the production of double haploids from microspores has been assigned a high priority in many plant breeding companies since the rapid production of homozygous plants is of major commercial value in many crop species. Double haploids would: (a) permit the efficient selection of desirable gene combinations and (b) enable new traits to be incorporated into product lines or hybrids more quickly. The currently analyzed clones associated with energy metabolism of the cell under stress conditions may prove to be of importance to understand induction of cell proliferation in general and embryogenesis in particular.

COOPERATIVE ACTIVITIES

Throughout the year a number of meetings were held between the participating laboratories.

All laboratories in the project participated at two joint meetings of the group in 1992. Apart from the presentation of current results, discussions took place on future interactions between subgroups, such as joint publications, common use of probes for *in situ* hybridization, exchange of Brassica *lines* and cooperation on 2D gel analysis. Indeed all these interactions were strengthened and expanded throughout the year.

The group of A. van Lammeren has cooperation with other teams of the T-Project outside group 5. V. Onckelen, Palme, Venis and Klambt on *in situ* detection of plant growth regulators and their binding proteins or receptors.

Cooperation with Czech colleagues is also carried out through the TEMPUS project teams of the T-Project outside group 5, for example on the quantification of DNA contents of nuclei and mRNA analysis.

EUROPEAN DIMENSION

Because of the subject nature of the research, it is by necessity a transnational collaboration. The group works well together and thus allows quicker transfer of knowledge and technology within the EC and beyond. The personal contacts have meant improved confidence and willingness for future cooperation. This is already reflected in the fact that 4 of the group members participate in two other EC projects including student exchanges. The difficulties with such projects are the geographical distances between participating laboratories with consequent greater requirements for travel money, telephones etc. More money should be set aside for short term student travel between participating groups. It would also be helpful to carry out such international projects over 4-5 year periods rather than the present 3 year contracts.

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T-PROJECT

“ANIMAL CELL BIOTECHNOLOGY”

Animal cell biotechnology

GENERAL COORDINATOR:
F. GANNON, University College Galway, Galway, IRL

A GENERAL OVERVIEW

(see also specific reports on the following pages)

During the past decade society has learned that the therapeutic products, vaccines and diagnostic reagents which it requires for improved health care and lifestyle will come increasingly from the Biotechnology sector. Genetic engineering techniques have allowed scientists to isolate and express genes of interest and hybridoma technology has provided monoclonal antibodies which have improved the specificity and reproducibility of diagnostic tests. It is easy to focus attention on the products or on the complexities of recombinant DNA technology required to isolate genes which encode lifesaving proteins. However an essential element in making these products is the host cell which provides the components and environment necessary for their expression. Biotechnology companies have choices which they can make as regards the host organisms between bacteria, insect or animal cells. With the aspiration to make products from biotechnology as identical as possible in all aspects to those that occur in nature, attention has focussed increasingly on the modifications which occur to the proteins after the translation in the cell of the DNA sequence into a chain of amino acids. Bacteria are not capable of performing these reactions and most agree that animal cells are the best choice when complex proteolytic and glycosylation reactions are required to yield a product which corresponds best to the original protein.

Animal cells grown in culture have a long and illustrious history in biotechnology. They are the basis of the very significant monoclonal antibody industry and their large scale culture has also been essential for the provision of many of the widely used vaccines. They also have been necessary for the expression of complex recombinant proteins such as erythropoeitin (EPO) which has dramatic effects on those suffering from anemia, tissue plasminogen activator (TPA) which provides effective emergency relief for those with acute myocardial infarctions and related heart trauma, or Interleukin 2 (1L-2) which is a potentially potent drug in the treatment of cancer.

However despite these successes and industrial experiences there are many aspects of the expression of therapeutic products in animal cells which would benefit from new approaches to improve the productivity and quality of product from this host cell. It was for this reason that the EC decided to establish a T Project on Animal Cell Biotechnology. This precompetitive project is built primarily on the skills of the genetic engineers. Its aims in this context are to provide vectors which will result in the placing of the gene constructs that one wishes to express in a preselected locus. Two general solutions are envisaged for this:

(i) targeted integration of the construct into a chosen locus on the chromosome or
(ii) novel vectors which will result in the maintenance of the gene in an extrachromosomal mode by the use of artificial chromosomes.

A second aim of the genetic engineering groups is to improve the level of production in the animal cells and the ease with which this expression is controlled by the development of new and inducible vectors.

Some of the solutions to this problem include the transfer of controls systems well established in bacteria to animal cells and the development of *in vivo* gene amplification methods that use inoccuous compounds as the controlling elements.

The genetic engineering work is fruitless unless it is corporated into cells that grow in a predictable manner. In this T project different cell types with established reputations in Industrial contexts are studied with particular emphasis on the matching of the environment in which they grow to the demands placed on them as they produce large amounts of proteins of interest.

Finally the quality of the products must be assured. As the major justification for the use of animal cells is the fact that the primary structure of proteins can be correcty modified, it is appropriate that projects address the well highlighted topic of post transnational glycosylation in cells and the equally crucial but less studied area of precise proteolytic processing of primary protein products.

Although each aspect of the work can be presented as a separate entity, the integration of the work will be essential if full commercial benefit is to be achieved. Some projects address this need by internal integration of different aspects. Others are more specialist in nature with the information obtained in those being transferred to other groups at the meetings of the T Project.

SUB-PROJECTS

Formally the T Project involves 6 different sub-groups. However, two N Projects are *de facto* part of the T Project as they are appropriate to it, can contribute to its success and benefit from the interaction with the other groups. The aims of the different sub-projects (indicated by the coordinator) can be summarised briefly as follow:

Hauser (N Project): This is a project which integrates the genetic aims of targeted integration to high performance loci in animal cells with the behaviour of different cells grown in culture under varying conditions of aggregation and immobilisation.

Gannon: This is another vertically integrated project which aims to obtain strong and inducible promoters of CHO, target their integration into a readily amplifiable genetic locus, develop matching cell culture conditions which can also act as the trigger for the induction of expression and, finally, monitor the quality of glycosylation of the proteins produced under the conditions.

Donini (N Project): The aim of this project is the development of artificial chromosomes for use in animal cells. These will provide a predetermined locus from which the gene of interest will be expressed. Success in this project requires knowledge on centromeres, telomeres and origins of replication with widespread impacts on topics of research broader than this T Project.

Rommelaere: This project aims to exploit a small non-integrating and non-pathogenic virus as an alternative extrachromosomal vector. This virus has also got interesting anti-cancer effects which further warrant its inclusion for study.

Bradbury: This project uses a novel bacteriophage system for the production of antibodies. By adapting this for expression in animal cells grown in culture the products will be improved by post translational modification. A further important element will be the targeted knock-out by the antibodies of functions in different cellular compartments with the consequences of greater information on animal cell physiology.

Maggi: The start-point of this project is also been a well understood inducible bacterial system which is transferred to a variety of animal cells. In addition this system involves the use of the estrogen receptor gene, the product of which is a well studied transactivation factor. The combination of these two components provides novel possibilities with consequences for the production of proteins from animal cells and the study of gene control mechanisms.

Conradt: This project analyses the very important question of post-translational modifications involving glycosylation. In addition to the detailed description of the glycosylated chains and pathways, this project studies specifically the consequences of cell culture conditions on these parameters.

van de Ven: Specific proteolysis of the primary protein products are the subject of this project. The enzymes that are essential for this are still being discovered and their function, specificity and control of expression are the topics of research including the influence on them of large scale cell culture.

HIGHLIGHTS / MILESTONES

Each of the subprojects present the highlights of their work to date in the individual reports. No attempt will be made to reiterate these points in detail. An overview of these however would retain the following elements:

- A novel bicistronic vector for gene expression in mammalian cells has been developed. This will be useful when proteins with two submits are required (Hauser).
- A wider range of proteolytic endo peptidases have been isolated and characterised. This new information will aid in providing therapeutic products from Animal Cells (van de Ven).
- The feasibility of using phage vectors for the expression of libraries of antibodies has been demonstrated (Bradbury).
- Conditions have been established for the growth of CHO cells in serum free medium (Gannon).
- A novel vector system, based on bacterial genes has been developed for use with mammalian cells. Very fine control is possible with this promoter permitting variable and inducible expression of products (Maggi).
- A diverse amount of new information on telomeres, centromeres and origins of replication in different cell systems has been accumulated. When integrated, these date will provide the basis for the non empirical construction of artificial chromosome. Information on origins of replication have already been successfully integrated into vectors used by other groups (Donini).
- The possibilities of using a non-pathogenic parvovirus as a vector for targeted expression of proteins in cells has been examined (Rommelaere).
- Exciting new information on the impact of cell culture conditions on glycosylation profiles of proteins expressed in BHK cells has been described. This will guide future work on the commercial production of glycosylated proteins (Conradt).

WIDER CONSIDERATIONS

Industries based on Animal Cell Culture are major contributers to employment, wealth and the well being of the community. Until this T project, they had never

worked together in a coordinated manner to address scientific, regulatory, patent and other topics of mutual interest. Because of the efforts of those involved in the EC T Project, a new grouping of industries that addresses these topics has emerged. This industrial platform (Animal Cell Technology Industrial Platform — ACTIP) involves the leading pharmaceutical companies in Europe and meets every six months to discuss and monitor developments in this sector. A more coherent and well informed group emerges from these contacts and this will help to strenghten this industry in Europe. An update on progress in the T Project is also provided to ACTIP at each meeting by the overall coordinator.

Another benefit from this T Porject has been a booklet on Animal Cell technology (Blue Book in the ELWW series). This will be provided to policy makers, scientists and all other interested parties and will thereby disseminate information widely on this important topic.

As a scientific exercise, there is a growing awareness of the interlinkages which are fruitful between researchers working on very different aspects of the same problem i.e. how to improve the performance and quality of biotechnology industries that use animal cells grown in culture. The project is still young and the roads which lead to this goal have not yet met. But the directions which should be taken are now well indicated and significant improvements are anticipated by the end of the project.

COOPERATIVE ACTIVITIES

Unlike some other T Projects, the subprojects for Animal Cell Biotechnology were considered and selected independant of each other. This T Project was not constructed initially as a coordinated entity. Inevitably the initial phase of the project reflects internal interactions between the members of the sub-projects rather than bilateral linkages. Progress towards a fully integrated project was started by a meeting of all T Project contractors in Ireland in May 1992 followed by another meeting in Portugal in February 1993. At the latter meeting there were parallel sessions on vectors, cell culture and post translational modifications in which contractors from the different subprojects worked with colleagues specialist in these areas. The inherent interdisciplinary nature of the project was also recognised and built upon by a plenary session at which all aspects of the projects were discussed. Arising from these sessions a final meeting took place in which inter-project cooperations were discussed. Many of these were identified and plans established to work on them in the next phase of the project. Details of the interactions in the sub-projects are provided in the individual reports.

EUROPEAN DIMENSION

This project is truly European with 35 contractors from 10 countries. In addition it links research groups in universities and institutes with the relevant industries both by the participation of industries directly in the projects and by interactions with the industrial platform. The combined efforts of ACTIP, the scientists and actions such as the Blue Book and an descriptive folder also provide information to a wider European audience on this section of Biotechnology.

Control of recombinant protein glycosylation under defined cultivation conditions (BIOT CT-920304)

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OBJECTIVES

According to the original workplan, in the first year of the project scientific work centered at the comparison of the N- and O-glycosylation of three recombinant model glycoproteins produced under different cell culture conditions. The developement of an oligosaccharide-'mapping procedure' for N-glycans and, at the cellular level, a detailled analysis of the lipid-linked oligosaccharide intermediates of cells cultivated under defined cell culture conditions was planned.

The isolation of high producer cell clones and the comparison of corresponding products (with respect to oligosaccharide structure characteristics) from the cell lines obtained with those of parental cell lines was intended.

The results obtained lead us to consider also novel genetically engineered model glycoprotein variants (containing e.g the natural N-glycosylation sites of human EPO and glycosylation domains of human antithrombin III) as a tool for studying protein N-glycosylation from recombinant mammalian cell lines (see below).

MAJOR PROBLEMS ENCOUNTERED

For some cell lines the productivity decreased when running long-term cultures (in stirred perfusion reactors) under different culture conditions for 3-5 weeks). However, sufficient product for subsequent carbohydrate structural analysis was obtained when using larger amounts of culture supernatant for product analysis.

RESULTS

Analysis of the glycoprotein product:

BHK-21 cells were grown in 2-liter continuously perfused stirred bioreactors in suspension as well as on microcarriers both in the presence and absence of fetal calf serum.The purified product was thoroughly characterized by western-blotting, amino acid sequence analysis as well as by carbohydrate mapping using HPAE-PAD analysis of the PNGase F liberated N-glycans and methylation analysis.Thus, proximal α1-6fucosylation, the sialylation degree as well as the antennarity of N-glycans from products obtained under different cultivation conditions have been compared:

(i) cells grown in suspension secreted a higher proportion of N-glycosylated protein when compared to cells grown on microcarrier.

(ii) terminal sialylation and proximal α1-6 fucosylation of N-glycans was found to be lower in the product secreted from cells grown in the presence of 2% fetal calf serum than in the product harvested from serum-free culture supernatants.

(iii) the presence of fetal calf serum in the culture medium affected the degree of O-glycosylation of the model protein.

(iv) the ratio of biantennary and triantennary N-glycans differed in the product from suspension cultures when compared to that on cells grown on microcarrier both in the presence as well as in the absence of FCS.

Analysis of cellular parameters:

The lipid-linked precursor oligosaccharides and the total cellular glycoprotein bound oligosaccharides was examined under different cell cultivation conditions, e.g. as described above.

In the presence or absence of FCS the same oligosaccharide-PP-Dol pattern was detected in BHK cells under glucose-rich conditions only Glc_3Man_9, Glc_2Man_9 or Glc_1Man_9 were detected; in the glycoprotein fraction Glc_1Man_9 or Man_9 oligosaccharides were predominating.

These patterns were the same for cells grown under suspension or grown on microcarriers. In contrast, when grown under glucose-free conditions, microcarrier attached cells and suspension cells showed a totally different oligosaccharide-PP-Dol pattern with mainly Glc_3Man_9 and Glc_2Man_9 for the suspended cells and Man_2 -Man_9 and Glc_1Man_9 for attached cells. Interestingly, the differences detected in the lipid-donors were not observed in the cellular glycoprotein fraction which contained Man_4 -Man_9 and Glc_1Man_9 under either conditions.

Changes of cell culture conditions over a short time period (for 1-2 days under perfusion conditions; e.g. omission of amino acids, source of energy supply, presence/ absence of FCS) were shown to have no effect on the final glycosylation pattern of the recombinant glycoprotein products.

Proteolytic degradation:
A dipeptidyl-peptidase-like activity is presumably responsible for the removal of NH_2-terminal A-P sequence of our model proteins; subsequently, removal of small amino acids (Thr, Ser) leads to truncations of the product and thus increases the heterogeneity of the recombinant proteins.

Comparison of high producer vs. low producer cell clones:
No differences in the oligosaccharide pattern of the recombinant glycoprotein product was detected in cell several clones that produced about 0.05 µg, 1 µg or 10 µg of protein/ 24 hours when grown in tissue culture flasks.

Analysis of novel recombinant BHK-21 cell lines secreting variant glycoproteins:
Novel genetically engineered glycoprotein variants secreting cell lines (protein variants that contain 12-15 amino acid residues adjacent to the N-glycosylation consensus sequences from human AT III and human EPO) will be investigated in the second period in order to allow for more generalizations to be made with respect to the effect of cell culture conditions on the glycosylation pattern of glycoproteins.

HIGHLIGHTS / MILESTONES

The results obtained indicate that under well defined and controlled cell culture conditions (such as applied in the present study by using stirred perfusion bioreactors) different glycoforms of recombinant therapeutic proteins can be obtained with carbohydrate stuctures of predictable physiological properties (e.g. antennarity and sialylation state of oligosaccharides on glycoproteins are discussed to profoundly affect their *in vivo* half-lives).

The quality intracellular precursors for N-glycosylation of proteins are affected by cell culture conditions which therefore is an important parameter for the glycosylation degree of the final recombinant glycoprotein product.

WIDER CONSIDERATIONS

Methods for cultivation of recombinant mammalian cell lines for the production of therapeutic glycoproteins are being evaluated with respect to the final glycosylation pattern of the desired product. Investigations include: at the level of characterisation of the final product the carbohydrate structure analysis of glycoproteins and at the cellular level a detailled analysis of physiological parameters such as concentrations and turnover of oligosaccharide precursor that can be expected to affect the glycoforms/glycan structures of products when using different cultivation conditions. A final aim of the project is to enable the 'modelling' of animal cell culture to achieve the production of reproducible and defined glycoprotein therapeutics with a given cell line and furthermore, to allow for predictions to be made with respect to final glycoform-products. The results obtained so far let us conclude that the quality of products only marginally affected by short-term changes in cell culture conditions. Long-term alterations in cell culture can be designed generating glycoprotein products with predictable glycan structures and hence with defined physiological properties.

COOPERATIVE ACTIVITIES

Two members of the Lille group visited the GBF. Experiments have been carried out together with members of the GBF group (two weeks).
A member of the GBF group visited the Laboratoire de Chimie Biologique for the exchange of results and discussion of further activities.
Glycosylation analysis (by the GBF group) was carried out for one laboratory participating in the BRIDGE programme.
Standard oligosaccharides for 'mapping of carbohydrates' have been made available to other laboratories participating in the BRIDGE programme.

JOINT PUBLICATIONS

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Optimization and validation of virus-based linear vectors (BIOT CT-920305)

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PARTICIPANT:
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OBJECTIVES

The aim of this project is to construct new expression vectors that may be used as anticancer agents or constituents of artificial minichromosomes.

During the reporting period we have tried to
- elaborate a packaging cell line for recombinant MVM vectors,
- increase production of existing recombinant MVM viruses,
- test the functioning of putative cellular origins of replication in linear MVM-based vectors.

MAJOR PROBLEMS ENCOUNTERED

The isolation of 'packaging cells' proved rather tedious due to the absence of a rapid screening method. Each clone has to be tested by transfection for its efficiency of recombinant virus production.

RESULTS

For the construction of our linear vector we have started from the molecular clone of MVM(p) (Minute Virus of Mice). When this molecular clone is transfected into permissive cells, the viral DNA is excised from the pBR backbone and replicates autonomously as a virus.

MVM transcription occurs from two promoters: P4, the early promoter, controls the expression of two overlapping genes coding for non-structural proteins; the non-structural NS-1 protein plays a role in the cytotoxicity and the replication of the parvovirus and it trans-activates the second promoter, p38, that controls the expression of two capsid proteins.

Two types of vectors have been tested in our laboratory, p4cat and p38cat, on which the foreign gene (here cat) is inserted downstream from P4 or P38 respectively. The first type of vector only retains the parvoviral termini and the P4 promoter. It can accomodate inserts of up to 4.5 Kb but it is replication deficient since it lacks NS-1 protein. The second type of vector can accomodate only smaller inserts, up to 1.6 Kb, but it will replicate autonomously.
These two types of vectors were transfected into transformed human fibroblasts and cat expression was measured. Transactivated P38 is a stronger promoter than P4, their activities are comparable to those of the RSV LTR and the SV40 promoter respectively. This expression is not dependent on replication of the vector. Both vectors lack the VP genes, coding for capsid proteins.

One possible application of these parvoviral vectors could be the immunotherapy of cancer. Given the oncotropism of parvovirus MVM it could be used to specifically deliver foreign genes into tumour cells. To this ends, we have cloned the interleukin genes IL2 and IL4 under the control of the P38 promoter. In these

constructs, 1.5 Kb of MVM DNA have been replaced by the 0.5 Kb IL genes. We could package the recombinant viruses by cotransfecting them with a replication deficient MVM DNA into transformed human cells.

However, the resulting stocks of MVM/IL2 were contaminated by wild-type MVM that was produced through recombination between MVM/IL2 and helper DNA. This problem could be circumvented by using 'packaging cells' that express capsid proteins from integrated genes. One persisting problem is the low titre of MVM/IL2 virus stocks (10^3 particles/ml). We could increase this titre by an order of magnitude when cotransfecting simian cos-1 cells. Since virus production can be increased by transfecting appropriate host cells we have tried to establish cos-1 packaging cells that express MVM capsid proteins. We have not yet been able to isolate an efficient packaging cell line but we continue the screening of clones we have already isolated. Moreover results obtained in our laboratory indicate that the type of insert and therefore probably DNA structure influences the yield of chimeric virus production. We have modified the MVM/IL2 genome by adding 1 Kb of virus sequences to restore the length of wild-type MVM. Preliminary results indicate that this longer vector yields approximately 10 times more virus than the original vector. So that we are now able to produce infectious MVM/IL2 virions with titres of about 10^5 particles/ml in cos-1 cells.

The group of S. Riva (Pavia) has purified and cloned short DNA sequences that were shown to belong to the subset of genome replicated at the onset of S-phase in human promyelocytic HL-60 cells. One of these (pB48, 1560 bp) was utilized to isolate a larger genomic region (L30E 13.7 kb). They have shown that L30E DNA derives from a transcribed region of the human chromosome 19, band p13.3. The 13.7 kb region was found to contain two closely spaced and nonoverlapping genes, one of which corresponds to a B-type nuclear lamin (lamin B2). It was proved by dot blot hybridization that the replication of the L30E region is activated within the first minute of the S-phase in HL-60 cells suggesting the presence of an origin of replication inside it. The origin was localized by means of a new quantitative pCR technique developed in their laboratory. HL-60 cells were synchronized at the G1/S border by two successive blocks of DNA synthesis with aphidicolin. After the release from the block, cells were labelled for 10 min. with ^{3}H-BrdUrd and then DNA was extracted from nuclei, size fractionated and immunopurified with anti-BrdUrd monoclonal antibodies. A fixed amount of immunopurified nascent DNA was mixed with increasing amounts of the competitor template, identical to the corresponding genomic sequence except for a 20 bp insertion in the middle so that the respective amplification products can be resolved by gel electrophoresis. The mixed DNA sample was then subjected to pCR amplification with pairs of oligonucleotides synthesized on the basis of the sequence of interest. As the number of competitor molecules increases the ratio between the two bands changes. The ratio of the amplified products faithfully reflects the initial relative concentration of genomic and competitor DNA. The results of these experiments show that the number of BrdUrd DNA molecules deriving from the pB48/pBN1 central region of L30E is significantly higher than that deriving from the flanking sequences supporting the conclusion that the pB48 region is very close to a DNA replication origin from which replication fires bidirectionally. In first approximation, the origin region spans the end of the lamin B2 transcription unit and the beginning of a second unit that codes for a 850 nt polyadenylated mRNA. The sequence of 600 bp separating the two transcription units contains a promoter which comprises a protected sequence homologous to the binding site for several bHLH DNA binding proteins such as USF/MLTF or the MYC/MAX complex. These findings emphasize a possible linkage between

transcription and initiation of DNA replication similarly to what is observed in viral replication origins where cis-acting transcriptional elements are overlapped with those controlling activation of DNA replication. Our resuts suggest that in the L30E replicon, initiation of replication occurs either at a single site or in a relatively restricted region within approximately 3 Kb. The quantitative PCR technique presented here will be employed for a more precise mapping of the replication start site within the single copy L30E DNA. L30E DNA is therefore a promising component for the construction of a vector that replicates autonomously in mammalian cells. The final aim of the BRIDGE project is the development of such a vector by utilizing other key elements such as scaffold attachment sequences and telomeric ends (of both parvoviral and human source) developed by other participants.

HIGHLIGHTS / MILESTONES

A parvoviral vector has been constructed that transmits a functional foreign gene (here IL2) into permissive target cells. This virus retained the oncotropic host range of the parental virus and could therefore be used to specifically target the expression of genes coding for cytokines or toxic compounds to tumour deposits.

On the other hand an MVM vector will be used to test the replicative properties of L30E DNA upon transfection into human cells. This L30E DNA has been isolated during the reporting period and it was shown to contain a bona fide mammalian origin of replication.

WIDER CONSIDERATIONS

Minute Virus of Mice (MVM) and the closely related parvovirus H-1 have several properties that make them interesting candidates for the construction of transducing vectors:

(i) many different cell types present receptors for these viruses but their expression is mainly limited to transformed cells (oncotropism),

(ii) their linear single-stranded DNA does not integrate,

(iii) they are non pathogenic for adult laboratory mice, and experimental infection of adult humans with H-1 was asymptomatic,

(iv) finally, the genome of MVM has been cloned into plasmid pBR322, rendering the DNA accessible to genetic engineering.

We propose to use the oncotropic properties of this parvovirus to obtain targeted expression of genes that interfere with the survival of tumour cells, such as for instance lymphokines. Increasing the local expression of certain lymphokines can be expected to stimulate cells of the immune system that react against tumour cells. We have replaced part of the genes coding for capsid proteins by the human IL2 gene which is expressed under the control of the P38 late promoter of MVM(p). Upon transfection into permissive human (NB-K, MRC-5V1) or rat (HSNLV, FREJ4) cells, the recombinant MVM-IL2 DNA is excised from the pBR backbone, amplified and, in the presence of VP proteins, packaged into recombinant viral particles. VP proteins can be provided either by a helper plasmid or by genes inserted into the host cell. The recombinant viruses are able to transfer fully functional IL2 genes to permissive target cells and retain the oncotropic host range properties of the parental virus.

The yield of infectious virus produced is dependent on the host cell used as well as on the structure of the recombinant viral DNA. We have obtained titres of about 10^5 particles/ml using simian cos-1 cells as host cells for transfection with a recombinant DNA that retains as many viral sequences as possible (i.e. a vector that is close to the size of wt MVM DNA).

The structure and organisation of mammalian chromosomal replicons and the molecular mechanisms underlying the activation of new rounds of DNA replication during the S-phase are still largely unknown. This is in part due to the difficulty to develop in mammalian cells the kind of functional assay that allowed the identification of ARS of yeast. To overcome this limitation we developed a procedure to physically isolate DNA fragments encompassing chromosomal origins of replication from cells synchronized at the G1/S border. This procedure proved to be effective and yielded a DNA fragment that was shown to contain a bona fide origin in the chromosomal context. Such a fragment is a promising tool for the construction of innovative vectors such as linear vectors containing this human origin flanked by telomeric sequences (parvoviral or chromosomal). This molecule should replicate under cell cycle control (unlike the existing virus-based vectors) and could be interesting in view of a gene therapy approach.

COOPERATIVE ACTIVITIES

The work reported here has brought about the exchange of scientific material and visits of 1 Italian scientist to Brussels and 3 Belgian scientists to Pavia, mainly to discuss experimental protocols and a joint publication.

EUROPEAN DIMENSION

MVM vectors with an SV40 origin of replication have been tested in Brussels. The parvoviral NS-1 protein does not interfere with the functioning of this exogenous origin of replication. Given these results the L30E origin, isolated in Pavia, will now be inserted by the Italian group into the MVM vector. The replication of the resulting construct will be characterized by the Belgian group. The replication of constructs containing the L30E origin and human telomeres will also be tested. Human telomeres will be provided by other participants in the BRIDGE T Project: Donini (Rome) and Cook (Edinburgh).

This collaboration has brought together two laboratories working in quite different domains. The joint project has allowed each of the two laboratories to profit from the specific competences of the other group.

PUBLICATIONS

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Improvement of production of bioactive proteins by genetically engineered animal cells using the novel furin-class of mammalian endoproteolytic processing enzymes (BIOT CT-910302)

COORDINATOR:
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G. MARTENS, University of Nijmegen, Nijmegen, NL
J. VAN MOURIK, CLB, Amsterdam, NL

OBJECTIVES

1. K.U. Leuven: Proprotein processing using the novel furin-class of processing enzymes:

A. To obtain insight in the complexity and diversity of the *fur* gene family, studies will be focused upon the identification, molecular cloning and characterization of additional *fur*-like genes (various species) and on alternatives to generate diversity.
B. Molecular analysis of the regulation of expression of these genes and the synthesis and post-translational modification of the corresponding gene products.

2. University of Bremen:Development of extrachromosomal vectors for introduction and amplification of foreign genes in animal cells:

A. Isolation and purification of Double Minutes.
B. Attempts to make the DNA more recombigenic.
C. Re-introduction into recipient cells.

3. University of Cambridge: Expression of active forms of the furin-like prohormone converting endopeptidases:

A. To generate sequence-specific polyclonal and monoclonal antibodies against PC1, PC2 and furin by immunization of animals with hybrid proteins generated by expression in a prokaryotic system and by use of peptide antigens.
B. To express PC2 at high levels in eukaryote cells in an active form which will provide material for the study of the catalytic and physicochemical properties of the protein.

4. Holland biotechnology bv: Production of bioactive proteins by animal cells in large scale cell culture:

A. Developing and producing monoclonal antibodies.
B. Research pertaining to large scale production of furin and related enzymes in mammalian cells.

5. University of Perugia:Expression of cloned genes into mammalian cells using retroviral- and EBV-derived vectors:

A. Isolation and characterization of the SHC locus.
B. Characterization of the in vitro and in vivo binding properties of the SHC SH2 domain.
C. Evaluation of the role of the SHC protein during mitogenic signal transduction.
D. Evaluation of the SHC oncogenic potential.

6. University of Nijmegen: Identification of genes involved in sorting, post-translational modification and secretion of peptide hormones:

A. Isolation and characterization of cDNAs encoding proteins functioning in the regulated secretory pathway.
B. Cloning, cDNA sequence analysis and characterization of PC2-related proteins from Xenopus intermediate pituitary cells.

7. CLB Amsterdam: Processing of precursor proteins for blood clotting factors:

A. Site-directed mutagenesis of factor VIII at the endoproteolytic processing site Arg1645-Arg1648; effects of sequence alterations on efficiency of cleavage.
B. Effect of furin on the processing of factor VIII.

RESULTS

The concerted research of this animal cell biotechnology program is focused upon sorting, post-translational proprotein processing and other modifications, secretion, development and use of expression systems, and large scale cell culture. Research is performed in an integrated manner both at the laboratory and the consortium level. In the first phase of the program, progress is made according to plan in the participating laboratories. For more details of the results, see section HIGHLIGHTS/MILESTONES.

HIGHLIGHTS/MILESTONES

Research in the participating laboratories has led to the following results:

K.U. Leuven

— Full length cDNAs were obtained for the furin-like enzymes PC1, PC2, PC4, PC6 and PACE4. They were cloned in mammalian expression vectors and biosynthesis was studied in mammalian cells.
— Two FUR-like genes were identified and characterized in other species, DFUR1 and DFUR2. The DFUR1 gene encodes three structurally and functionally distinct proprotein processing enzymes. The new members are called Dfurin1, Dfurin1-X and Dfurin1-CRR. This enzyme diversity is the result of alternative splicing.
— Characteristics of the promoter region of the human FUR gene were defined.
— Computer-assisted molecular modelling was used to further define the 3-D structure of the subtilisin-like catalytic domain of human furin. The role of negatively charged side chain residues on the substrate binding face of the enzyme was studied by site-directed mutagenesis. A series of 13 mutants was constructed.
— Analysis of furin mutants in which amino acid residues of the catalytic triad and the oxyanion hole were mutated revealed differences in substrate processing and autocatalytical processing of furin.

University of Bremen

- Construction of a plasmid containing neo^R as a selectable marker and a prefixed target site for DNA integration. The neomycin resistance gene, the SV40 early promoter and the megalinker of meganuclease SCEI were cloned in phagemid pOG45.
- Cell lines were transformed with the recombinant plasmid and transformed clones isolated.
  Using PCR technology, integration of the recombinant plasmid in the transformed cell lines was studied.

University of Cambridge

- cDNA sequences encoding fragments of the catalytic domain, N-terminus and C-terminus of the enzymes PC1 and PC2 were subcloned into the prokaryote expression vector pGEX-3X and the hybrid proteins so generated used to raise antisera. High titre sera directed at the catalytic domain were obtained that proved useful for immunofluorescence microscopy, immunoelectron microscopy and immunoprecipitation.
- An assay system was developed for the rapid assay of PC1 and PC2 based upon proinsulin as substrate and using a monoclonal antibody generated to a peptide spanning the cleavage site in proinsulin for separation of the product and substrate for the reaction. This assay permitted kinetic measurements.
- Secretion and activity of PC1 and PC2, synthesized in heterologous cells, were studied. Optimal conditions were defined which should be applicable to situations where large scale production is required.
- The post-translational fate of PC2 was followed in pancreatic islet tissue using a combination of pulse-chase radiolabelling experiments and microsequence analysis of the mature protein.

Holland biotechnology bv

- A hollow fibre culture system (artificial kidney) was developed for the large scale production of proteins produced by animal cells. The following parameters were investigated: a) the amount of cells necessary to start; b) the pH versus CO_2 concentration; c) the amount of fetal calf serum within and outside the fibres; d) the immunoglobulin production in the course of time.

University of Perugia

- The SHC locus was structurally and functionally characterized. It encodes three proteins which are variably expressed in all tissues tested. These proteins contain an SH2 domain in their carboxyterminal regions. As a result of alternative splicing, the amino-termini are different and contain a glycine/proline-rich region that is 50% homologous to proto-collagen alpha 1.
- Several lines of evidence suggest that SHC is involved in mitogenic signal transduction. SHC is rapidly phosphorylated in tyrosine upon growth factor stimulation and it is physically associated to the activated receptor. SHC can stably bind to the GRB2/SEM5 protein product, involved in RAS activation. SHC overexpression can induce neoplastic transformation in murine fibroblasts and neuronal differentiation in PC12 cells.
- In expression studies, a two step procedure was used to avoid recombination of the virus; 1: transfection of the murine ecotropic psi2 packaging cell line; 2: infection of the amphotropic PA317 packaging cell line with supernatants of the transfected cells. A wild type amphotropic retroviral vector was used; this gag^+

LN vector was designed by Miller and colleagues. It is composed of two LTRs, the gag^+ packaging site, a multiple cloning site, an internal SV40 promoter driving the expression of the neomycin resistance gene. In an alternative approach, cytoplasmic replicating EBV-related vectors, generating high copy numbers, were used.

University of Nijmegen

- Differential hybridization screening was used to identify genes in Xenopus intermediate pituitary that are involved in sorting, post-translational modification, and secretion. One of the proteins of interest is the neuroendocrine polypeptide 7B2. It was found that this protein is exclusively present in the regulated secretory pathway of neurons and endocrine cells. Interaction of 7B2 with pro-opiome-lanocortin and the involvement of 7B2 in the sorting and processing of pro-opiomelanocortin and other prohormones is studied.
- The prohormone convertase PC2 was cloned from Xenopus intermediate pituitary cells. Furthermore, the expression pattern of PC2 in the pituitary gland of black- and white-adapted animals was studied with non-radioactive in situ hybridization. It was found that PC2 is co-expressed with pro-opiomelanocortin.

CLB Amsterdam

- The following Factor VIII mutants were constructed: Factor VIII-del(868-1526, which is a mutant that lacks the potential cleavage site at position 1313; Factor VIII-del(741-1668), which is a mutant that lacks both the potential cleavage sites at positions 1313 and 1648.
- Preliminary results from furin-mediated processing studies indicate that proteolysis of full length Factor VIII does occur; no proteolysis was observed with mutant Factor VIII-del(741-1668).
- It was found that sulphation is an important post-translational modification step for binding of von Willebrand factor and Factor VIII.

WIDER CONSIDERATIONS

A wide variety of biopharmaceutically important mammalian proteins are synthesized in nature by highly specialized cells; they are often synthesized first as parts of biologically inactive precursor proteins, from which they are excised and, after sometimes further post-translational modification, they are finally secreted by these cells in their properly folded and biologically active form. These post-translational processing steps are often numerous and highly complex in nature and occur in particular cellular compartments. In most cases, these processes are too complex to be mimicked properly by simple or genetically modified micro-organisms and, therefore, the use of the highly specialized processing and secretory machinery of animal cells is at present the only good alternative. To efficiently produce such proteins by animal cells, it is not sufficient very likely to simply express cloned DNA under control of strong promoters. Animal cells should be genetically modified to improve their production capacity at various levels and, therefore, critical genes that define the post-translational modification and secretion machinery of such cells should be identified and used for genetic engineering.

One important step in post-translational processing is often endoproteolytic processing of precursor proteins at restriction cleavage sites consisting of multiple basic amino acid residues; responsible enzymes, such as furin, have recently been identified and they are already shown to be versatile tools in post-translational pro-

cessing of precursors of a variety of proteins. Furin or furin-like enzymes play a key role in the production of most neuropeptides and polypeptide hormones, growth factors, growth factor receptors, enzymes, blood clotting factors, etc. They are also involved in the processing of precursor proteins of viral pathogens such as HIV and influenza virus; processing of these viral proteins is critical for infectivity of these viruses. Molecular microdissection of the post-translational modification and secretory machinery of animal cells and the use of this knowledge to improve production of biopharmaceutical proteins may prove to be of vital importance for future biotechnology.

COOPERATIVE ACTIVITIES

In the context of this research program, there are intensive cooperate activities between the University of Leuven, University of Cambridge, University of Nijmegen and the Central Laboratory of the Blood Transfusion Service of the Dutch Red Cross. Holland biotechnology bv, the University of Bremen and the University of Perugia were to a lesser extent involved in the cooperate activities in the first phase of the program. Cooperative activities have included exchange of materials for joint experiments and mostly bilateral meetings. Furthermore, three plenary meetings were held. One plenary meeting was held in Leuven, Belgium, just before the start of the program, one in Clifden, Ireland (May 25-27, 1992) and a third one in Sintra Portugal (February 14-17, 1993).

EUROPEAN DIMENSION

The recent discovery in Europe of furin, the first mammalian subtilisin-like proprotein processing enzyme, and, later on, the furin-like enzymes opened new perspectives for the production of biopharmaceutical proteins by animal cells. To pursue these in an efficient way, combination of the new know-how with existing expertise in various scientific areas (gene expression, post-translational modification, secretion) was required. This was difficult to achieve on a national level. The main benefit of this internationally-coordinated research program is that it enabled the quick mobilization in Europe of the laboratories and expertises required for this particular animal cell biotechnology program.

LIST OF JOINT PUBLICATIONS/PATENTS WITH TRANS-NATIONAL AUTHORSHIP

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University of Cambridge

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University of Nijmegen

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Development of an integrated vector system for the expression of immunoglobulins in different cellular compartments of mammalian cells (BIOT CT-920306)

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OBJECTIVES

No objective was to have been completed by 6 months. The following were to have been partly completed (in brackets, the total number of months allocated).

1. The development of the phage (and phagemid) vectors for the creation of the antibody libraries (12 months).
3. The study of the stability of different forms of antibody binding domains (single chain, FAb, single chain attached to constant regions, complete antibody) within the cellular compartments of mammalian cells. This will be done with antibodies presently under study (Y13/259, anti ras p21 and NC1, anti substance P) (24 months).
4. The testing of targeting signals as applied to antibody domains (12 months).
5. The creation of 'targeting signal cassettes', which by the addition of appropriate sites will allow straightforward cloning into the integrated vectors (12 months).
6. The development of mammalian plasmid expression vectors (in 1), containing the targeting cassette to direct the antibody to the appropriate cellular compartment (4), and expressing the form of antibody found to be most stable within the cellular compartment under study (3) (24 months).
9. Functional analysis of cell lines expressing antibody domains directed against the selected intracellular or extracellular antigens presently under study, and others which may be selected from a phage library. General vectors will be used for this goal when they become available (24 months).

RESULTS

Work is progressing well in the three laboratories. Most of the objectives described above (1, 3, 4, 5, 9) are being carried out in one or other of the laboratories, being part of the research interests of the individual laboratories. The development of the bacterial expression of antibodies is being done by CAT while the study of the expression of different antibody forms in eukaryotic cells is mainly being done by SISSA and SIRS.

The largest task of this project, the creation of the integrated vector system, is a project which is being carried out very much in collaboration. Most vectors available for antibody expression have evolved from the pSV2 series. These have not been optimised for antibody expression and it is extremely difficult to express antibodies cloned using the phage system in these vectors. In ensuring the simplest passage from phage antibody selection to mammalian expression we have discussed the requirements, sites and configuration of the vector system extensively, and

have now designed the vectors on the computer using real DNA sequences and the strategy we intend to use.

HIGHLIGHTS / MILESTONES

1. This has been completed.
2. This is still in progress. It has been shown that normal and ScFv Y13/259 antibodies are very stable in xenopus oocytes.
4. It has been shown that nuclear localisation signals will direct antibodies to the nucleus as well as to the cytoplasm. Further signals are under study.
5. This is in progress.
6. This is still in progress. The most difficult part of the project, the design of the vectors, the choice of the sites, the choice of the promoters and selectable markers has been done. The vectors have been assembled on the computer (using the program CAD gene) and the assembly of true pieces of DNA has started.
9. This is in progress.

A kit for the cloning of mouse antibodies using the phage system is now commercially available. It is expected that these vectors, when developed, will also be commercially available as 'an add on module'.

WIDER CONSIDERATIONS

This project unites two important technologies. The first is the derivation of antibodies using the phage display system. Since 1975 monoclonal antibodies have been isolatedf by the immunisation of mice and the fusion of spleen cells to myeloma cell lines. The phage antibody system will supercede this method with a number of advantages.

1. It should be quicker and simpler: we expect a researcher to be able to buy a single pot library from which any antibody specificity can be isolated.
2. The derivation of a phage monoclonal antibody will occur simultaneously with the cloning of the DNA encoding that antibody, allowing the further easy manipulation of the antibody for expression in any cell line.
3. The use of mice for immunisation is expected to be reduced.
4. The derivation of the truly human (as opposed to humanised) antibodies will be greatly facilitated.

The second technology is the use of cloned antibodies as tools in tissue culture cells and in transgenic animals. Once the cloning of the DNA sequences encoding useful antibodies is made easier (as it will be when antibodies are isolated using the phage system), we expect the expression of antibodies in different cellular compartments to be widely used in the study of many different scientific problems. Furthermore, the possibility of developing transgenic animals which express antibodies directed against pathogens may become a relatively simple way of obtaining disease resistant livestock which express normal proteins.

This project also addresses one of the possible criticisms of the phage antibody system. Once the vector system is completed, a researcher will be able to easily express his antibody as a normal human antibody (including all post-translational

modifications such as glycosylation) in human tissue culture cells. This will be very important for antibodies destined for human therapy.

COOPERATIVE ACTIVITIES

In the six months since we started this project the members of the project have met four times to discuss the exact details of the vector system and have communicated numerous times by telephone and fax. There have been two meetings with the other members of the T project (once in Ireland and once in Portugal), as a result of which, we have received DNA samples from one group (Bujard) and are waiting to receive samples from two others (Grummt and Hauser). The research performed by these three groups will be used in the development of our vcctor systcm. In addition to thc 'physical' help provided by these groups, we have had numerous useful contacts with the other members of the T project.

EUROPEAN DIMENSION

The main benefit of internationally coordinated research of this type is the possibility of collaboration which cannot be funded by nationally derived funds. Another specific advantage of EC funds is that collaboration is not limited to academic groups. The collaboration of academic groups with industrial ones tends to be useful for both parties. The difficulties of European collaboration are those of distance. In Europe the lack of a cohesive European air travel policy means that the costs of travel (compared to those of USA) are often prohibitive. High telephone costs cause similar problems.

The specific benefits of the European collaboration of this T project are described in the 'cooperative activities' section above. One of the benefits of this specific T project which I would like to emphasise is the organisation of meetings to which all members of the T project attend. This has introduced me to fields and scientists which I would not normally come into contact with, and in this sense, quite apart from specific help I have received, has been enriching both scientifically and culturally.

Construction of permanently transfected cells expressing steroid hormone receptors (BIOT CT-920308)

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OBJECTIVES

— Construction of regulatable vectors containing either the human estrogen or progesterone receptor cDNA.

— Testing of the transfection methods most suitable for each cell line.

— Setting up the protocols for the rapid analysis of the levels and activity of the expressed estrogen and progesterone receptors.

MAJOR PROBLEMS ENCOUNTERED

The promoter originally proposed did not function as exepected. Another heterologous regulatory system was therefore developed. In this system the extent of control of the expression of a gene in mammalian cells covers more than 4 orders of magnitude.
This system, which functions at the transcriptional level, was proven to be applicable in more than 20 different cell lines.

RESULTS

1. The human estrogen receptor was inserted in the regulatable vector developed in Heidelberg. The construct was analyzed by restriction mapping in order to verify the absence of any rearrangements and the direction of insertion of the estrogen receptor mRNA.

2. Protocols ensuring the efficient transfection were set up for two cell lines: a neuroblastoma cell line (SK-N-BE) and a hepatoma cell line (HEPG-2). Several difficulties were encountered for the transfection of the neuroblastoma cell line, finally these difficulties were overcome by the use of electroporation. The HEPG-2 cells line could be efficiently transfected by lipofection. With regards to Hep G2, the plasmid harbouring the transactivator (tet repressor fused to the activator protein VP16), the plasmid containing the transactivator (tet repressor fused to the activator protein VP16) driven by the CMV promoter was stably transfected into the hepatoma cell line and selected for G418 resistance. The best stable transformant was then transfected with the human estrogen receptor, inserted in the plasmid vector pUD10-3 (pUHDHego) and co-transfected with an estrogen responsive element (ERE) inserted in the long terminal repeat of the Mouse Mammary Tumor Virus (LT-MMTV) upstream of the reporter gene (Luciferase or ß galactosidase, plasmid pGL2ERE).

3. Protocols for the rapid quantitative analysis of estrogen and progesterone receptor protein DNA and hormone binding activities were set up. In particular the receptor protein levels can be detected (with antibodies provided by G.Greene) by: Western analysis, very sensitive immunoenzymatic assay and

immunocytochemistry; the hormone binding activity can now be measured in whole cells and a sensitive nitrocellulose filter binding assay was set up in order to detect ER affinity for specific fragments of DNA.

HIGHLIGHTS/MILESTONES

Two major achievements can be mentioned:

a) The first concerns the development of a very novel vector allowing the regulation of the expression of mammalian proteins by heterologous promoters. The most interesting feature of the present vector system consists in the possibility to regulate the expression of a mammalian protein over a wide range of concentrations. A wide application of this control system in animal cell cultures as well as in transgenic animals can be expected. In our study in particular, the possibility to finely regulate the levels of steroid receptors will facilitate the understanding of the importance of intracellular receptor levels in the moodulation of the transcription of selected promoters.

b) The second concerns the generation of a neuroblastoma cell line expressing the estrogen receptor. In such cell line the activated estrogen receptor determines the morphological differentiation of the cells. Furthermore, these cells upon exposure to low concentration of 17β-estradiol stop growing and start expressing proteins which are specific of differentiating neurones: tau and synaptophysin. The cell system generated besides representing a very valid tool for the analysis of estrogen action of neural-derived cells, opens new conceptual orizons for the possible role of this sex steroid in the development of nervous cells. In addition, this cell system could represent a novel system for the identification of ER synthetic agonists or antagonists provided of tissue-specificity of action.

WIDER CONSIDERATIONS

The possibility to generate cell lines of various origine with any type of receptors has a series of very interesting consequences:

The first one consists of a conceptual progress in the understanding of how a cell works; in fact it has been surprising to see that often enough a cell may acquire the complete response to a factor only by being able to express this factor's cognate receptor. This observation would suggest therefore that the machinery necessary for the trasduction of the signal is present in the cell despite of the fact that the cell does not express a specific receptor. This could be due to the fact that the transduction mechanisms are general and the unique feature determining the responsiveness of a cell to a factor is the presence of its cognate receptor. The possibility to transfect a wide range of cells with specific receptors will enable us to understand the specific role that these receptors can play in different tissues and will facilitate our understanding of the tissue specificity of action of hormones.

The other consequence resides in the fact that this technique will enable us to construct very refined models for drug screening. The major advantage of these novel screening systems resides in the fact that they will limit the use of experimental animals and will provide a mean to study the activity of drugs on homologous (human) cells. Interestingly enough, these systems used as screening tools promise to be not only more reliable that the previous screening tests based on animals, but also much less expensive and time consuming.

COOPERATIVE ACTIVITIES

During the meeting of the T-Project participants in Sintra, Portugal a series of preliminary approaches were undertaken in order to start a series of exchanges of materials and collaborative projects. In particular:

1. several groups asked to utilize the vector system developed in the laboratory in Heidelberg by Bujard's group;
2. several experiments were planned to be carried on with the laboratory of F.Gannon;
3. the neuroblastoma cell line expressing ER generated in Milan by Maggi's group will be utilized to test the expression of a new neural/endocrine protein (NSP) cloned by the lab. of Prof. Van de Ven in Leuven;
4. the possibility of a collaboration with the lab. of Hoogenboom has been discussed. The aim of the collaboration would be to utilize the combinatorial library of antibodies developed by Hooggenboom's group to identify estrogen-induced growth factors in cells of neural origine.

EUROPEAN DIMENSION

The internationally-coordinated research does not present particular difficulties. On the other hand, the advantages of the international dimension are in the possibility to interact with other european laboratories focused on complementary research goals and to discuss with them the results of the studies carried on. It would be impossible to interact with such a varied and competent group of scientists within a single Country.

PUBLICATIONS

Ma Z.Q., Spreafico E., Pollio G., Santagati S., Cattaneo E. nad Maggi A. Activated estrogen receptor mediates growth arrest and differentiation of a neuroblastoma cell line. Proc. Natl. Acad. Sci. (USA), in press.

Targeted inducible amplified homologous expression system from quality products for animal cell in culture (BIOT CT-910307)

COORDINATOR:
F. GANNON, U.C.G., Galway, IRL
PARTICIPANTS:
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M. CLYNES, Dublin City University, Dublin, IRL
J. LEHMANN, University of Bielefeld, Bielefeld, D
W. Mc DOWELL, M.R.C. Collaborativc Ccntrc, London, UK
C. ROSSI, University of Brescia, Brescia, I

OBJECTIVES

The objectives for the first 6 months as in the technical annex to the contract are as follows:

- Transfer of the selected CHO cell line to all groups.
- Establishment of culture conditions for the cell line.
- Preparation of mRNA from CHO grown under on condition.
- Preparation of a cDNA bank using this mRNA.
- Identification of cDNA clones which correspond to high copy number mRNA.
- Transfer of the D1.3 expression vector to the selected CHO cell line.
- Preliminary analysis of Glycosylation profile of D1.3 expressed in CHO.
- Isolation of the CAD gene from CHO.
- Establishment of conditions for electroporation of CHO cells.

MAJOR PROBLEMS ENCOUNTERED

There were some delays in the initial transfers of the cell lines and RNA which were to be used by all the participants. The various legal problems which caused this have now been solved.

RESULTS

With reference to the objectives for the first 6 months (see above):

(1) The cell line was CHO grown and characterised by K. Bergemann of Thomae, a subcontractor to J. Lehmann and transferred to all partners;

(2) Culture conditions including serum free media have been optimised by Clynes (Batch culture) and Lehmann (Continuous culture);

(3) mRNA prepared from CHO grown in the presence (Clynes) or absence (Bergemann) of serum has been characterised and transferred to Gannon;

(4) cDNA libraries of high complexity have been prepared from both mRNAs (Gannon);

(5) cDNA clones which correspond to high copy number mRNA have been isolated and some sequence data obtained (Gannon);

(6) the antibody producing plasmids have been transferred to CHO cells (Lehmann);

(7) A glycosylation profile of proteins synthesised in CHO has been obtained (McDowell);

(8) The CAD gene from CHO has been isolated and sequenced (Bianchi);

(9) The conditions for the successful electroporation of vectors into the CHO cells have been established (Rossi).

HIGHLIGHTS / MILESTONES

This project has three general components: Genetics, Cell Culture and Post translational modification of the product. All three sections are performing ahead of the agreed schedule. This is a major highlight at the start of the project.

The groups active on genetic aspects have laid the foundation for targeted integration into an amplifiable locus by the isolation of the CAD gene from CHO (Rossi and Bianchi). This group has also optimised the conditions for the transfer, by electroporation, of a marker gene (neo). Using RNA from CHO [provided by Clynes and Bergemann (a sub contractor to Lehman)], cDNA libraries have been prepared, screened and sequences that correspond to mRNA present in high copy number isolated (Gannon). The same group has also identified candidates for genes that are expressed in CH0 lines grown in the absence but not the presence of serum.

The groups active on topics related to cell culture (Clynes and Lehmann), in addition to providing RNA, have also defined conditions under which CHO cells will grow in the absence of serum in batch (Clynes) or continuous (Lehmann) culture. Furthermore, as part of the work to define the impact of various parameters on cell growth, Clynes reports a surprising tolerance by the CHO cells of high concentrations of Ammonium.

The post translational modification work (McDowell) focussed on the establishment of refined analytical methods for the detailed study of glycosylation status from CHO.

WIDER CONSIDERATIONS

An underlying aim of this project is to provide quality products from Animal Cells in a safe and efficient manner. These concerns are addressed by:

(a) developing vectors which will express the genes of interest under the control of CHO(rather than viral) promoters,

(b) targeting the site of integration of the novel DNA to a preselected locus on the chromosome and thereby avoid the possibility of unexpected consequences attendant to loci selected in a random manner,

(c) increasing productivity by the use of a safe trigger for genetic amplification,

(d) describing cell culture conditions that are independent of the use of serum, a product known to vary from batch to batch and in which many constituents are not defined,

(e) obtaining a correctly glycosylated protein which is the best substitute for product obtained from nature.

Work on all of these topics has been successfully undertaken in the first months of this project. Collectively and as separate components, the know-how and

materials which are beginning to emerge from this work will have important commercial benefits.

COOPERATIVE ACTIVITIES

All members of the group have met at the T project meetings in Clifden Ireland (May, 1992) and Sintra, Portugal, (February 1993).

Materials transferred include:

Cell line CHO DUKX(K1) Lehmann to all others members
CHO K1mRNA: Cells grown with serum ... (Clynes to Gannon)
CHO ssmRNA: Cells grown with no serum.. (Lehmann to Gannon)
CHO PALA sensitive ... (Bianchi to Lehmann)
CHO PALA resistant .. (Bianchi to Lehmann)
Antibody expressing plasmids for use as a readily monitored product (McDowell to all).

EUROPEAN DIMENSION

This project involves partners in 4 different and distant countries. The skills of the partners are very strongly complimentary and the techniques involved very diverse. This global and integrated approach is possible only with this E.C. funding.

T-PROJECT

“HIGH RESOLUTION AUTOMATED MICROBIAL IDENTIFICATION (HRAMI)”

High resolution automated microbial identification (HRAMI) (BIOT CT-910294)

COORDINATOR:
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3. D. BITTER-SUERMANN, University of Hannover, Hannover, D
4. D. COLLINS, Institute of Food Research, Reading, UK
5. K. SCHLEIFER, Technical University of Munich, Munich, D
6. P. GRIMONT, Pasteur Institute, Paris, F
7. M. HÖFLE, GBF, Braunschweig, D
8. K. KERSTERS, Rijksuniversiteit Gent, Gent, B
9. N. KENNEDY, Computer Applied Technologies, Dublin, IRL
10. R. DE WACHTER, University of Antwerpen, Antwerpen, B

OBJECTIVES

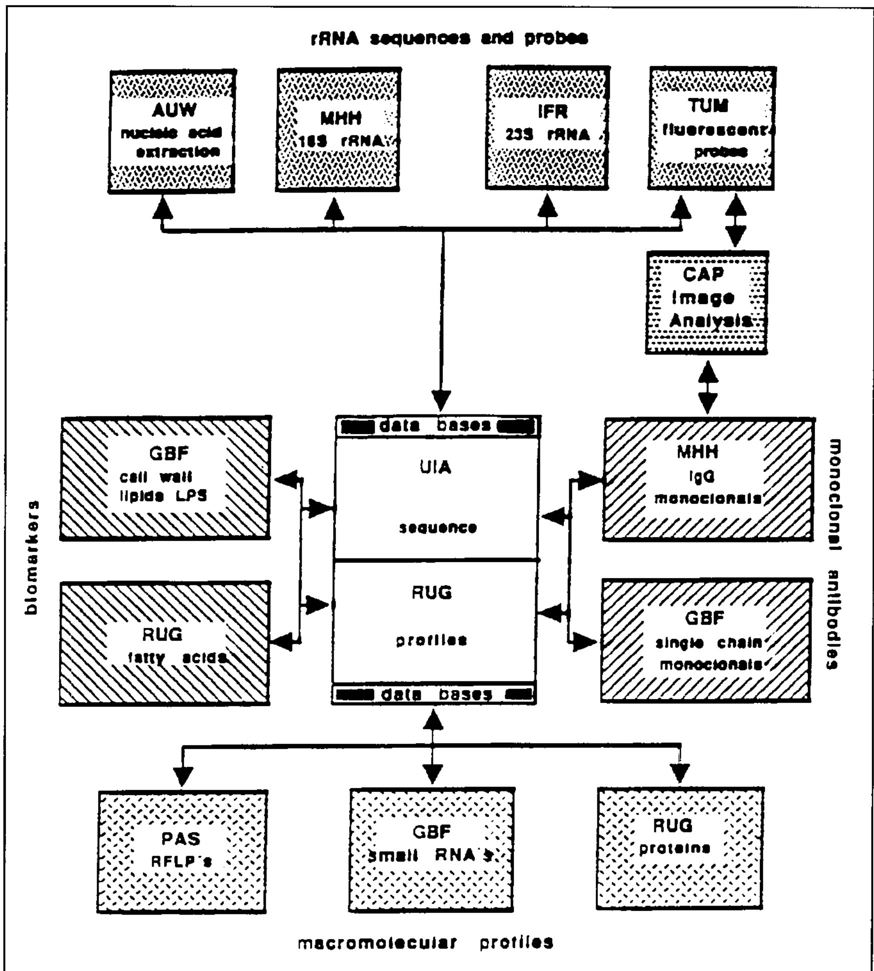
The main objectives of the research consortium are:
1. to **develop high resolution automated molecular methods** for the rapid identification of microorganisms,
2. to **assess the utility and to compare the efficiency** of the different methods developed, and
3. to exploit them to **expand our microbial taxonomy base**, in particular, for 'non-culturable' microorganisms.

A multidisciplinary research team of 10 participants is developing automated technology in the fields of molecular genetics, immunology, analytical chemistry, instrumentation and separation science, and applying this technology to the rapid, accurate identification of microorganisms. More specifically:
1) Ribosomal RNA sequences,
2) Chemical 'biomarkers',
3) Macromolecular 'profiles' and
4) Stable antigens are being analyzed to characterize commercially-important and environmentally-relevant standard sets of microorganisms (core strains).

The results will be compared and evaluated with respect to identification utility and analytical facility. Those analytical systems found to be useful will be used to expand taxonomic data bases (i.e. MINE) and will be evaluated with respect to their applicability to the identification, quantitation, and sensitive detection of microbes in environmental samples and, hence, their utility for exploring microbial community structure and for assessing the possible impact of the introduction of genetically modified microorganisms on target microbial communities. The HRAMI participants are grouped and, interact with one another, according to the following organogram.

ORGANOGRAM OF THE T-PROJECT

High Resolution Automated Microbial Identification (HRAMI)



CONTRIBUTION OF SINGLE PARTICIPANTS

PARTICIPANT 01: Antibodies, Biomarkers, 16S rRNA Sequences
K. T. Timmis, H. L. Fredrickson, E. Moore, GBF, D

OBJECTIVES & MAJOR PROBLEMS ENCOUNTERED

In this initial year of the HRAMI research project the research effort at the GBF was initially planned in 2 areas, lipid chemotaxonomy and recombinant antibodies. The lipid taxonomy work was scheduled to develop and optimize a system for supercritical fluid extraction — chromatography and mass spectrometry. The immunochemical

approach was intended to explore newly-emerging recombinant antibody technology as a means of producing rapidly and inexpensive monoclonal antibodies, of a single isotype, against stable, taxonomically-useful bacterial antigens. Whereas, the former activity could be rapidly initiated and has progressed satisfactorily, delays were encountered in the hiring of a suitably-qualified person to do the recombinant antibody work. For this reason, although the work has now started, no progress for the first year is reported. On the other hand, significant progress was made in a third area, namely the determination of 16S ribosomal RNA gene sequences of *Pseudomonas* core strains, an area requiring additional input that was readily achieved through expansion of an existing GBF group.

RESULTS

1. **Biomarker**: The first phase of our lipid chemotaxonomy research project focused on the implementation of existing, and the development of new chemical procedures for analyzing microbial natural products and assessing the taxonomic usefulness of the information derived. This work focused mainly on three instrumental analyses. We have developed a rapid analytical system, based on supercritical fluid chromatography (SFC), which is able to resolve almost all commonly encountered bacterial isoprenoid quinones, including those with unsaturated isoprenoid chains (Fig. 1). This system is a significant improvement over the standard HPLC method because of its ability to resolve structural isomers. The ability to interface the SFC with a mass spectrometer will enable the generation of chemical structural information. We have now analyzed more than 100 of the HRAMI RNA Group I strains with this method.

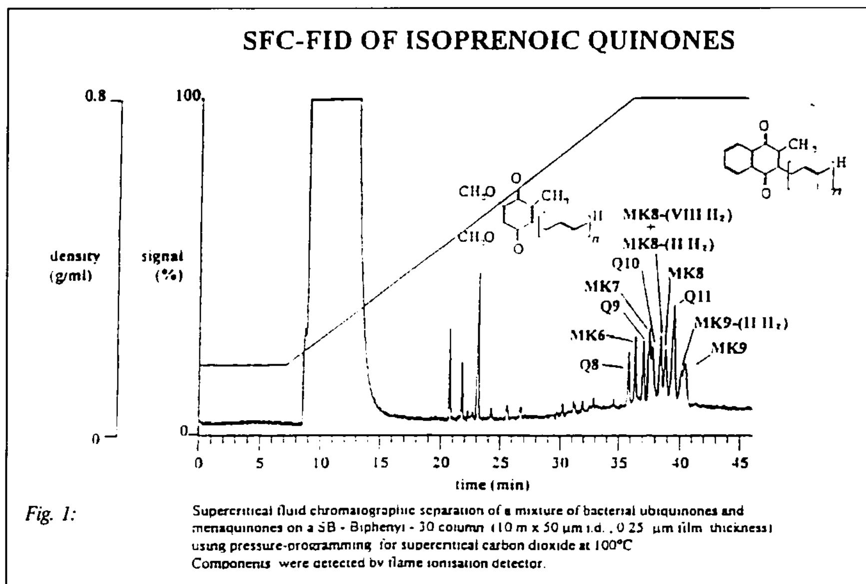


Fig. 1: Supercritical fluid chromatographic separation of a mixture of bacterial ubiquinones and menaquinones on a SB - Biphenyl - 30 column (10 m x 50 μm i.d., 0.25 μm film thickness) using pressure-programming for supercritical carbon dioxide at 100°C. Components were detected by flame ionisation detector.

We have begun to explore the possibility of using Diffuse Reflectance Infrared Fourier Transformed (DRIFT) spectrometric analysis of intact polar lipids, with a multivariate numerical analysis, as a means to identify microorganisms. The initial application of this approach to *Sphingomonas* and *Pseudomonas* strains has shown that this approach can rapidly identify microorganism to the genus level.

An automated, high resolution, gas chromatographic (GC) system, designed for high sample throughput, was established in our laboratory. The hardware and software of this system have been optimized for the reproducible resolution of mixtures of fatty acid methyl esters (FAME) commonly found in bacteria. Additionally, we have established a parallel GC system (except that it uses He as carrier gas) which is directly interfaced with a Hewlett Packard MS ENGINE mass spectrometer (MS). The GC-FID is used to generate the primary chemotaxonomic data and the GC-MS is used to confirm the structural identity of the compounds, as indicated by their GC retention indices. Using this system, and working in conjunction with K. Kersters (participant 08) on the HRAMI core strains which they provided (to date over 200), we are evaluating the existing commercial technology of microbial taxonomy based on total lipid fatty methyl ester analysis and identifying factors which limit its usefulness.

2. **rRNA Gene Sequences**: Methods were developed, whereby, genomic DNA is isolated from pure cultures and the 16S rRNA gene, or portions of the 16S rRNA gene, are amplified by the polymerase chain reaction (PCR) using primers for conserved regions of the sequence. The amplified DNA is sequenced directly, using fluorescent-dye label for cycle-sequencing reactions, and automated sequencing determination on Applied Biosystems, Inc. model 373. The complete 16S rRNA gene sequences for many of the recognized species of the rRNA Group I, as well as some from related species and strains (approximately 20 sequences in total) were determined and made available to the HRAMI partners.

PARTICIPANT 02: Nucleic Acid Extraction, 16S rRNA Sequences

A. Akkermans, I. Blok, AUW, NL

OBJECTIVES

1. Improvement of nucleic acid extraction techniques from soil samples.
2. Quantification of rRNA-specific probe signals to enable monitoring specific target organisms in population dynamic studies and field release experiments.
3. Sequence analysis of rRNA genes from non-culturable microorganisms with the aid of the PCR to enable specific probe development.

MAJOR PROBLEMS ENCOUNTERED

Due to difficulties in finding a qualified candidate for the project, we have started the project with a delay of 6 months in November 1st, 1992. During the first period from November 1992 — April 1993 we have focussed on the improvement of the nucleic acid extraction method and the sequence analysis.

RESULTS

Results from previous studies indicate that general standard nucleic acid extraction procedures cannot be applied without rigourous additonal purification procedures. This is due to coextraction of components, such as, humic acids and polyphenols, which are abundant in the soil. These components interfere with conventional filter hybridization techniques and inhibit sensitive enzymatic amplification reactions (e.g. PCR, NASBA). Our aim is to selectively remove these interfering components prior to hybridization and amplification. Hereto we have designed a selective 16S rRNA extraction procedure. This procedure utilizes a sandwich hybridization format, in which 16S rRNA target molecules are linked to paramagnetic beads via multiple rRNA-targeted capture probes. Hybridizations are carried out in concentrated solutions of the chaotropic salt guanidine thiocyanate, in which cells are

lysed, proteins (including nucleases) are denatured, and nucleic acids are unwound and stripped from cellular matrices, as well as other components which are present in the soil. After the hybridizations are completed the probe-target hybrids are captured on paramagnetic beads and magnetically separated from unhybridized nucleic acids and other components. The captured targets are washed several times and the purified 16S rRNA is finally eluted from the paramagnetic beads in a low salt buffer and used for further analysis. This extraction method is universal in design and enables, by the inclusion of specific primers (or reporter probes), the quantitative detection and specific amplification of 16S rRNA target sequences.

In a model system with *Frankia* 16S rRNA as target, we are currently testing and optimizing this new extraction method, in combination with specific amplification methods. Our aim is to develop the method for quantitative use with fluorescent or chemiluminescent oligonucleotide probes as an alternative for radioactive probes.
Our aim to develop specific oligonucleotide probes for the detection of recalcitrant micro-organisms was focussed on three groups of organisms, i.e. nitrifying bacteria, segmented filamentous bacteria (SFB's), and *Frankia*-like actinomycetes.

A region of 600 nucleotides of the 16 S rRNA of a Nitrosospira strain isolated from acid soil was sequenced. Further analyses are in progress to complete the whole sequence. In addition, we have sequenced a region of 1000 nucleotides of the 16S rRNA gene of Segmented Filamentous Bacteria (SFB's) and analyzed the phylogenetic position of these organisms. SFB's cannot be cultivated in monoculture, but have recently been propagated from single cells in germ free mice. Eubacterial 16S rRNA gene sequences were amplified by the PCR, cloned and subsequently sequenced. Closest relationships were found with *Clostridium butyricum* and *Clostridium sticklandii* clusters, indicating that SFB's can be classified as a new group of anthropogenic symbiotic clostridia. SFB's are known to occur as morphologically distinct symbionts in the ileum of mammals and birds, but never have been isolated and identified. Our sequence data enables the design of SFB primers to be used to amplify related sequences from the intestine flora of other animals. Furthermore we will develop specific SFB probes for *in-situ* hybridization studies.

HIGHLIGHTS / MILESTONES

We have identified and analysed unculturable symbionts (SFB) in the ileum of mice.

PARTICIPANT 03 (part A): Monoclonal Antibodies

D. Bitter-Suermann, MHH, D

OBJECTIVES

1. Selection and isotype switching of monoclonal antibodies.
2. Design of individual ELISA's.
3. Development of a pan-bacterial ELISA.

MAJOR PROBLEMS ENCOUNTERED

The sensitivity and sequence of the set of ELISA's for analysis of natural samples contaminated with bacteria from broad pan-bacterial range to family- and genus-specific, and finally, subgenus-specific narrow range has to be improved.

RESULTS

1. During the last year, including the meetings in Hannover and Amsterdam, we have focused finally on four monoclonal antibodies (mab) against the most relevant bacterial species presenting an environmental health risk in water systems. These are:

   a) The mab 898, against the enterobacterial common antigen (ECA) of the whole family *Enterobacteriaceae*, with *E. coli* as the main representative for fecal contamination.

   b) The mab 2125, against a heat-shock protein (HSP 60)-epitope of the whole genus *Legionella*, with the most relevant and pathogenic *Legionella pneumophila* colonizing warm water systems.

   c) The mab 2528, against an HSP 60-epitope of, probably, the whole *Pseudomonas*r-RNA group I, with *P. aeruginosa* as the most pathogenic species in aquatic habitats for immunocompromised persons.

   d) These three isotype-switched (to IgG 2a) mabs finally were complemented by a mab 900 against a common epitope of nearly all bacteria (with the exception of a few *Archaebacteria*) located on the elongation factor Tu (Ef-Tu). This pan-bacterial antibody serves as an indicator for bacterial contamination, followed by the set of the three others which, according to the national German and European water regulation rules for examination of bacterial contamination in water, have to be absent or below certain concentrations, depending on the type of water.

2. Each of the four ELISA assays for the detection of the respective bacterial antigens was optimized separately, the ECA ELISA being the best elaborated, and was accepted in January 1993 as a German pre-standard for detection of *Enterobacteriaceae* in drinking water. At the moment a national evaluation study is running. Both the *Legionella*-specific and the *Pseudomonas*-specific test systems were further improved by colony-blot- and dot-blot-techniques. Both epitopes of the HSP's were cloned and characterized.

3. In the future the pan-bacterial detection assay, with Ef-Tu as an indicator for bacterial growth even of non-culturable bacteria, has to be optimized with regard to sensitivity. We started with an epitope library by cloning the proper Ef-Tu gene sequence into the M13 bacteriophage for expression within the coat protein III. This antigen presenting phage will then be used in a capture ELISA with mab 900 as capture antibody and the natural Ef-Tu protein as competitive antigen.

HIGHLIGHTS / MILESTONES

Discovery of a family-specific mab for *Enterobacteriaceae* and genus-specific mabs for *Legionella* and *Pseudomonas*.
Demonstration of the applicability of a mab against elongation factor Tu as a universal antibody against all prokaryotes including *Archaea*.
Successful design of ELISAs for all 4 antibodies and evaluation of their environmental applicability.

PARTICIPANT 03 (part B): 16S rRNA Sequencing
E. Böttger, MHH, D

OBJECTIVES

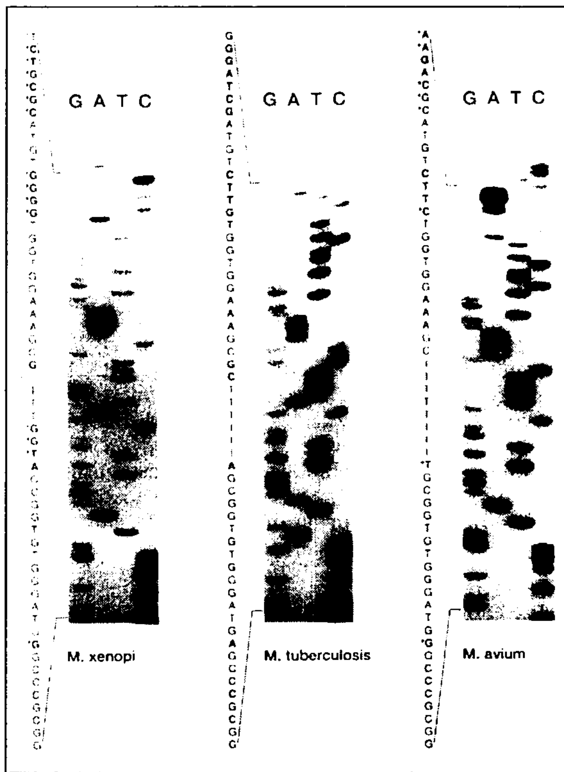
1. Establishing of solid-phase sequencing techniques.
2. Identification utility and analytical facility of 16S rRNA sequencing.
3. Characterization of microbial diversity and identification of novel and uncultured microorganisms.

RESULTS

We developed a technique for rapid sequence determination of 16S rRNA genes. The procedure is based on the presence of highly conserved regions within this molecule, which serve as primer target sites in PCR to amplify 16S rRNA gene fragments from virtually any microorganism. In order to allow an automated sequencing procedure, several steps of the strategy had to be refined. Biotinylated primers and solid phase sequencing methods have been employed to facilitate rapid 16S rRNA sequence determination. The technique was first established for manual sequencing protocols. In collaboration with M.Collins (participant 04) the solid phase sequencing strategy is now adjusted for automated sequencing procedures (1).

As a model system we have focused on the genus *Mycobacterium* in order to evaluate the molecular methods targeting the 16S rRNA gene with respect to identification utility, analytical facility, microbial diversity and characterization of uncultured microorganisms. Mycobacteria are fastidiously growing microorganisms which are widespread in nature. Standard biochemical protocols for identification include complex and time consuming procedures and often give ambiguous results. In addition, identification by phenotyping contains an inherent problem: the phenotype of a species is not an absolute stable property but exhibits quite remarkable variability. This intraspecies variability often results in the inability to characterize and classify isolates as these cannot be assigned to an established taxon on the basis of their biochemical and phenotypic properties. In contrast to phenotypic and biochemical features, the 16S rRNA sequence of a species is a stable property. Based on species-specific signature regions within the 16S rRNA molecule we have proposed a genotypic approach for identification of mycobacteria (see Fig. 1 on next page).

Refinement of several steps involved in the sequencing procedure (nucleic acid extraction, solid phase sequencing) provided the basis for implementation of 16S rDNA sequence determination for identification of mycobacteria at the MHH in 1992. Our efforts were mainly directed at the improvement of 16S rRNA sequence determination, allowing a feasible and readily applicable method for routine identification of fastidiously growing bacteria, already demonstrate that 16S rRNA sequencing probably represents the single most powerful marker for a genotypic based approach for identification. 16S rDNA sequence determination has establish several of biochemically-unidentifyable isolates as novel species and has described a steadily expanding group of mycobacteria which are characterized by a unique phylogenetic position. Our results indicate the potential of 16S rRNA sequencing to identify the nature of microorganisms which cannot be identified by more traditional techniques, and demonstrate that 16S rRNA sequencing will increase our understanding of microbial diversity, as its discriminating speciation capability far exceeds that of standard biochemical identification techniques (2-6).



Part. 03 — Fig. 1: *Direct sequence determination of amplified 16S rDNA gene fragments using solid phase sequencing techniques for identification of microorganisms. Depicted are the results of sequencing reactions for Mycobacterium xenopi, M. tuberculosis and M. avium. Note that each species is characterized by a unique nucleotide sequence.*

In theory amplification primers targeting highly conserved regions within the 16S rRNA molecule permit amplification of any microorganism of interest — including noncultivables. However, this strategy is hampered by the frequent contamination of reagents used for the amplification reaction, in particular Taq polymerase, with exogenous bacterial DNA. Based on 8-methoxy-psoralen and long-wave UV light we have developed a procedure to eliminate contaminating DNA in Taq polymerase as well as in the reagents used for amplification. The elimination of contaminating DNA facilitates the use of a broad range amplification approach for definitive analysis of microbial communities and is especially suited for direction and identification of microorganisms that cannot be cultured (7).

HIGHTLIGHTS / MILESTONES

— Implementation of solid-phase sequencing techniques
— Identification of hitherto unknown microorganisms
— Elimination of contaminating DNA within PCR reagents

PARTICIPANT 04: 23S rRNA sequencing
M. D. Collins, R. Hutson, IFR, UK

OBJECTIVES

The specific objectives were to improve the methods for direct, automated sequencing to the point of efficiently applying these methods to the sequence analysis of large-subunit rRNAs, analysis of selected bacterial strains by 23S rRNA sequence determinations, and the sequence application of such sequence data to the development of hybridization probes to be used for detection and identification.

MAJOR PROBLEMS ENCOUNTERED

The predominant problem in determinations of 23S rRNA gene sequences is the size of the gene. This creates problems in amplification of the gene by PCR, as well as requiring additional time, effort and expense for the sequence determinations and analyses. Thus, major emphasis has been placed upon improving the existing methods of DNA sequencing and automation.

RESULTS

In this study we have developed a set of conserved/semi- conserved oligonucletide primers to facilitate the PCR direct sequencing of the large-subunit rRNA genes of bacteria. Their applicability in 23 rRNA sequencing has been demonstrated in a number of gram positive bacteria (i.e. *Clostridium botulinum* group, lactic acid bacteria) (1-5). The use of this technique in developing highly specific gene probes for the economically important 'mastitic pathogen' *Streptococcus uberis* has been comprehensively studied. *Streptococcus uberis* is serologically and biochemically very heterogenous, and it is currently exceedingly difficult to distinguish this species from other streptococci, particularly *S. parauberis*. The complete large-subunit rRNA gene sequences of *S. uberis* and possibly related streptococcal species have been determined. Diagnostic 'signatures' in the large- subunit rRNA have been identified and used for the design of highly specific gene probes to differentiate *Streptococcus uberis* from *S. parauberis* and other 'pyogenic' streptococci. These probes are proving valuable in epidemiological studies of strains associated with clinical mastitis or subclinical infection and from the environment.

Good progress has been made in improving direct PCR sequencing methodologies. Using these techniques sequence reads of up to 600 bases have been obtained for both small- and large-subunit rRNA. Improved manual sequencing protocols have also been developed which allow for the rapid and simple direct sequencing of PCR products and plasmid DNA (1).

As part of a collaborative effort within the programme on comparing the resolution and applicability of different identification approaches, work has commenced on sequencing diagnostic regions of the large-subunit rRNAs of Pseudomonads. Two hypervariable regions (approximate positions 55 — 464 and 1029 — 1854) have been selected for full sequence analysis. Sequencing studies have been completed for the first region. These data will be evaluated by other participants within the programme as potential targets for oligonucleotide probe design.

An additional objective of the project was to increase the available numbers of small- and large-subunit rRNA sequences. Approximately 20 16S rRNA, 3 18S rRNA and 10 23S rRNA sequences have been deposited in the rRNA sequence database of the University of Antwerpen (participant 10) and are now available to the scientific community.

HIGHLIGHTS / MILESTONES

Establishment of direct, automated, DNA sequencing to PCR-amplified large-subunit genes.

*PARTICIPANT 05: **Fluorescent Probes, Automated Probe Design***
K. H. Schleifer, R. Amann, W. Ludwig, TUM, D

OBJECTIVES

Comparative sequence analyses of 16S and 23S rRNAs are the basis for a phylogenetic classification of prokaryotes. These sequences contain conserved, to highly-variable, regions and are ideal for designing nucleic acid probes. Software should be developed for an automatic alignment of the sequences, for error check, signature analysis, reconstruction of phylogenetic trees, and probe design. Methods should be developed for the identification and *in situ* detection of culturable as well as non-culturable bacteria. *In situ* analysis of attached microbial communities will be carried out with fluorescently-labeled nucleic acid probes in an epifluorescence microscope and flow cytometers will be used for a rapid analysis of cell suspension.

MAJOR PROBLEMS ENCOUNTERED

Automated image analysis of fluorescently labeled cells seems to be more difficult, as expected.

RESULTS

Published rRNA-targeted oligonucleotide probes and their optimal hybridization parameters have been compiled in a data base. An editor, including tools for automated alignment and secondary structure analysis of rRNA sequences, has been developed and is currently being improved. Software for automated probe design, based on aligned rRNA sequences, is under development. Full length 16S rRNA sequences of *Nitrobacter* spec. str. 255 and K34, *Nitrospira* spec. str. 295, *Ilyobacter tataricus, I. polytropus, pelobacter propionicus, P. acetylenicus, Ruminococcus pasteurii, Paracoccus denitrificans, Thiosphaera pantotropha, Thiobacillus cuprinus* and 23S rRNA sequences of 14 Gram-positive bacteria with a low DNA G + C-content, *P. denitrificans, Pseudomonas diminuta, P. stutzeri, Rhodopseudomonas palustris, Bradyrhizobium lupinii, B. japonicum, T. cuprinus, Vibrio vulnificus, Nannocystis exedens, Stigmatella aurantiaca* have been determined (1,2).

A combination of PCR-assisted 16S rRNA sequence-retrieval and fluorescent oligonucleotide probing was used to reveal the phylogenetic status of *Sarcobium lyticum*, a hitherto uncultured intracellular parasite of small amoebae (6).

A new approach to microscopically detect and identify individual cells of *Bacteria* and *Archaea* was developed. Ribosomal RNA-targeted oligonucleotides were 5'-end labeled with the enzyme horseradish peroxidase (HRP) and used for whole cell hybridization. Specifically bound probe was visualized by the enzymatic formation of an intracellular precipitate from the substrate diaminobenzidine. Penetration of the enzyme-labeled probe into whole fixed cells of gram-negative bacteria required their pretreatment with lysozyme/EDTA, whereas permeablity of some archaebacterial cells was improved by addition of the detergent SDS to the hybridization buffer. Hitherto, we did not achieve penetration of enzyme-labeled probe into gram-positive bacteria and yeast. This method should be a valuable tool

for the identification of microbial cells in environments with elevated background fluorescence or in cases where an epifluorescence microscope is not available (3).

We have optimized the combination of fluorescent rRNA-targeted oligonucleotide probing and flow cytometry which is a very promising method for a high resolution automated analysis of mixed microbial populations (4). Fixed cells of bacteria and yeasts were hybridized in suspension with fluorescein- or tetramethylrhodamine-labeled oligonucleotide probes complementary to group-specific regions of the 16S ribosomal RNA (rRNA) molecules. Quantifying probe-conferred cell fluorescence by flow cytometry we could discriminate between target and non-target cell populations. Hybridization conditions, kinetics, and posthybridization treatments were critically examined. Intermediate probe concentrations, addition of detergent to the hybridization buffer, and a posthybridization washing step were found to improve the signal to noise ratio considerably. We could demonstrate a linear correlation between growth rate and probe-conferred fluorescence of *Escherichia coli* and *Pseudomonas cepacia* cells. Oligonucleotides labeled with multiple fluorochromes did show elevated levels of nonspecific binding and could therefore not be used to lower the detection limits which still restrict studies with fluorescing rRNA-targeted oligonucleotide probes to well growing microbial cells. Two probes of different specificities — one labeled with fluorescein, the other with tetramethylrhodamine — could be applied simultaneously for dual color analysis.

Based on comparative analyses of 16S and 23S ribosomal RNA sequences sites specific for the alpha-, beta-, and gamma-subclasses of *Proteobacteria* were located. Short oligo-deoxynucleotides complementary to these signature regions were evaluated as potential nucleic acid probes for the differentiation of the major subclasses of *Proteobacteria*. Hybridization conditions were optimized by the addition of formamide to the hybridization buffer and high stringency post-hybridization washing. Single-mismatch discrimination of probes was further improved by blocking nontarget probe binding sites with competitor oligonucleotides. Nonisotopic dot-blot hybridization to reference strains demonstrated the expected probe specificities, whole cell hybridization with fluorescent probe derivatives allowed the identification of individual microbial cells (5). These probes have been applied to the in situ monitoring of population distribution and dynamics in complex microbial communities like activated sludge.

HIGHLIGHTS / MILESTONES

Development of an improved editor and first success with automated alignment of rRNA sequences. *In situ* identification of a nonculturable *Legionella* killing amoeba. Designing of probes specific for α-, β- and γ-subclasses of *Proteobacteria*. Optimization of flow cytometric identification of microbial cells in mixed suspensions.

PARTICIPANT 06: rRNA Gene Restriction Patterns
P.A.D. Grimont, PAS, F

OBJECTIVES

Our contribution to HRAMI is to develop a reliable and automatized system of species and strain identification based on rRNA gene restriction patterns. Bottlenecks are automated DNA extraction providing a material suitable for endonuclease digestion, optimized transfer and hybridization of DNA fragments,

mobility measurement of video-captured band patterns, relationship between length and mobility of fragments, and development of a data base containing rDNA fragment sizes with associated tolerable error.

RESULTS

Automatic DNA extraction and purification: We have developed a protocol for performing 21 steps in 75 minutes using AutoGen50. AutoGen540 is now routinely used.

Determination of rRNA gene restriction patterns: We have reported earlier on the technical improvements yielding well resolved banding patterns (including vacuum transfer and acetylaminofluorene-labeled rRNA as a probe). These patterns can also be obtained to extract and purify 8 DNA samples in 75 minutes. The machine has been well accepted by our technicians and students who use it intensively to produce all DNA samples needed for our project.

Determination of rRNA gene restriction patterns: We have reported earlier on the technical improvements yielding well resolved banding patterns (including vacuum transfer and acetylaminofluorene-labeled rRNA as a probe). These pattern can also be obtained with a mixture of two digoxigenin-labeled oligonucleotides. These oligonucleotides correspond to universally conserved sequences at both extremities of *rrn* operons.

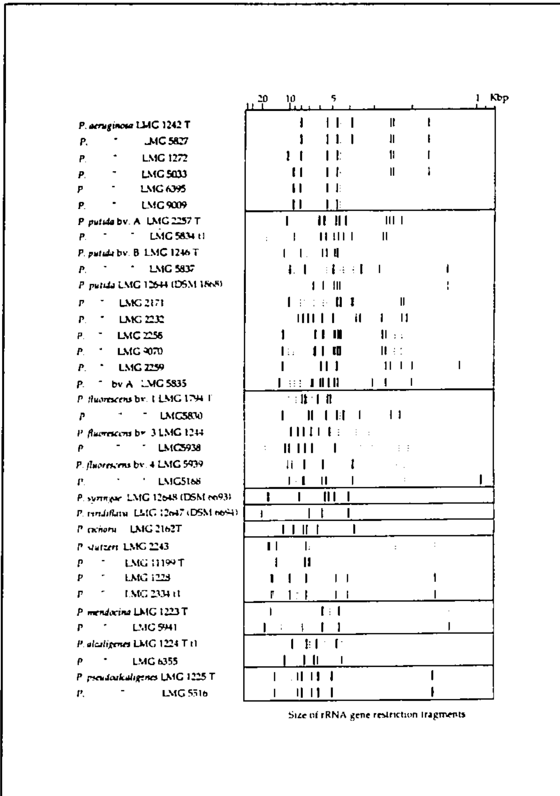
Patterns have been determined for several groups of bacteria including the 'core strains' distributed by participant 08 (RUG). A total of 19 restriction endonucleases were tested on a few representatives of the genera *Pseudomonas, Flavobacterium* and *Cytophaga*. Best results were obtained with *Sma*I, *Hind*III, *Sac*I, and *Hinc*II. The results obtained with a collection of *Pseudomonadaceae* strains showed the relative homogeneity of *P. aeruginosa* (pointing out a strain which was misnamed in the Collection) and the wide heterogeneity of *P. fluorescens* and *P. putida* (Fig. 1). Several species showed characteristic fragments allowing identification at the species level: *P. aeruginosa, P. stutzeri, P. mendocina, P. alcaligenes, P. pseudoalcaligenes, P. cepacia, Acidovorax delafieldii, Comamonas acidovorans, C. terrigena, C. testosteroni, Hydrogenophaga palleronii, H. pseudoflava,* and *P. diminuta*. In the *Flavobacterium-Cytophaga* branch, the following species can be identified by the presence of characteristic fragments: *Flavobacterium breve, F. gleum, F. odoratum, Shingobacterium mizutae, Cytophaga johnsonae, Flexibacter columnaris, Weeksella virosa.*

Videocapture: An image capture system (Visio-Mic^{TM}, Genomic Collonges-sous-Salève, France) including a CCD camera, a 386 PC equipped with a vidocapture card, an enhanced VGA monitor and a video copy processor, is used to capture rRNA gene restriction patterns in the form of a TIFF file where each byte of the raster data correspond to a pixel (thus allowing 256 shades of gray). The TIFF file, saved on a diskette, can be used with an appropriate software on either an Apple Macintosh or an IBM compatible machine. Image capture takes only a couple of minutes (including adjusting settings).

Analysis of captured data: A program has been written by P.A.D. Grimont to read TIFF files, detect lanes and bands, calculate migration values, interpolate fragment size accurately, and provide a schematic representation of fragment migration. All needs and constraints of data capture have been evaluated. Sources of variation are identified. A program for data base creation and screening has been developed. At the moment this report is written, databases for the identification

of some bacterial groups (e.g. *Escherichia coli*) are being built and the first trials of automatic identification are encouraging.

Exploitation prospects: Our techniques and programs will allow Reference Centers to perform more acurate identifications and to characterize and identify industrial strains possibly released in the environment.



Part. 06 — Fig. 1: *Ribotypes of different species of fluorescent* Pseudomonas *spp. obtained with* Sma *I restriction enzyme.*

HIGHLIGHTS / MILESTONES

— Successful automated DNA extraction for rRNA gene restriction pattern analysis.
— Restriction pattern analysis by digoxigenin-labeled oligonucleotides.
— Software development and evaluation for rRNA gene restriction pattern analysis.

PARTICIPANT 07: Low-molecular-weight RNA Profiles

M. Höfle, E. Katsivela-Hussein, GBF (MPI), D

OBJECTIVES

1. Development of high resolution LMW RNA profiles by conventional electrophoresis.
2. Comparison of high resolution LMW RNA profiles of core strains.

3. Establishing of an electronic data base for high resolution RNA profiles.
4. Application of capillary electrophoresis to LMW RNA.

MAJOR PROBLEMS ENCOUNTERED

Set up of a normalized, PC-based data base of LMW RNA profiles is in an early stage and capillary electrophoresis is in a preliminary phase.

RESULTS

1. **Development of high resolution LMW RNA profiles**: The limiting factor in the application of the analysis of low-molecular-weight (LMW) RNA (5S rRNA and tRNA) to the taxonomic classification and identification of bacteria was the resolution of the tRNAs. With the newly developed high resolution gels 40% to 50% more tRNA bands could be obtained. Progress in terms of purification of tRNA was made by using purification columns with an ultrafiltration membrane (cut-off 100 000 dalton). In terms of rapidity a fast screening gel technique was developed that allowed the rapid screening of up to 60 strains on the same gel.

2. **Comparison of high resolution LMW RNA profiles of core strains**: High resolution electrophoresis of LMW RNAs was then applied to 11 species of the genus *Pseudomonas* (only from RNA group I) including several core strains per species. On the high resolution gels all reference strains of the same species showed the same molecular weight profiles, i. e. the banding patterns of their tRNAs were identical. The banding pattern of all species were different except the following two pairs: *P. chlororaphis* and *P. aureofaciens; P. mendocina* and *P. pseudoalcaligenes*. These two pairs are also closely related according to DNA-DNA homology and 16S rRNA sequence comparison, respectively. Furthermore, one of the *P. putida* reference strains, DSM 3936, was identified as a *P. fluorescens*.

With the standard LMW RNA technique a set of 77 nitrous oxide-producing isolates from the central Baltic was screened (1). The bulk of the isolates (almost 80%) could be identified as *Shewanella putrefaciens*. The rest of the strains (18) belonged to 12 different genotypes often represented by a single isolate.

3. **Establishing of an electronic data base**: Data were catured from high resolution gels via an automated gel scanner. An electronic data base of all core strains was established but mathematical normalization of single gel runs caused problems. Newly developed profile analysis software by RUG (participant 08) might solve these problems.

4. **Application of capillary electrophoresis to LMW RNA**: Capillary electrophoresis was applied for the first time to a set of low molecular-weight reference molecules (pd(A)25-60, $tRNA^{Phe}$ (77nt), $tRNA^{Tyr}$ (89nt), 5S rRNA (120nt)). Different running conditions were tried resulting in the best separation of oligonucleotide and tRNA standards on a 3% acrylamide filled capillary. A major analytical problem encountered was the instability of the largest molecule (5S rRNA) during electrophoresis under the given running conditions. Newly developed capillaries and different running conditions might solve this analytical problem.

HIGHLIGHTS / MILESTONES

— Improvement of resolution for tRNAs by 50% and increasing of the rapidity by a factorof 3 for electrophoretic LMW RNA profiles.
— Establishing of an electronic data base on high resolution LMW RNA profiles.

— Demonstration of the applicability of LMW RNA profiling to a set of marine isolates.

PARTICIPANT 08: Protein and Fatty Acid Profiles, Strain Management

K. Kersters, M. Vancanneyt, RUG, B

OBJECTIVES

1. Selection and distribution of core strains
2. Software development
3. Cellular fatty acid analysis (FAME)
4. Whole-cell protein electrophoresis (SDS-PAGE)
5. Biolog System

MAJOR PROBLEMS ENCOUNTERED

Taxonomic evaluation of all data is still in an early stage.

RESULTS

1. **Selection and distribution of core strains**: In order to facilitate the selection of well-characterized core strains, an integrated strain data base was constructed for bacterial taxa for which 16S and/or 23S rRNA sequence data are available. Core strains were selected in the following microbial groups: pseudomonads, *Flavobacterium/Cytophaga* complex and enterococci. LMG distributed approximately 220 different core strains to the participants GBF, IFR, MHH, PAS, RUG and/or TUM.

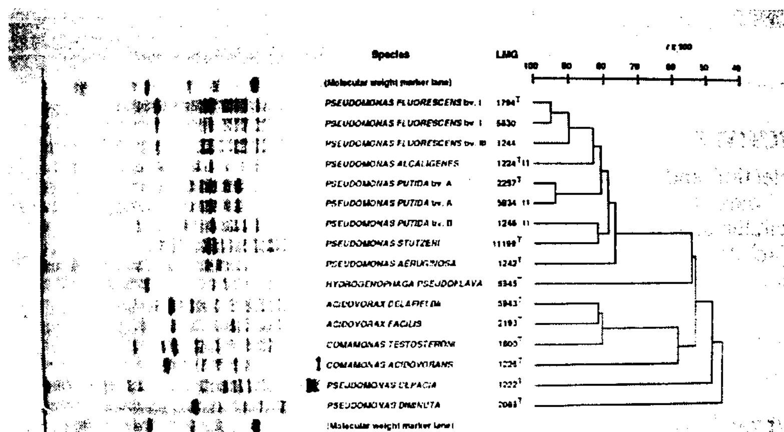
2. **Software development**: Improvement of software was made for data capture and alignment of protein profiles. New software was developed for the numerical comparison of BIOLOG data.

3. **Cellular fatty acid analysis (FAME)**: The cellular fatty acid composition of 170 selected core strains of the pseudomonads, *Flavobacterium/Cytophaga* complex and the enterococci was studied. A variety of fatty acids was detected, including branched and straight-chain acids, cyclopropane and hydroxy fatty acids. Within these taxa an overlap of profiles occurred at the infra-specific level and even among some species. The fatty acid pattern usually correlated well with groupings at higher taxonomic levels e.g. for the pseudomonads each of the major rRNA subgroups seem to have a characteristic fatty acid profile in which the hydroxy fatty acids are of particular taxonomic value. Profiles were stored in a database and will be used for the identification of new isolates.

4. **Whole-cell protein electrophoresis (SDS-PAGE)**: The electrophoretic separation of cellular proteins of core strains of the pseudomonads, *Flavobacterium/Cytophaga* complex and the enterococci was most useful for the detection of taxonomic relationships at and below the species level (Fig. 1 on the next page). Valid deductions on the genotypic relatedness levels could not be made. Within some species a considerable protein electrophoretic heterogeneity was shown. Sometimes, this heterogeneity was correlated with the infraspecific grouping. Strains of some species, showing qualitative and/or quantitative unique protein patterns, were characterized at the strain level. All fingerprints were stored into a database.

5. **Biolog system:** The ability to oxidize 95 different carbon sources yielded metabolic fingerprints. The majority of the pseudomonad strains studied were identified to the species level. In some cases taxonomic information was obtained above and/or below

the species level. The groupings obtained with the Biolog system were compared with the results obtained by SDS-PAGE and FAME analysis. A database was constructed.



Part. 08 — Fig. 1: *Each of the 16 pseudomonad strains is characterized by a specific and reproducible protein fingerprint. These are grouped by calculation of similarity coefficients between each pair of protein patterns. The dendrogram showing the similarities between the protein fingerprints was obtained by the UPGMA clustering technique.*

HIGHLIGHTS / MILESTONES

— Improvements of software for data capture.
— Accumulation of extensive protein, fatty acid and Biolog data
— Preliminary comparison of the efficiency of the different phenotypic techniques for identification of microorganisms.

PARTICIPANT 09: Computerized Image Analysis
N.P. Kennedy, CAP, IRL

OBJECTIVES

The work proposed was divided into 5 work packages as follows:
WP1. Investigate the analytical requirements.
WP2. System requirements definition.
WP3. Configuration and commissioning of system at CAPTEC premises.
WP4. Prototyping and experimentation.
WP5. Evaluation.

MAJOR PROBLEMS ENCOUNTERED

1. The original workplan schedule was dependant on a start date which was not achieved by the project. The resultant delay will require a project extension beyond the original contract end date of 31st Oct 1993.
2. The time period required to establish effective technical collaboration with our linked participant in the Technical University of Munich was initially underestimated. This delayed the CAPTEC workplan, but the rate of progress is now satisfactory.

RESULTS

Within the HRAMI project, CAPTEC is performing research into methods of computerized image processing and analysis which are appropriate for the quantitation of fluorescence emitted by bacterial cells stained with fluorescent labels. This work is being performed in collaboration with the TUM (participant 05).

Work packages 1 to 3 have been completed. WP4 is ongoing, and WP5 has not yet begun.

WP1 The user requirements were determined following several working meetings. Some of the most salient software features desired include the ability to read colour image files in TIFF format, correct for background variation. and enable interactive exclusion of non-target or artefactual objects.

WP2/3 The relevant sections of the imaging system at TUM have been duplicated in CAPTEC, enabling easy transfer of data.

WP4 In order to facilitate development of the prototype software to address the user requirements, a variety of representative images were obtained from TUM. Software adaptation and development is proceeding to solve some of the basic problems inherent in these before measurement procedures can be tackled satisfactorily.

Image processing: Some image processing operations (*eg* stretching, brightening, *etc*) render some later measurements invalid (*eg* of fluorescence intensity). However, other operations are essential, such as background correction and detection of artefacts.

Background subtraction: An important preliminary to the analysis of images is the subtraction of background artefact. The optimal method by which this is achieved depends, in part, on the objective of the subsequent analysis. In images of bacteria labelled with fluorescent oligonucleotide probes from TUM, the background fluorescence is unevenly distributed in the image and the primary objective is to estimate intensity of fluorescence rather than to detect cellular margins. Local, pericellular background subtraction is inappropriate in this case — although this is the usual method used when the next operation is edge detection. All areas of the image which contain objects are identified before background intensity is measured in the remaining areas. It is then possible to subtract background artefact, taking it's distribution across the image into consideration .

Detection and exclusion of non-target fluorescent objects: Where samples have been treated with dual fluorescent labels and an image is acquired during excitation of one tag (say fluorscein), a problem can be the 'contamination' of the image data by faint fluorescence from the other tag (say rhodamine). Where fluorescence per image is being measured, it is important to eliminate such contamination. Similarly, flocculated amorphous material may have autofluorescent properties. Such artefactual data must also be excluded from measurement. Both of these sources of non-target fluorscence can be identified using a scatterpolt of the image colour content (*eg* red on green), allowing the user to select an appropriate colour vector for the measurement of fluorescence. This facility improves the specificity of measurement.

Image Analysis: The principal requirements are the ability to count objects of interest and to measure some of their morphometric and fluorescent properties.

Initial efforts have been directed at straightforward images of standard fluorescent latex beads, before progressing to images of bacteria.

Counting, measurement of dimensions and fluorescent intensities: Automatic detection and counting of objects is a standard requirement of many image analysis applications. Difficulties arise where objects of interest are not distinguishable from each other (closely apposed, clustered, overlapping, *etc.*). An example of the measurement functions of the prototype software was done successfully with standard latex beads.

PARTICIPANT 10: rRNA Data Base Management

R. de Wachter, J.-M. Neefs, UIA, B

OBJECTIVES

— Putting a database on the structure of small and large ribsomal subunit RNA at the disposal of other HRAMI participants.
— Further updating of the database with sequences published in scientific journals and international sequence databases.
— Development of programs to automate sequence retrieval from sequence libraries and alignment in our database.
— Construction of phylogenetic trees derived from a large number of sequences at request.

MAJOR PROBLEMS ENCOUNTERED

Due to the exponential increase of rRNA sequences published in literature or deposited in nucleotide sequence libraries, sequence retrieval and alignment in our own databases will have to be further automated.

RESULTS

Growth of the database on rRNA structure: The small ribosomal subunit RNA (SSU rRNA) database (started in 1984) and the large ribosomal subunit RNA (LSU rRNA) database (started in 1991) contain all published sequences or sequences deposited in nucleotide sequence libraries (GenBank and EMBL), as well as those contributed by the HRAMI research partners. The sequences are stored in the form of an alignment for optimal similarity and contain the postulated secondary structure in an encoded form. Newly entered sequences are aligned with those already present, and the secondary structure pattern is transposed to them, by means of a set of dedicated programs. Manual adjustment of the alignment with a dedicated editor follows if necessary. New programs are written to further automate both the search of new sequences in nucleotide sequence libraries and their alignment in our databases.

a) **a) SSU rRNA**: In April 1993, the database contained 1803 complete and partial structures, distributed as follows: 364 *Eukarya*, 65 *Archaea*, 1,260 *Bacteria*, 30 plastids, 84 mitochondria.

b) **LSU rRNA**: This database contained (April 1993) 201 structures distributed as follows: 41 *Eukarya*, 14 *Archaea*, 53 *Bacteria*, 13 plastids, 80 mitochondria. The secondary structure model of LSU rRNA is not yet as detailed as the SSU rRNA model, especially for *Eukarya*. Sequence alignment is therefore somewhat less dependable, but comparative study of the secondary structure of LSU rRNA is in progress.

c) **Contributions of the HRAMI partners to the database**: 16 SSU rRNA sequences were obtained directly from MHH, 25 from IFR, 4 from TUM. 21 LSU rRNA sequences have been received by HRAMI partners were retrieved from nucleotide sequence libraries. The total number of sequences contributed by HRAMI contractors this far is 350.

Software package for phylogenetic tree construction: A software package (TREECON) has been developed for the reconstruction of phylogenetic trees from nucleic acid sequence alignments. The program runs on IBM-compatible microcomputers. This package is at the disposal of the HRAMI partners, and it performs the following tasks: distance matrix calculation, tree construction, bootstrap analysis, rooting, and drawing.

Variability calibration of alignment positions: Some descrepancies can be observed between SSU rRNA-derived phylogenies and the classically accepted evolutionary divergence among Eukarya. These discrepancies are caused by the fact that conversion of sequence dissimilarity into evolutionary distance does not take account of local variability differences in the nucleotide sequence. A method was developed to quantitatively measure the variability of alignment positions. After this variability calibration, different parameters can be used to convert dissimilarity into evolutionary distance for different parts of the alignment, resulting in a more accurate distance computation. This method will shortly be applied to bacterial SSU rRNA sequence alignments.

HIGHLIGHTS / MILESTONES

- The SSU RNA database increased from 520 sequences at the onset of the project to 1803 at this moment. The LSU rRNA database was started with the project and now counts 201 sequences.
- The database is available on-line and off-line to all interested HRAMI partners
- The TREECON software package for phylogenetic tree construction is now available.
- Phylogenetic trees of larger size, to be constructed on a VAX workstation, are supplied upon request.

GENERAL REPORT FOR ALL PARTICIPANTS OF THE T-PROJECT HRAMI

WIDER CONSIDERATIONS

Microorganisms have major positive and negative effects on mankind and his environment. For example, they are the basis of many biotechnological processes, infectious diseases, plant and animal nutrition, elemental cycling in the biosphere, and deterioration of materials. Our ability to benefit from the positive and minimize the negative consequences of microbial activities is often impeded by our inability to identify the relevant microorganisms due to the lack of adequate technology. Existing taxonomic systems have been made antiquated by the large numbers of apparently new microbial taxa being discovered, and by demands from legal and industrial concerns for systems which would be fast and unambiguous. Useful taxonomic/identification systems are a prerequisite for exploring the phylogenetic and metabolic diversity of microorganisms in nature, and for the development of new biotechnological applications therefrom. There is, therefore, an urgent need to develop and automate new bioanalytical systems designed to rapidly obtain and process taxonomically useful information from relevant microbial components. In the context of Biosafety the development of rapid

automated methods to identify microorganisms will facilitate risk assessments of genetically modified microorganisms (GMM's) that have been developed for release into the environment. Such methods will allow better characterization of microbial consortia in target ecosystems and more sensitive tracking of GMM's, and their genes of interest, and hence, permit a more sensitive monitoring of potential perturbations of such consortia as a result of the added GMM's.

COOPERATIVE ACTIVITIES

Three meetings of the HRAMI partner laboratories have been held since the beginning of the funding period: Munich — May 1992, Hannover — November 1992, and Amsterdam — April 1993. The fourth has been scheduled to link with the BRIDGE Biosafety Meeting in Granada — October 1993.

EUROPEAN DIMENSION

The HRAMI T-Project has enabled the expertise and resources of some of the leading European laboratories in the area of microbial identification to focus on common research problems. In the short year that this project has been funded the individual researchers have shown a strong common interest in the scientific issues that have arisen. They have been able to work together to reduce redundancies in planned research and share results, which stimulates the research of the other partners.

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Summary

The Commission of the European Communities is implementing several priority actions specifically designed for improving the competitiveness of European biotechnology. One of these actions aims at the establishment of a Community network for training and research and has been executed from 1982 to 1989 in the framework of two successive Community programmes: the Biomolecular Engineering Programme (BEP; 15 million ECU; April 1982-March 1986) and the Biotechnology Action Programme (BAP; 75 million ECU; 1985-1989). These programmes aimed therefore at establishing a supportive infrastructure for biotechnology research in Europe and the elimination of bottlenecks which prevent such exploitations of materials, data and methods originating from modern biology. The current programme BRIDGE (**B**iotechnology for **I**nnovation, **D**evelopment and **G**rowth in **E**urope) covers the period 1990-1993, with a budget of 100 million ECUs. The research activities in the biotechnology programme BRIDGE are conducted via two different types of projects: N-projects and T-projects. Following three successive calls for proposals in 1989 and 1990, 69 N-projects and 7 T-projects were launched during 1990. These encompass 579 participating organizations (388 for the N-projects and 191 for the T-projects) from 11 Member States and 5 EFTA countries. A 'BRIDGE catalogue of contracts' (EUR 14278 — Editor: B. Nieuwenhuis) listing the participating laboratories together with a description of their objectives was published in 1992. On a regular basis the project coordinators introduce a progress report covering the scientific results achieved in the different laboratories, the evidence of an increasing integration of work and additional relevant considerations. These contributions are assembled in this '1993 report' which, following the 1992 edition (EUR 14298 — ed., A. Vassarotti), presents a complete overview of research progress achieved under the EC BRIDGE programme over the period April 1992-March 1993.

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