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 (1990-1993)

Progress Report 1992

Edited by
A. Vassarotti

EUR 14298 EN

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 (1990-1993)

Progress Report 1992

Edited by
A. Vassarotti

Published by the
COMMISSION OF THE EUROPEAN COMMUNITIES

Directorate-General
Telecommunications, Information Industries and Innovation

L-2920 Luxembourg

Cataloguing data can be found at the end of this publication

Luxembourg: Office for Official Publications of the European Communities, 1992

ISBN 92-826-3997-5

Printed in Belgium

TABLE OF CONTENTS

AREA D: PRE-NORMATIVE RESEARCH

— *In vitro* evaluation of the toxicity and pharmacological activity of molecules

— Biosafety

INTRODUCTION

A glance at recent history:

The year 1992 is the 10th anniversary of the existence of Community R&D programmes in Biotechnology. Indeed it was in 1982 that the pioneer programme for Biomolecular Engineering (BEP) was launched to help developing, through integrated transnational efforts, the tools needed to bring molecular and cellular biology applications to agriculture and agrofood industry. While (only) 103 laboratories participated in BEP, some 262 laboratories were initially included in the ensuing Biotechnology Action Programme (BAP 1985-1989). To further reinforce the Community R&D effort in Biotechnology and to enable Spanish and Portuguese laboratories to join the activities, the BAP programme was revised in 1988, bringing the overall number of laboratories to 378 (with the 116 new contracts, signed after the revision, covering the period 1989-1990). At present, in the framework of the BRIDGE programme, as many as 579 laboratories are engaged in transnational collaborations.

A different way to look at the amplification of the EC effort in biotechnology R&D over the past 10 years would be to focus on the size of the budgets allocated. The conversion into yearly expenses is not straightforward but a reasonable indication stems from two extreme figures: 2 million ECU/year in 1982 compared with about 55 million ECU/year in 1991. A decade has been, therefore, sufficient to witness a quantative change by more than an order of magnitude.

BRIDGE:

The current programme BRIDGE (Biotechnology Research for Innovation, Development and Growth in Europe; 1990-1993) with a budget of 100 Million ECU and implemented with the help of CAN-BRIDGE (see table, p. XII), covers the following research areas:

Information Infrastructure	-	Processing and analysis of biological data
Enabling Technologies	-	Protein design; molecular modelling; biotransformation; genome sequencing;
Cellular Biology	-	Industrial microorganisms; plants and associated microorganisms; animal cells;
Pre-Normative Research	-	In vitro evaluation of the activity of molecules; safety assessment of GMO's.

The corresponding research activities are conducted in BRIDGE via two types of projects: N-projects and T-projects.

The N-projects (N for Network) are built upon the previous successful BAP experience with the European Laboratories Without Walls (ELWW). Basically the N-projects are carried out by groups of European laboratories (on average 5.6 per project) which, through complementary and highly integrated approaches, are devoted to the removal of specific gaps in knowledge and know-how. Although still limited to a fraction of the lifetime of the N-projects (in most cases about one year out of three) the progress reports in the following pages provide sound illustrations of the "plus value" resulting from those integrated approaches.

The T-projects (T for Targeted) aim at the removal, through significant investment of skills and resources (on average almost 30 laboratories and 100 staff per project), of important bottlenecks resulting from structural and scale constraints. Seven T-projects are implemented in BRIDGE:

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

The two latter T-projects, which were the subject of later calls for proposals and therefore only started after Autumn 1991, are not covered in this 1992 report. For the remaining 5 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 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 are open independent structures, called upon the initiative of the industries and interested in the research carried out in a given T-project. They communicate freely with the contractors and, in certain cases, dissect 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.

Finally, two novelties introduced in BRIDGE and shared by all specific programmes under the IIIrd Framework Programme of Community R&D activities need to be underlined:

- Closest ties with EFTA countries - Although not funded by the EC, laboratories from EFTA countries participate in the different projects: 17 laboratories from 5 EFTA countries are collaborating in BRIDGE.
- Decentralisation - 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 preparation and submission of proposals to the Commission also covers, once a proposal is selected and thereafter implemented, several other administrative and scientific duties.

- The progress reports in the following pages, for instance, have been prepared by 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.

D. de Nettancourt,
Head of Division Biotechnology.
Directorate-General for Science,
Research and Development.

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. Catalogue of BAP achievements - (1990). A. Vassarotti and E. Magnien eds, Editions Scientifiques Elsevier, Paris, 242 pp.

  Biotechnology R&D in the EC. Catalogue 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. Catalogue of BAP achievement 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:
  Biotechnology Research for Innovation, Development and Growth in Europe (1990 - 1993). Catalogue 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.

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

Dr. D. de Nettancourt,
CEC, DGXII - F2,
Rue de la Loi 200,
B-1049 BRUSSELS,
Belgium.

COMPOSITION OF THE COMMITTEE OF ADVISORY NATURE FOR BRIDGE

CAN BRIDGE

BELGIUM
J. De Brabandere
J. Hutschemackers
(N. Nolard)
(M.C. Lenain)
(V. Lories)
(B. Lebegge)

DENMARK
L. Kjærgaard
O. Norén
(I. Petersen)
(T. Kjems)

FEDERAL REPUBLIC OF GERMANY
R. Wandel
E. Warmuth
(H. Autzen)
(H. Lang)
(S. Kieffer)

GREECE
C. Goulas
L. Margaritis

SPAIN
A. Albert
J. Ramon Pellon
(M. Candela)
(S. de la Concha)

FRANCE
P. Printz
(S. Béranger)
(M. Lelong)

IRELAND
L. Donnelly
J. Ryan

Italy
A. Albertini
M.G. Sadurny
(G. Magni)

LUXEMBOURG
P. Decker

THE NETHERLANDS
W. Harder
K. Planqué
(J.J. Dekkers)

PORTUGAL
F.J. Carvalho Guerra
J.M. Novais
(A. Xavier)

UNITED KINGDOM
R. Dietz
E. Linton
(C.G. de Grouchy)
(M.E. Nugent)
(D.G. Lindsay)

COMMISSION
H. Tent
D. de Nettancourt

SECRETARIAT
E. Magnien

() Substitutes or experts

BRIDGE

N-PROJECTS

AREA : A

INFORMATION INFRASTRUCTURE

Title	**Continuation of the expanded services of the EMBL Data Library.**
Contract number	**BIOT CT-910254**
Official starting date	**1 March 1991**
Coordinator	**Graham Cameron, The EMBL Data Library, Heidelberg, Germany**
Participants	**The EMBL Data Library**

Objectives set for the reporting period

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.

Work was also planned to improve the efficiency of production systems to cope with faster data throughput and to take account of requirements generated by genome scale sequencing with automated sequencing machines.

Also anticipated were meetings of the European Advisory Committee and the International Advisory Committee, as well as a gathering in Japan of the collaborating databank groups (the EMBL Data Library, GenBank, DDBJ).

Major problems encountered

1. European network performance has often limited our ability to keep EMBnet nodes up-to-date, and required labour-intensive *ad hoc* interventions in the automatic procedures.
2. The Gulf War resulted in travel restrictions for some of our American collaborators which postponed the preceding collaborative meeting, with this in mind the collaborative meeting planned for February 1992 has been put off until May.
3. The European Advisors did not meet in August 1991 as planned, but held a meeting before the meeting of the international meeting in March 1992.

Results

Increased data throughput has been the most striking recent development in the Data Library. Hardware and software developments and refined data processing procedures meant that at the end of 1991 we were entering data three times faster than at the start of the year. The four releases planned during the year were successfully delivered.

The nucleotide sequence database grew by about 50% in 1991 to over 75 million base pairs and is now over 80 million base pairs. The growth is summarised in figure 1.

Data management

Major new investments in hardware for the Data Library have included the purchase of a MicroVAX 3100 as a database server and six DECstation 2100 RISC workstations. This resulted in huge savings on software licenses and made available the better user interfaces possible with the workstations. This, with software developments using a new version of ORACLE, resulted in substantial productivity improvements. Porting of our major applications to a client/server environment based on workstations and the MicroVAX during 1992 should give even better performance.

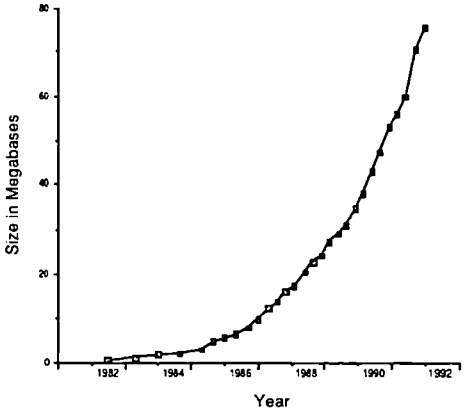


Figure 1. Nucleotide Sequence Database growth

Data Distribution

Quarterly releases of the databases continue to be distributed on magnetic tape and CD-ROM. CD-ROM has now comfortably overtaken magnetic tape as the distribution medium of choice, and the number of customers continues to increase. Some new indices have been added to allow user software to efficiently access the databases on the CD-ROM. A collaboration with Oxford University Press resulted in several additional databases described in the Nucleic Acids Research 1991 Sequence Supplement being distributed on the EMBL CD-ROM.

Fileserver

The EMBL file server continued to be popular, with requests for SWISS-PROT sequences and Brookhaven structural data increasing dramatically and, of course, the constant flood of requests for latest nucleotide sequences and the free molecular biological software. More than 170 programs are now available.

The Mail-FastA database searching service is in increasing demand, with an average of more than 30 searches per day. Unfortunately the less computationally-intensive Quicksearch service has not proven as popular and it will probably be replaced by the equally efficient and more widely accepted BLAST algorithm.

A useful by-product of rapid availability of the data is that we receive more feedback and input from researchers who will, for example, notify us if journal publications contain data we have not yet released.

EMBnet

EMBnet continued to flourish with the 15 nodes receiving daily updates. EMBnet is funded under a separate BRIDGE contract.

Data from genome projects

In 1991 the Data Library incorporated the first data from major genome sequencing projects. Both the European yeast chromosome III and the British C. elegans sequencing projects submitted data in the course of the year.

Sequence data in patents

Persistent discussion with the European Patent Office (EPO) into ways in which sequence data included within patent applications can be rendered public seems to have borne fruit. It is hoped that in early 1992 a contract can be finalised whereby the EPO provide the financial resources for the database work to be carried out at EMBL. The backlog of data should be made publicly available via the usual means during the coming year.

Patent applications with their first priority in the USA will be processed by the NCBI, with whom data will be exchanged.

Sequence Analysis Research

Rapid sequence comparison

The EMBLSCAN tool which was developed in 1990 to rapidly locate database entries similar to a new sequence was enhanced for distribution on our CD-ROM as well as being incorporated into the normal production procedures of the Data Library. This helps to match published sequences and submissions and avoid duplicate entries in the database.

Multiple sequence alignments

Higgins' CLUSTAL multiple sequence alignment program, which has become something of a standard, has been completely rewritten in collaboration with Alan Bleasby (Daresbury, UK). A single, menu driven program called CLUSTALV was produced which runs on a wide range of computers. Many new features were added, including the ability to align old alignments with each other and the calculation of phylogenetic trees.

The tree in figure 2, from Waters *et al* (1991), was calculated using CLUSTALV and shows relationships between the rRNAs of six species of *Plasmodium*, the organism that causes Malaria. The tree is based on genetic distances calculated from small-subunit rRNA sequences. Bootstrap confidence limits are shown under each grouping in the tree. The main conclusion from the tree is that *P.falciparum*, the most serious cause of Malaria in humans, is related to the species parasitising birds. This arose from a lateral transfer event, where a parasite changed host between birds and humans.

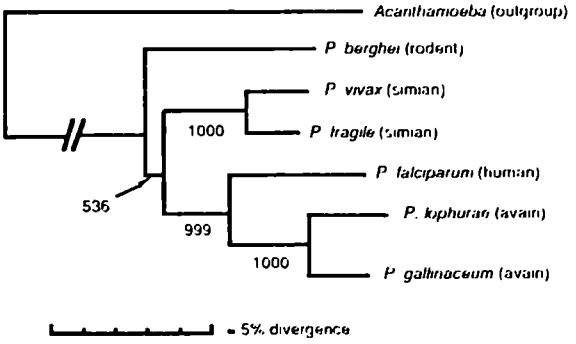


Figure 2. A phylogenetic tree of six Plasmodium species derived from SSU rRNA sequences. Branch lengths are proportional to estimated sequence divergences. Figures below each grouping show bootstrap confidence limits from 1000 bootstrap samples.

Simultaneous Database Access

In 1991 Peter Sibbald joined the group as an independent researcher. His first project is to explore using multiple databases simultaneously. It is becoming clear that databases are already and will become even more distributed. Updates to those databases are going to be more frequent and the complexity of the data stored will grow.

This work has received support in equipment contributions from the Digital Equipment Corporation as part of their Scientific Innovators Program.

Highlights/ milestones

We have now seen the first data from major genome sequencing projects entering the database and began to face up to the challenges that this poses.

Wider considerations

Improvements in the efficiency of the EMBL Data Library enable it to provide convenient access to a comprehensive collection of sequence data. Today's database, however, at 80 million base pairs, is less than 3% of the size of a human genome (20% of the data are human). Efforts to sequence the human and other genomes will have profound effects on the scale and complexity of the database task. Increased understanding will make the information useful in diverse areas, resulting in rising demand for the Data Library's services and a need for innovative solutions in new application areas. The Data Library must have the stability to provide good services today and the resources and vision to develop the services of the future.

Cooperative activities

International advisory committee

The fourth meeting of the International Advisory Committee for Nucleotide Sequence Databases took place in Washington March 1991, and the fifth in Heidelberg in March 1992.

International Collaborations

GenBank/DDBJ — The collaboration with the GenBank and DNA Database of Japan (DDBJ) groups and daily data exchange between these groups continued, with the annual collaborative working meeting being held in Heidelberg (24-28 June 1991). The main topics of the meeting were the feature table and more detailed aspects of common annotation conventions.

The National Center for Biotechnology Information (NCBI) — From October 1992 NCBI will manage the GenBank project. With this in mind we did much in 1991 to solidify the collaborative links with NCBI, both through a formal agreement between the two organisations, and through informal interactions. Three of the EMBL staff paid a one week visits to NCBI in the course of the year to discuss practical aspects of the collaboration such as editorial standards and data exchange mechanisms.

The Martinsried Institute for Protein Sequences (MIPS) — 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.

The Genome Data Base (GDB) — In view of the importance of good links to mapping information, we reinforced our collaboration with the Genome Data Base from Johns Hopkins University. In August 1991 one week course on the use of GDB was offered in collaboration with staff from CEPH in Paris, the DKFZ from Heidelberg and the IMP in Vienna and Johns Hopkins and Utah.

Genome informatics centres

The importance of genome project data is well recognised, and in order to improve the communication between genome initiatives and the public databases, a workshop was organised with representatives from several European genome projects to discuss specific problems of data exchange and representation. Procedures were discussed to allow the automatic incorporation of data from such projects into our database. Future such consensus meetings with broader perspectives are planned.

List of joint publications with trans-national authorship

Data Library Staff (1991) EMBL Data Library Release Notes and User Manual, Releases 26,27,28,29, 30.

Bairoch, A. and Data Library Staff (1991) SWISS-PROT Release Notes and User Manual, Releases 17,18,19,20, 21.

Bairoch, A. and Boeckmann, B. (1991) The SWISS-PROT protein sequence data bank. Nucl. Acids Res. 19, 2247-2249.

Fuchs, R. and Cameron, G.N. (1991) Molecular biological databases: The challenge of the genome era. Prog. Biophysics Mol. Biol. 56, 215-245.

Fuchs, R. and Higgins, D. (1991) The EMBL Data Library. In: Collins J. and Driesel A.J. (eds) Genome analysis: from sequence to function. Huethig Buch Verlag, Heidelberg, pp. 149-150.

Pelandakis, M., Higgins, D.G. and Solignac, M. (1991) Molecular phylogeny of the subgenus Sophophora of *Drosophila* derived from large subunit of ribosomal RNA sequences. Genetica, 84, 87-94.

Powell, R., Higgins, D.G., Wolff, J., Byrnes, L., Stack, M, Sharp, P.M. and Gannon, F. (1991) The salmon gene encoding apolipoprotein A-I: cDNA sequence, tissue expression and evolution. Gene, 104, 155-161.

Sharp, P.M., Lloyd, A.T. and Higgins, D.G. (1991) Coelacanth's relationships. Nature, 353, 218-219.

Waters, A.P., Higgins, D.G. and McCutchan, T.F. (1991)*Plasmodium falciparum* appears to have arisen as a result of lateral transfer between avian and human hosts. Proc. Natl. Acad. Sci. USA, 88, 3140-3144.

Other publications/ patents

Bilofsky, H., Informatics in Molecular Biology. "Medical Informatics Europe 1991" K.-P. Adlassnig, et. al. (Eds.), Springer-Verlag, 28-31; 1991.

Fuchs, R. (1991) MacPattern: Protein pattern searching on the Apple Macintosh. CABIOS 7, 105-106.

Fuchs, R., Weber-Schaeuffelen and Zinke, H. (1991) Computer in der Molekularbiologie. In: Bertram, S. and Gassen, H.G. (eds) Gentechnische Methoden. Gustav Fischer Verlag, Stuttgart pp. 307-315.

Stoehr, P. (1991) Sequence databases from EMBL and developments in electronic access. "Proceedings of the 1991 Chemical Information Conference" H. Collier (Ed), Infonortics Ltd, Calne, 187-195.

Stoehr, P. and Cameron, G. (1991) The EMBL Data Library. Nucl. Acids Res. 19, 2227-2230.

TITLE: CarbBank

CONTRACT NUMBER: BIOT-CT90-0184

OFFICIAL STARTING DATE: 01.02.91

COORDINATOR: Prof. K. Bock, Carlsberg Lab., Dept. Chem., Copenhagen, DK

PARTICIPANTS: Prof. H. Paulsen, Uni Hamburg, Dept. Org. Chem., Hamburg, FRG

Prof. J.F.G. Vliegenthart, Uni Utrecht, Dept. Bio-org. Chem., Utrecht, NL

OBJECTIVES SET FOR THE REPORTING PERIOD:

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 (01.02.91 - 31.01.92) was to increase the number of oligosaccharide structures included in the database in order to reach a complete coverage of the current literature in the near future. The objective for the reporting period was to release a version of the database with at least 3000 records.

Furthermore, the development of tools to link the structural database to spectroscopic information like ^{1}H- and ^{13}C-NMR data was planned as well as a better connection to other databases.

MAJOR PROBLEMS ENCOUNTERED:

A major problem was the collecting of data by manually extracting the oligosaccharide structures and the annotation from the original literature. This problem was solved by using the Chemical Abstracts Services as a tool to get access to literature in the carbohydrate field as well as to extract relevant information.

RESULTS:

In January 1992, the latest version of the *CarbBank* program and the structural database CCSD with about 8000 records was released.

In the beginning of the project the task of collecting the structural data was performed manually by a systematic survey of the existing carbohydrate literature. The input of data into the database was actually a manual procedure which is very time consuming.

In the course of this project the computer program *CASON* was developed in Copenhagen to translate the systematic name of a compound as used in the CA registry file to a structural representation as used in *CarbBank*. The Chemical Abstracts (CA) ONLINE service was utilized

to search for specific structural elements of N- and O-linked oligosaccharides. The search resulted in about 2900 entries which were extracted and subsequently converted using the *CASON* program. These data were divided under the 3 participating groups and verified against the original literature. Detected errors like misspellings, structural errors and wrong specification of aldose/alditols were corrected. Some structures which were not sufficiently characterized were not incorporated into *CarbBank*. An error report was send to CAS.

In exchange for the computer program *CASON* Chemical Abstracts agreed to release all entries from CAS containing oligosaccharide structures with more than two monosaccharides for our purpose of building up the *Complex Carbohydrate Structural Database*. Thus by October 1, 1991 we received about 30000 records from CAS covering the carbohydrate literature from 1966 - 1991. About 15000 were suitable for translation into a format which can be read by *CarbBank*. About 4000 records were already in CCSD, so that the remaining 11000 have to be verified and checked against the original literature by the curators. This is currently in progress at the participating laboratories in Utrecht, Hamburg and Copenhagen. The contract with CAS allows us to workup the backlog of already published oligosaccharide structures as well as the ones which will be published over the next five years in a manageable manner.

Due to this more automatical approach of collecting and verifying data our goal of covering the whole carbohydrate literature and including relevant structures and references into CarbBank will be achieved during the planned timeframe.

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*. 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 a ^{13}C-NMR module has been appended to the database. The database of NMR tables of carbohydrate structures has been extended and includes now a total of 508 ^{1}H-NMR records and 237 ^{13}C-NMR records. The corresponding database management program has been improved and the user interface has been changed to be IBM SAA/CUA compliable.

HIGHLIGHTS:

Due to the contract with Chemical Abstracts the number of structures (8000) currently included in the database is much higher than anticipated at this point of time.

COOPERATIVE ACTIVITIES:

Meetings:

November 1991	Maastricht (NL) - Symposium "Bioinformatics in the 90's" and BRIDGE meeting Prof. J.F.G. Vliegenthart, Prof. H. Paulsen, A. van Kuik, R. Stuike-Prill, A. Kleen.
February 1992	Hamburg (FRG) - *CarbBank* meeting Prof. K. Bock, Prof. J.F.G. Vliegenthart, Prof. H. Paulsen, A. van Kuik, R. Stuike-Prill, A. Kleen.

Otherwise 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.

A cooperation with the US National Library of Medicine / National Center for Biotechnology Information (NCBI) will result in the distribution of *CarbBank* on a CD-ROM together with other databases, like amino acid (PIR) and nucleotide sequence (GenBank) databases. This will significantly increase the number of distributed copies of the database. Currently, the integration of *CarbBank* into the database retrieval software 'Entrez' developed by NCBI is in progress. After completion an easy access of different databases using the same software enviroment will be possible.

Recently, a cooperation with the amino acid sequence databases MIPS and PIR has been set up to find a way of cross referencing these databases with *CarbBank*.

PUBLICATIONS:

CarbBank Version 2.2 with the database CCSD5.

A ^{1}H-NMR database for complex carbohydrate structures. J.A. van Kuik and J.F.G. Vliegenthart (1991), *TIGG* **3**, 229-230.

TITLE:	Protein Sequence Databank
CONTRACT NUMBER:	BIOT-CT90-170
OFFICIAL STARTING DATE:	01/01/1991
COORDINATOR:	H.W. Mewes, Max-Planck-Institut f. Biochemie W-8033 Martinsried, Germany

OBJECTIVES SET FOR THE REPORTING PERIOD:

Development of software tools for rapid distribution of protein sequence data within Europe and informatics support of the annotation process.

MAJOR PROBLEMS ENCOUNTERED:

We encountered two major problems in achieving the goals set. The first problem is the situation on the labor which is market is very unfavorable to recruit qualified computer scientists. The project requires a sound knowledge of both programming techniques and modern biology. The second problem is the poor performance and high costs of European networks, which did not permit an efficient use of the resources provided by the on-line system. The current connectivity and bandwidth of academic data communications lag far behind the technical development. The US gained a considerable edge in both resources available for bioinformatics and the network infrastructure.

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 informatics support of the data distribution and the development of a formal Sequence Database Definition Language (SDDL) in collaboration with PIR-Washington.

1. **New methods for data distribution:**

Rapid distribution of protein sequence data becomes increasingly important. The traditional distribution by magnetic media is too slow and not frequent enough to fulfill the needs of the user community. Releases distributed on magnetic media or on CD-ROMs may miss hundreds of new entries due to the delay in release production and distribution (usually 1-2 months). Local implementations of the data need require skilled personnel and considerable hardware resources, which are costly.

Communication software opens access to remote data on network systems. The network performance is not yet satisfactory in many cases. The storage of data at a single node is not feasible. We investigated an intermediate solution: data should be distributed by a number of EMBnet nodes, covering 'local' needs. Our approach will provide nodes with an appropriate software to parse transactions and to install the data in a searchable database management system (INQ/ATLAS).

The project in progress is part of a collaboration with the Technical University of Munich (K. Heumann, Department of Informatics, headed Prof. Dr. Bayer).

Data distribution through networks is looked at as a special type of a distributed application. The key concept of communication is a client/server model (see fig. 1). The

data distribution targets are databases within Europe. Therefore, it is necessary to use wide area networks (WAN) as transport media. The software should work as well on local area networks (LAN). TC/IP is implemented a protocol using public network and local ethernet connections. A typical feature of WANs is that only one-to-one communication is supported. This is the main difference from LANs which can easily use broadcast concepts where one-to-many communication is done. In a WAN communication, there are many possible sources for errors, for example transport and communication errors and run-time errors at the remote systems. For both types error logging and error recovery are needed. In open networks it is important to protect subnet partners from an unauthorized access. Maximum security is achieved if authentication is extended from only identifying the communication partners to identifying the functionality of the application and the type of data that the remote partner verifies to process. It is important to assure maximum software portability to allow the application to operate in inhomogenous networks. Therefore, software is layered on a general purpose communication mechanism that hides the underlying network from the application software. The communication mechanism used is the remote procedure call (RPC). A remote procedure call functions like a local procedure call, but the caller is resident on a different node.

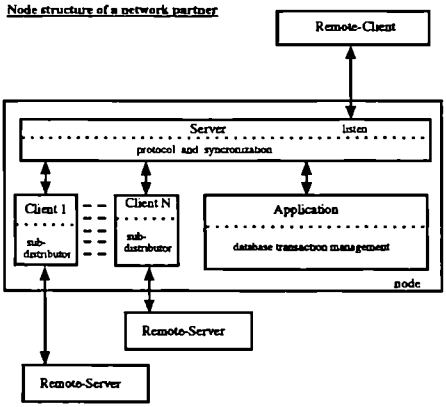


Figure 1 Client/Server concept for data distribution

2. Data exchange between nodes of the PIR-International

During 1991, a mechanism of updating protein sequence data that relies on the frequent use of a transaction protocol through the INQ (IN-Query-System) became fully operative, leading to a rapid increase of the number of entries accessible to the users. Entries are updated daily and the dataset PIR3 is downloaded weekly to the on-line system. The following data are inserted into INQ:

- Data from journal scanning, verified by double input
- Data from EMBL translations verified by double input

- Data from EMBL translations marked as 'unpublished' (no double input possible)

The data must fulfill the following requirements:

1. The title line must be complete.
2. The biological source must be checked against the taxonomy database.
3. The reference must not conflict with any other reference in the database.
4. The accession number must be unique.

All updates of entries are automatically submitted to the INQ-database.

An efficient complete annotation of protein sequences must be done by protein families. This requires the exchange and frequent update of protein entries between the databanks. Based on the experience gained with the INQ system, we are working on the methods for reservation and updating of entries on distributed nodes. The system has to fulfill the requirements of a distributed database, a problem that has not yet been solved by commercial systems. We intend to build up the system on top of a commercial, object-oriented database management system. The development of such a complex software to fulfill special needs of the protein sequence databanks is in an early stage.

3. User-support and on-line system

The on-line system provides users with free access to the latest versions of the major databanks. Currently, the system holds 238.645 sequences in 13 databases which are concurrently accessible interactively by the XQS system. The latest PIR-International protein sequences are updated weekly. Sequence comparisons to search for homologies can be performed as batch jobs submitted by electronic mail. Searches in the databases are also possible through a file-server system. MIPS has dedicated powerful resources to support external users (3 VAX computers and one DEC-Station 5125).

4. Progress in protein classification

One of the handicaps in the usage and the future development of the present PIR data bases is the lack of a complete sequence classification: only 25% of the sequences contained in the PIR1 data base have already been classified by NBRF and MIPS. The other 75% are gathered in the two databases PIR2 (partially annotated) and PIR3 (preliminary entries).

The goal of the cooperation was to extend the classification of PIR1 to the sequences of PIR2 and PIR3 in order to reach a homogeneous structure the whole data base using the existing Family Alignment Data Base and the corresponding software tools developed at the Max Planck Institute for Experimental Medicine in Göttingen. For the period Nov. 91-Feb. 92 we expected to automatically classify approximately 50% of the total data base.

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 in PIR2 and PIR3 according to the classification rules of PIR-International

This set of programs closed the life-cycle of the Family Alignment Data Base (FAMALN-DB) which now consist of 4 major phases:

1. - Migration to new release
2. - Scanning the sequence data base for new homologies
3. - Multialigning new or modified families
4. - Classification of PIR2/PIR3 sequences

Several programs had to be modified to account for the PIR-Classification of the sequences and to build up the corresponding PIR-Data Base indexes. The PIR-Classification and the family classification used by the Göttingen group are not a one to one mapping. Using an independent classification scheme, we were able to detect several inconsistencies in the PIR classification. Most of these have been cleaned up in Rel. 31 of the PIR-DB or are going to be cleaned up in the next release. The more important use of the family classification was to create a preliminary classification of many PIR2 and PIR3 sequences. At the end of Rel. 30 life cycle in Feb. 1992 we were able to classify:

in PIR1:	8798 (done by PIR)
in PIR2:	6168 (this project)
in PIR3:	5095 (this project)

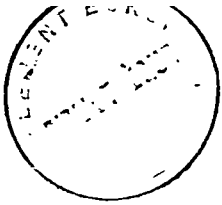
Total: 20.061 sequences = 59% of total 33.989 sequences

In summary, the major aim of the project has been achieved. In conjunction with the new XQS-System of PIR, indices allow an unified view of the full PIR-International data base, using the superfamily classifications.

5. FASTA Database

MIPS compares every protein sequence added to the dataset against all other sequences available. The information is valuable for the protein classification and the annotation based on the principle of sequence similarity. In addition, most sequence comparisons performed by external users repeat comparisons that are already performed at the time the sequence was added to the dataset.

The number of possible one to one comparisons for the current set of 50.000 sequences is 1.25 million. The task of comparing all available sequences with themselves is feasible within approximately 40 days of run-time on an ULTRIX system. Storage requirements will be moderate. Such a database requires a flexible data structure to store results from newly added sequences. Sequence similarities are symmetric between each pair of sequences compared. Therefore, the FASTA search of a new sequence must be written to the records of all other similar sequences to result in an up-to-date database of protein relations. However, this database will be highly interesting not only for the internal purposes of the databank. Once set up, updating will require only moderate efforts with respect to CPU-time and allow the user to retrieve protein similarities extremely rapidly. Currently the set of 36.150 sequences available in the PIR datasets are compared.



6. Development of a new format/database structure

An important step for the development of the sequence databanks was achieved with the definition of a standardized format for sequence data exchange. The evolution from individual sequence data entries to complex interlinked data structures requires the extension and formalization of the data definition.

The CODATA format possessed two major disadvantages: First, the syntax was formally defined, but not the semantics. Second, the rigid definition of the format structure required the adaptation of the existing sequence databanks, which is an unattainable goal.

The representation of complex biological information in digital computers is only possible with the application of well-defined semantics, serving for an exact description of the data interdependencies. A certain feature, e.g., a modified site, is only valid in relation to the one-dimensional array of the sequence. These relations are displayed as numeric coordinates, they can also be used to transform one sequence into another (e.g. to describe sequence discrepancies). The numeric values have no intrinsic meaning, the information is only generated by the combination of two or more different data types. Object-oriented languages provide mechanisms to handle these complex data types as operations ('messages') of classes to generate transient objects (in contrast to the stored, 'persistent' objects). The currently implemented database systems handle semantics by specialized software, written on top of standard applications provided by the databases management system (DBMS). A formal description of the relations does not exist. The software must 'know' the relations of certain data fields (tables) and has to interpret data dependencies, rendering software data and schema dependent. Every change in the schema has to be reflected in changes to the software. Although protein sequence data and the associated information require only a limited number of datatypes for their description, a large number of different, multiple relations between the elements exist. The organization of the databank as 'components' allows an independent development of complex data structures, like the taxonomy, the superfamily classification, the genetic information or the complex protein architecture.

In collaboration with Dr. D.G. George of PIR-Washington, the theoretical concept of a sequence definition language was used to address the current problems by

1. incorporating high-level data types that include a functional definition of the data
2. providing a data declaration section that allows the organizational structure and information content of the database to be formally defined
3. providing a mechanism that allows interdata dependencies to be resolved selectively and unambiguously
4. introducing a mechanism for unambiguous reference to information in other database components and thus providing for their interlinkage.

This approach allows data verification by strong datatyping and a formal control of the data set. Its realization will be of basic importance for the success of sequence data processing.

HIGHLIGHTS/MILESTONES

New methods for data exchange and distribution of protein sequences have been explored. The concept of data distribution over wide area networks has been investigated. A new method for protein family classification has been applied. An advanced sequence data description language is under development.

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. During the past 4 years, the amount of data available increased by a factor of 5.4 fold. In the future improved methods for data processing and distribution will be applied. Structuring the database into protein families and development of new concepts for the organization of biological data will largely improve the usefulness of the database in the future.

COOPERATIVE ACTIVITIES

P. Kreisl participated at the EMBnet meeting on Crete (May 1991).

H.W. Mewes participated at the Sectorial Meeting of the BRIDGE bioinformatics group at Maastricht NL, Nov. 1991.

OTHER PUBLICATIONS

George D.G., Orcutt B.C., Mewes H.W., and Tsugita A.; 'An Object-Oriented Sequence Database Definition Language (SDDL)', submitted to Protein Sequences and Data Analysis

TITLE: Promotion of EMBnet: Computer Network for Bioinformatics in Europe

CONTRACT NR.: BIOT CT91/0273

OFFICIAL STARTING DATE: 01.04.1991

COORDINATOR: Prof. Cecilia Saccone, CNR, Rome, Italy

PARTICIPANTS: Aarhus University, H. Ullitz-Möller, Denmark
University of Helsinky, J.C. Roos, Finland
Université René Descartes, G. Cremer, France
DKFZ, S. Suhai, Germany
Daresbury Laboratory, A. Bleasby, United Kingdom
Inst. of Mol. Biol. and Biotech., C. Savakis, Greece
CSMME - CNR, C. Saccone, Italy
University of Nijmegen, J.H. Noordik, Netherlands
Biotechnology Centre Oslo, E. Paulssen, Norway
C.S.I.C., J.M. Carazo, Spain
Uppsala Biomedical Centre, G. Magnusson, Sweden
Biozentrum Basel, R. Dölz, Switzerland

OBJECTIVES SET FOR THE REPORTING PERIOD:

Taking into account that the aim of this project is to reinforce EMBnet through the consolidation of the existing node activities, stimulating collaboration and promoting new initiative in biocomputing, the objectives selected for the initial period were:

a) the drafting of a feasibility study on connection improvement; a task force for telecommunications was appointed;

b) setting up of a bulletin board - conferencing system

c) study on the technological tools for data distribution

d) planning of specialized courses and workshops

e) hiring of an EMBnet resource project assistant to help in consolidating the existing group activities, with the aim of stimulating further collaboration in biocomputing, networking and training.

A Funding Committee (FC) was created to coordinate and supervise all activity relevant to the above-mentioned objectives. This Committee is formed by Prof. Cecilia Saccone (CNR, Italy); Martin Bishop (HGMPRC, United Kingdom); Reinhard Doelz (Biozentrum, Switzerland); Peter Stoehr (EMBL, Germany); Mats Sundvall (BMC, Sweden) .

MAJOR PROBLEMS ENCOUNTERED:

EMBnet RESOURCE PROJECT ASSISTANT

This position was advertised in NATURE (vol. 352, 4 July, 1991) and 27 applications were received. The features of applicants were the following: average age 33 years, and previous experience in accademic institutions, with more or less long stages outside the original countries.
It can be noted that the majority had a biological background and little knowledge in computer sciences. A pool of possible candidates (namely Peter Norbert Saurugger from Austria, David W. Featherston from Canada, Mark A. Vandeyar from Britain and Jorn Wolters from Germany) was then selected and they were interviewed in Bari on the occasion of the first Funding Committee meeting on 15th October 1991. No one of them was estimated suitable for the job. The Funding Committee decided then to hire a more experienced person with managerial skills. A call for proposals was circulated in January, describing in detail the tasks of this person. During the second Committee meeting, held in Heidelberg last March 8th, the hiring of Prof. Albert M. Kroon was decided on the basis of his previous experience as full professor of biochemistry at the State University Medical School of Groningen and at the University of Amsterdam, as well as on his work as managing and research & development director at Haarlem Allergen Laboratories B.V., and at the HAL Group both in the Netherlands and abroad. He also has some experience as advisor for lifesciences policy in the College van Bestuur, the main board of administrators of the University of Amsterdam.
This caused a delay in the implementation of the job for the project assistant. However, the attempt was instrumental, in our opinion, to assess the profile of the bioinformatic scientist available on the market.

RESULTS:

TRAINING PROGRAM FOR EMBNET/BRIDGE

A scheme for the future training programm was decided. This programm included: one ad hoc seminar per annum, with a level of funding analogous to that for EMBO or FEBS workshops; practical courses; annual business meetings. For all courses and seminars, precedence has been given to those organised by more than one country.

IMPROVEMENT OF DATA DISTRIBUTION

a) A Pilot Project for the Development of improved Data Exchange Mechanisms has been started by Rainer Fuchs at the EMBL. The project was financed with ECU 6000 and will have a duration of six months. Since the data are received by the different nodes on various protocols (DECnet or TCP/IP) and they are further manipulated for local distribution according to the locally available database management and sequence analysis software, it is necessary to develop standards for interfacing the EMBnet data distribution mechanism to software which acts on the delivered data.
b) The acquisition of Multinet TCP/IP software was financed. This software allows the improvement of the local communication standard as it is of no obstacle for a mixed environment VMS-UNIX, which are the two operative systems most widely spread in the EMBnet community.

BULLETIN BOARD - CONFERINCING SYSTEM

A bulletin board was set up for the exchange of experience among the EMBnet users, who can discuss topics of common interest directly on-line using a single e_mail address. Manager of this project has been appointed Rob Harper from the Finnish node.

MILESTONES: A comprehensive study was carried out by Reinhard Doelz on the EMBnet status and its possible developments. It listed the various services available on-line from EMBnet, with a detailed analysis of the connectivity performance - e.g. success rates and reliability rates for data transfer.

The study also included suggestions for improvement which took into account most of the available communication protocols and their pros and contras on EMBnet network. A chapter was devoted to hardware, users, funding and staff. and another to education, user services and databases.

Therefore, since this study gives a very accurate idea of problems as well as of opportunities for development of EMBnet as a whole, it will be used as feasibility study for further developments.

COOPERATIVE ACTIVITIES:

This project is, in its very structure, quite different from other BRIDGE funded programs in that it is based completely on cooperation of different countries, EEC and EFTA, and Institutions. Each single result is the fruit of this cooperation. Yet, EMBnet as an organization is open to contribution from any other institution and organisation; indeed, a 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 INSERIM in France.
Against this scenario, the Italian node of EMBnet isdiscussing joint research with the MIPS, bearing on the development of object-oriented databases. Contacts with NCBI have also been established.

TITLE:
Integrated data and knowledge base of protein structure and sequence

CONTRACT NUMBER:
BIOT–CT90–0271 (SMA)

OFFICIAL STARTING DATE:
1 April 1991

COORDINATOR:
Shoshana J. Wodak, Université Libre de Bruxelles, Bruxelles, BE *(partner 1)*

PARTICIPANTS:
Chris Sander, European Molecular Biology Laboratory, Heidelberg, DE *(partner 2)*
Janet Thornton, University College London, London, GB *(partner 3)*
Peter Gray, University of Aberdeen, Aberdeen, GB *(partner 4)*
Chris Rawlings, Imperial Cancer Research Fund, London, GB *(partner 5)*
Michel Moreau, BIM, Everberg, BE *(partner 6)*

OBJECTIVES SET FOR THE REPORTING PERIOD 1 July 1991 – 31 March 1992 :
All partners : Design of a standard schema of object types and relations describing protein structure and sequence.
Partners 1 (Brussels) and *3 (UCL)*: incorporate sequence and structure homology information into the respective databases.
Partner 4 (Aberdeen): Prototype implementation of an Object–Oriented front end.

MAJOR PROBLEMS ENCOUNTERED:
Partner 1 (Brussels):
In order to store data on protein sequence and structure homology, it was necessary to develop efficient ways of representing this data in the database. In particular, solutions to the problem of representing multiple sequence alignments (based either on pure sequence or structure comparisons) had to be devised.

Partner 3 (UCL):
To attain the objective of incorporating sequence and structure homology information into the database, it was necessary to develop the following computer programs:
a) a sequence alignment tool for identifying proteins with sequence homology;
b) a structural alignment tool which can be used either for superposing whole structures to identify distantly related proteins or to find common structural motifs which are not necessarily the result of divergent evolution.

To identify structural motifs, a method for generating local structure alignments also needed to be adapted.

Partner 4 (Aberdeen):
The main problem encountered was the long delay in providing funds. The services of Dr. Kemp might have been lost, had not the SERC given us a one–year grant to continue some other work. Work on the BRIDGE contract started only in October 1991, after attending the GBF–CAPE Symposium and BRIDGE meeting in Braunschweig.

Another problem has been the delay in acquiring a license for the SYBASE software, at a specially reduced price. However, a great deal of help was provided from Jean Richelle at UCMB, and we hope to have a running system soon.
We are experiencing IPR (Intellectual Property Rights) problems already in using protein data provided by other partners for pre-competitive work with a small biotechnology company in order to try out our system. The commercialization of the 'value added' databases is fraught with potential IPR problems which could seriously hinder their take-up. We are trying to structure our database so that program and data modules can be purchased from various sources for various platforms, but it is not going to be easy.

Partner 5 (ICRF):
A number of programs have been developed using the logic programing language Prolog to check the consistency and completeness of the topological description of protein structures used in the TOPOL database. Most of the problems encountered could be traced back to inconsistencies and incompleteness in the original Brookhaven files.

RESULTS:
All partners (more specifically a working group including members of partners 1, 3, 4 & 5):
Very good progress was made in the design of a standard schema of object types and relations describing protein structure and sequence. A report describing the schema and objects types on which consensus has been reached is circulating among all partners for comments. This mainly concerns the hierarchy of molecular structure elements such as macromolecule, subunits, quaternary structure, chain, ligand, solvent, various descriptions of secondary and supersecondary structure elements, residues, atom groups, atoms, and finally spatial proximity and specific interaction types made between atoms. A standard definition of such object types and the relations between them is not only necessary for the design of compatible databases, but also and foremost a prerequisite for any standard exchange protocol of macromolecular data, which is badly needed in the field.

Partner 1 (Brussels):
Extensions and enhancements of ALI, the user interface to SESAM: ALI contains metaknowledge about SESAM. This includes a list of SESAM tables, data fields, and their aliases, as well as the type and relational properties of the fields. Commands have been developed in ALI to introduce this metaknowledge interactively, thereby allowing the user to bring ALI up-to-date on changes made in SESAM. Further improvements have been made to the automatic query building algorithm in ALI. When querying SESAM through ALI, the user needs to specify only the fields he wishes to view. Those used for 'join' purposes in different tables are provided automatically by the algorithm. The latter furthermore optimizes the query operation by minimizing the number of tables to be accessed. The procedure whereby this is achieved is very general and works even for very complex queries.
Other enhancements include:
1) Reorganization of the MENU of ALI into a tree structure that reflects the hierarchic concepts of protein structure. This greatly facilitates the specification of data fields and conditions in accessing SESAM.

2) A set of PLOT and GRAPH commands to produce 2D graphic output (screen or printer) of any pair of numerical data fields extracted from SESAM.

Incorporation of data on protein sequence and structure homology: Having devised ways of representing multiple sequence alignments in SESAM, information on aligned sequences of 28 protein families including the Globins, Cytochromes–C's Lysozymes, microbial RNases, have been stored in SESAM.

Representation and incorporation of data on sequence patterns corresponding to common structural or functional motifs: An exhaustive repertoire of sequence patterns uniquely associated with secondary structures (α,β, Coil, Turn) at specific positions (Rooman & Wodak, 1988, 1990) and their respective territories in all known protein structures has been stored in SESAM.

Partner 2 (EMBL):

Two algorithms to extract from the database representative sets of protein chains with maximum coverage and minimum redundancy have been developed. The first algorithm focuses on optimizing a particular property of the selected proteins and works by successive selection of proteins from an ordered list and exclusion of all neighbours of each selected protein. The other algorithm aims at maximizing the size of the selected set and works by successive thinning out of clusters of similar proteins. Both algorithms are generally applicable to other databases in which criteria of similarity can be defined and related to problems in graph theory. The largest non–redundant set extracted from the current release of the Protein Data Bank has 155 protein chains. The selection may be useful in statistical approaches to protein folding as well as in the analysis and documentation of the known spectrum of three–dimensional protein structures (Uwe Hobohm *et al.*, in press).

Partner 3 (UCL):

A sequence alignment tool has been developed which performs all pairwise comparisons and generates protein families by single linkage cluster analysis. Sets of nonhomologous proteins can easily be derived by selecting a single representative from each family. A multiple sequence/structure alignment is also in progress, which will allow us to establish sequence and structure constraints for different families and folds. Such information could be stored in the databank in the form of templates, generated at different similarity levels.

To identify motifs, a method for generating local structural alignments has been developed. This can be applied to each family separately, extracting folding motifs for that family or across the databank giving universal motifs. It can search for all occurrences of known motifs or find new motifs. Plans for future work include an analysis of all families within the databank using these techniques. It concerns identification of new motifs and extensive characterisation (both sequence and structure) of new and established motifs. Research will also include assessing ways of storing such data in the databank.

We also actively participated in the standard schema design efforts (see *all partners*).

Partner 4 (Aberdeen):
We have coordinated the standard schema design efforts using the latest database modelling techniques (see also *all partners*) and produced a report of the working group sessions in Aberdeen (23–25 January 1992). The design standards described in this report have already been through the first stage of refinement through comments. The schema looks promising. It includes most of the types of macromolecular data stored by the partners, showing how they relate to each other. It is at an abstract conceptual level which allows different storage representations to be implemented by different partners, but without needing significant changes to application programs based on it. The schema is to be presented for a final stage of refinement at the EMBL meeting on 22–23 May 1992.
Our other work has been to prototype an extension to our object–oriented database which allows relational database storage routines to be used to store objects. The objects are stored in a separate module (partition) of our database, but can contain pointers to objects in existing modules. This will make it straightforward to use our Prolog search code and object–oriented software in conjunction with data taken from the SESAM database developed by *partner 1* (Brussels), as planned in our original proposal. The prototype test is encouraging.

Partner 5 (ICRF):
Progress has been achieved in the following areas of project tasks: Definition of the standard schema, addition/validation of data and knowledge and the development of user interfaces.
Definition of the standard schema (see also *all partners*): In common with other members of the project we 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.
Addition & Validation of data and knowledge:

a) A Rule–based Representation of Protein Folding Rules: Based on a Prolog representation of general protein folding rules in α/β proteins an interface to the TOPOL database and programs was implemented. By searching the TOPOL database using the folding rules as queries, proteins whose conformation could be shown to comply with the folding rules could be identified [1]. The results of the study helped to validate both the TOPOL representation of protein topology and the protein folding rules.
b) Validation of Protein Topology Data: A number of programs have been developed using the logic program Prolog to check the consistency and completeness of the topological description of protein structure used in the TOPOL database

Software and User Interfaces

a) New PAPAIN User Interface Modules: New graphical interfaces have been developed to the programs for predicting protein topology described in [1] and the protein topology diagramming tool extended to enable α/β topologies to be displayed

b) PROSITE and PROSEARCH Interface: An interface to the PROSITE database and the PROSEARCH programs for locating the position of sequence motifs in protein sequence data has been developed and integrated into the PAPAIN interface

c) X Windows User Interfaces: The PAPAIN suite of user interfaces providing graphical manipulation and visualisation of (i) protein topology diagrams (ii) protein secondary structure predictions (iii) protein sequence profile analysis and (iv) the TOPOL database as well as the developments mentioned above in [3a] and [3b] have been converted to run under X windows using the MOTIF window manager.

[1] Clark, DA, Shirazi, J., Rawlings, CJ (1991) *Protein topology prediction through constraint-based search and the evaluation of topological folding rules*, Protein Engineering, 4, 751-750

Partner 6 (BIM):

Counselling and guidance were provided (mainly to *partner 1*) for the following developments:

1) design and implementation of the database schema for representing information on multiple sequence alignments. In the adopted solution, a table relates pointers to individual residues of each protein to a unique pointer to the position in the alignment. This is general enough to allow representation of alignments for families with any number of members.
2) Procedures in ALI for accessing and viewing sequence mapped information in SESAM were extended to include motifs of secondary structure elements and to retrieve information on aligned sequences.
3) enhancements of the ALI querying performance, in particular in devising procedures for optimizing query design based on user specifications.

Help was provided to *partners 1* and *5* in negotiating acquisition of a SYBASE licence at a reduced price.

HIGHLIGHTS/MILESTONES:

Significant progress has been made towards reaching a consensus in the design of a standard schema of object types and relations describing protein structure and sequence.

WIDER CONSIDERATIONS:

Work done within this project on the design of standard schema of object types and relations describing protein structure and sequence is a prerequisite for the development of standard exchange protocol of macromolecular data, which is badly needed in the field. In particular, it should provide the templates and guidelines for the description of protein and nucleic acid data in ASN.1 or CIF formats. This in turn will allow for efficient communication between different databases independently of their internal design. Our efforts are thus of direct relevance to those of the IUCr in Chester UK who define standards for protein crystal structure data exchange format, and to those of the Protein Databank team at Brookhaven who are in the process of redesigning their database and exchange formats. Contacts have been established with T. Koetzle and E. Abola (Brookhaven Protein Databank) and G. Dodson and P. Fitzgerald (IUCr) in view of a closer collaboration.

COOPERATIVE ACTIVITIES:

Several joint meetings and workshops were organized within this reporting period:

1) *All partners* met for one day following the CAPE symposium on *Prediction and experiment: three-dimensional structures of homologous proteins* in Braunschweig (18–19 September 1991). At this meeting organizational matters and collaborations were discussed.
2) *Partners 1, 2, 4* and *6* attended the meeting in Maastricht (20–22 November 1991) on *Bioinformatics in the 90. Partners 1 and 4* gave presentations related to their database work. Following this meeting, the coordinator of the present project (*partner 1*) presented it at a half day BRIDGE–database workshop.
3) *Partner 1 (Brussels)* was host to a 2–day meeting (12–13 December 1991) devoted to laying the grounds to the standard schema design work and to discussing collaboration strategy with the Brookhaven Databank team. A list of problems encountered and improvements to be made to the Brookhaven databank format was compiled. The necessity for adequate protein structure data validation software was discussed and designated as the most concrete and timely area of collaboration with Brookhaven.
4) *Partner 3 (UCL)* organized a workshop/meeting, supported by EC and CCP11, in London on 25–26 March 1992. In this meeting, 100 participants gathered to discuss protein structure coordinate data, the new proposed Brookhaven format and data validation. Invited speakers included the head of the US Brookhaven Protein Data Bank, (T. Koetzle), the scientist in charge of changing formats at Brookhaven (E. Abola) and various European speakers and representatives from ESF (European Science Foundation) and IUCr (International Union of Crystallography). This meeting has taken a lead in coordinating the European effort to facilitate data input to Brookhaven and the development of a validation suite. Within the group at UCL, procedures to assess the stereochemical quality of protein structures [Morris *et al.* (1992) Proteins, 12, 345–364] have been developed. These will contribute to a combined European effort to validated the coordinate data currently in the Brookhaven Databank and future entries
5) *Partner 4 (Aberdeen)* acted as hosts to *partners 1, 3, 4 & 5* in Aberdeen (23–25 January 1992) for the standard schema design workshop.
6) Dr. Kemp (*partner 4*) and Dr. Orengo (*partner 3*) made short visits each to *partner 1* (Brussels) on different occasions.
7) The SESAM database of *partner 1* has been installed at EMBL and at Aberdeen.

LIST OF JOINT PUBLICATIONS/PATENTS WITH TRANS–NATIONAL AUTHORSHIP:
Nothing to report.

OTHER PUBLICATIONS/PATENTS:

Zhuoan Jiao and P.M.D Gray, "Optimisation Of Methods In A Navigational Query Language", Proc. 2nd International Conference on Deductive and Object–Oriented Database Systems, December 1991, Germany. This paper shows how queries calling methods in an object–oriented database can be optimised, with several examples taken from the protein database

Title:
Immunoclone and hybridoma database network for Europe.

Contract number:
BIOT-CT91-0257

Official starting date:
01 April 1991

Coordinator:
Louis Réchaussat, CERDIC, Nice Sophia-Antipolis (France)

Participants:
Associate contractors:
Alan Doyle, ECACC, Porton Down (UK)
Tiziana Ruzzon, IST, Genoa (Italy)
Patrick de Baetselier, VUB/IMB, Brussels (Belgium)
Hans Kurzwelly, DIMDI, Köln (FRG)
Subcontractor:
Hans Lucius, Max-Delbrück Centre, Berlin (FRG)

Objectives for the reporting period:
1. Development of a scientific and technical database on immunoclone descriptions ("Immunoclone Database" - ICDB), coming from:
   - Scientific literature curently published,
   - Patent applications to the European patent Office,
   - Industrial and commercial catalogues,
   - Participants'collections of hybridomas.
   Altogether, the target is an input capacity of 2,000 per month after 18 months.
2. Implementation of a stable consortium agreement, as a model for biological database networks 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;
   Electronic communication deficiency because of the lack of infrastructures and know-how in centres' environment;
   Conversion of the existing database file into ORACLE format longer than expected because of a rate of data modifications higher than expected;
   Retirement of the Central laboratory of the Netherlands Red Cross (Amsterdam).
2. Concerning centres:
   CERDIC: Nothing to report;
   ECACC: One coder has left;
   IST: Delays for the implementation of the equipment;
   VUB/IMB:Nothing to report;
   MDC: Nothing to report.

3. A review of communication problems with visit(s) to concerned site(s) is planned in April 1992.
An addendum to the contract has been signed, with DGXII contract office, stating on the retirement of the Central laboratory of NL Red Cross and the reimbursement of the EEC funds.

Results:

1. Database management implementation: the data input and validation software has been achieved in May 1991 and a final release was distributed to each centre in September.
2. Hardware and software installation: ORACLE software is installed in each centre (except ECACC). Due to local situations, the implementations are not as homogenous as it was expected, but it does not affect the objectives of the project:
   - CERDIC has access to 4 different versions of ORACLE (VMS Version on DEC/Vax, Unix versions on IBM/Risk 6000 and Sun/Sparc 690 and DOS version on microcomputer), thanks to the CICA (Centre International de Communication Avancée), where it is located;
   - VUB/IMB and MDC have the implementation initially planned;
   - IST has access to a UNIX and a DOS versions.
   - During the period ECACC was currently using ICDB on IBM/Stairs accessible from CERDIC. The access to ORACLE from CERDIC/CICA server will be possible.
3. Communication procedures: Electronic communications were made between centres through FTP, mail and modems. E-mail is used by all centres (ECACC is using the commercial E-mail BT-Tymnet), FTP connections are used by CERDIC and IST, and under test in MDC and VUB. The EARN Network has been also prospected. Most of the encountered difficulties will be solved in the early weeks of the second period of the project.
4. Data collection: Patent applications and commercial catalogues (from 175 major companies worldwide) were made avalaible and distributed for coding by CERDIC. Accessibility to scientific literature depends on local library facilities. They are better for CERDIC, ECACC and MDC than for IST and VUB/IMB.
5. Data processing implementation: Two training session took place in CERDIC, one in May 1991, for ECACC and IST who could support the travel expenses before EEC first payment, and a second in September. All the centres were producing validated data with the software loaded on their own workstation, in October 1991.
6. Data processing optimisation: Controls of quality are currently done on each centre production which appears to be good. There is no need, up to now, for any extension of software controls. During the second

period of the project, the centres will have to increase their production and reduce the elapse time between the reception of the tasks and the shipment of coded data. Facilities for the use of the database under ORACLE will be developed in connection with the loading of the database on local workstations.

7. User-friendly interface: Planned for the second period with DIMDI.

Highlights/Milestones:

1. Consortium agreement: In addition to the Bridge contract, associate contracts and subcontract, a consortium agreement was finalized in November 1991 and countersigned by each participant. This document states on rights and duties for input activities, sharing of tasks, copyright, sharing of royalties, local use of the database and distribution policy.
2. Production: The total production during the first 11-month period of the project was of 8673 new records. In addition, 2,900 other records were processed by CERDIC on scientific literature for the years 1984 to 1988.
3. Collection of hybridomas: The ECACC hybridoma collection was entered in ICDB.
4. Diffusion: During the period the database was accessible on-line on DIMDI in FRG, on Data-Star in Switzerland and on the computer host of Conseil Général of Alpes Maritimes (Minitel). The database was updated monthly. The updates are also published in a printed form and on floppy disks for microcomputers ("Immunoclones" journal). CERDIC and ECACC act as reference centres for ICDB. IST, MDC and VUB/IMB are willing to do so. The general feeling is that ICDB needs a wider publicity among the scientific community.
5. Expansion of the network: The Institute of Molecular Genetics (Prague, Czechoslovakia) joined the network as corresponding member.

Wider considerations:

1. Electronic communications in research community: As far as networking scientific activity is concerned, there is a need of reliable and cheap electronic communication links. Many research institution equipment and know-how are below the minimum required for such activities. This situation may induce serious restrictions in the transfer of scientific and technological knowledge between the scientific and the industrial communities.
2. Availability of biotechnology products: The data processing is a powerful tool for the identification of new scientific and industrial products for biotechnology. The European countries can take advantage to broadcast this information, worldwide. But it is very important to link this facility to an

improvment of the availability and of the quality standards of the described products. Some biological products, like monoclonal antibodies and cell line cultures have a strategic importance in the worldwide competition of pharmacology research and development. The combination of information networks and bioproduct ressources may have a very positive effect on the development of biotechnologies in Europe.

Cooperative activities:

1. Thanks to the DGXII/F organization, a plenary session of all project members met in Maastricht, after the international conference "BioInformatics in the '90s" and the Bridge meeting (November 1991).
2. The two training parties held in Sophia-Antipolis, at CERDIC gave the opportunity to meet the technical staff of the project (One week for 3 people in May and 10 days for 6 people in September 1991).
3. Joint participation of CERDIC and ECACC to the international BioExpo (Paris, April 1991), CERDIC, ECACC and IST to BioTech '92 (Genoa, April 1992).
4. In Belgium, a local network was created between laboratories that are currently involved in the generation of immunoclones. This network is supervised by the VUB/IMB and will serve as a mean to obtain data from the national collections and to promote ICDB within Belgian institutions and biotechnology companies.
5. Representatives of ECACC, IST and DIMDI attended to CERDIC Board meeting, Sophia Antipolis, France, in July and December 1991.

List of joint publications: Nothing to report.

Other publications:

A Doyle and R S James, Databases for Animal Cell Lines, Bioinformatics, 1992;1(1):8-11.

TITLE: ELECTRONIC LINKING SERVICES FOR BIOTECHNOLOGISTS AND MICROBIOLOGISTS IN EUROPE

CONTRACT NUMBER: GRANT

OFFICIAL STARTING DATE: January 1991

COORDINATOR: B E KIRSOP, MSDN, CAMBRIDGE, GB

PARTICIPANTS: MSDN is sole contractor.

OBJECTIVES SET FOR THE REPORTING PERIOD: New users, collaborating laboratories, databases and networks to be linked to the MSDN. Promotional material, publicity and training to be provided.

MAJOR PROBLEMS ENCOUNTERED: Nothing to report.

RESULTS: [Note: The MSDN has received a single grant under the BRIDGE programme and has no BRIDGE funded partners. Comments regards 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.]

1991 was a year of major developments for MSDN that have resulted in a strong base on which improved and expanded services can be built. The three major developments undertaken to enhance the services overall were 1) improved database hosting system, 2) improved software and 3) establishment of in-house invoicing and billing administration.

1. DATABASE STORAGE/HOSTING: Until now the relatively high charges for storing MSDN databases on the BT computer have inhibited collaboration by database producers. The MSDN has investigated a number of alternative options and is now using another database hosting system offering good, yet inexpensive, storage.

The storage costs on the new system are $100/megabyte data/year. This is a very attractive arrangement, since large databases can now be hosted for a very modest cost and small databases can be combined into 100 megabyte packages for cost purposes, so that storage is almost free. Moreover, databases developed by scientists without access to any resources (for example, East European organisations) can be stored at MSDN expense. It is anticipated that the new hosting arrangement will lead to considerable interest from scientific database developers to link their information to the network.

The new storage system allows greater flexibility, since charging arrangements (for example, addition of royalty on usage, charges for downloaded records) can be readily introduced. Changes to the charging arrangements with BT were always difficult to make and slow to implement and the possibilites for making different

charging procedures were limited.

The change to the new system is now underway and will become effective at the beginning of March 1992. Already, the databases previously maintained on the BT computer have been reformatted and transferred to the new computer, new documentation has been prepared for users, new demonstration disks have been developed.

The work involved in this development has been substantial and, because of the importance attached to the change, has taken priority over other activities. The new system allows great potential for future development, since cost considerations will no longer be a factor for determining whether or not to use the MSDN services. Already, a number of new, up-dated or enlarged databases have agreed to link their databases to the network.

NEW/UP-DATED/EXPANDED DATABASES FOR ADDITION TO MSDN UNDER NEW ARRANGEMENTS:

* Biotech Knowledge Sources (BKS), maintained by BioCommerce DataLtd, renewed monthly, listing publications, videos, software, databases and conferences in biotechnology.
* American Type Culture Collection databases (Bacteria and Media, Protists, Recombinant Materials, Cell Lines and Hybridomas), up-dated and expanded
* European Collection of Animal Cell Lines catalogue, greatly expanded to include human lines, DNA probes, hybridomas and human b-lymphoblastoid lines
* BIA databases.

ADDITIONAL DATABASES CURRENTLY UNDER DISCUSSION:
* Animal virus database
* IMI catalogue
* Czech Collection of Algae and Cyanobacteria
* Aubit Who-What-Where databases.

IMPROVED SOFTWARE: Since it now seems likely that a larger number of databases will elect to be hosted on the MSDN computer rather than accessed via electronic gateways, it is desirable that improved software should be introduced. The software used for the BT-hosted databases was acceptable, but changes to the software were difficult if not impossible to implement. It was not possible to customise the software for MSDN purposes.

New software called INFO has been developed by collaborators and made available to MSDN. The MSDN has worked with the software developers to customise INFO to meet the specialised needs of MSDN. INFO is extremely simple, yet flexible and will allow greatly facilitated database searching. As more databases become linked to the network, the value of this will be much appreciated by the user community. Following a period of testing and development, INFO is now ready to be made available to the MSDN simultaneously with the change-over to the new storage system. Already users have been mailed with a description of INFO and a

quick reference guide. Additionally, the new system has been described in online NEWS and introduced in the latest Newsletter. With the introduction of INFO, the MSDN is now in a position to provide improved searching of the Directory maintained by the MSDN (for locating strains of organisms with specific properties), eliminating the original two-stage procedure.

BILLING: Difficulties with invoices prepared by BT in the past have caused much effort on MSDN's part to track and correct. Again, the inflexibility of large organisations has been frustrating from time to time. A decision has been made to move invoicing in-house so that billing can be customised and errors can be rapidly tracked and corrected.

Software has been written that allows the raw BT usage data to be transferred to an invoicing package. For the last two months of 1992, invoices have been prepared and distributed from MSDN's invoicing office. To facilitate customer support in North America, a part-time support person has been hired to answer enquiries and help explain any billing or technical changes.

Again, this change has been very demanding in manpower and it is gratifying that the new system has been introduced successfully with a minimum of problems. Indeed, irregularities that existed in the BT invoicing procedures have been identified and corrected, to the advantage of MSDN. The new system enables MSDN to attach special messages, discounts or announcements with the invoices, which we anticipate will further improve the service.

These major service enhancements have been demanding in time and effort. We believe they reflect the growing maturity of the network and its ability to provide an increasingly valuable service to the scientific community.

TRAINING: In addition to the above activities, the MSDN has been involved in providing training in use of the services. The following people have spent between 1-4 weeks at the Cambridge office, receiving individual tuition:

Penny Noble - ECACC UK (1 week, January 1991)
Katja Frohlich - ICECC Germany (1 week, February 1991)
Carmella Belloch Trinidad - CECT Spain (4 weeks, April 1991)
Monika Gotowczyc - IOAB Poland (3 days, May 1991)
Teresa Barreto - IBET Portugal (4 days, December 1991)

In addition, a training course was held in Glasgow (April, self funded), in India (November, UNEP funded), and in Canada (September, Environment Canada funded). Further funds for training courses in the Cape Verde/Senegal region and in Beijing, China have been agreed by UNEP. A workshop in Moscow to finalise MSDN-linked networking arrangements has been postponed to April 1992, because of difficulties in the region. In the meantime electronic communication has been established with the Institute in Russia and Letters of Agreement have been exchanged.

PROMOTION: Up-dated User Manuals and Demonstration disks are prepared routinely and can be obtained by application to the Secretariat. A number of lectures and demonstrations have been given by MSDN in different regions of the world. They include:

CODATA meeting, Nancy - participation
ECCO meeting, Valencia - report
BIA Council meeting, London - demonstration
UNCED preparatory meetings, London - participation
IUBS Biodiversity conference, Amsterdam - participation
IUMS Biodiversity Workshop, Amsterdam - contribution
DISNET contractors meeting (DGX111), Amsterdam
Montreal UNESCO Conference - lecture
BIOFOCUS meeting, Montreal - participation and lecture
IBEX Exhibition, Washington/San Francisco USA - lecture/ demonstration
COBIOTECH E/W, Bratislava - lecture
BIOINFORMATICS in the 90's, Maastricht - lecture, demonstrn.

In addition, visitors have been received at Cambridge and visits made to other organisations. Discussions on future collaboration have taken place with the Leatherhead Food RA in the UK and with the World Conservation Monitoring Centre. Agreement has been made with Graham Cameron of EMBL to discuss ways of linking MSDN and EMBL services.

Throughout all these activities, new contacts have been made and new opportunities for collaboration have been discussed.

During 1991/2, three NEWSLETTERS have been published (attached).

IRRO WORKSHOP: At the end of 1990 UNEP invited MSDN to set up an information resource for the release of organisms into the environment. Funds for a workshop to discuss the needs and specifications for such a resource were received from UNEP, CEC, USDA, EPA and Environment Canada. The workshop was held on two sites (because of travel restrictions caused by the Gulf War). The separate meetings were linked electronically. There was unanimous agreement that a decentralised system be established to distribute information on GMO's and non-GMO's of micro- and macro-organisms. Proceedings of the Workshop have been published by UNEP and widely distributed (copy attached). Over 1000 copies have been supplied to CUBE office for distribution.

A follow-up meeting was held at REGEM2 conference in the UK. At this meeting an IRRO Steering Committee was set up and agreement taken to set up online Bulletin Board, NEWS and email using the MSDN system, but as a separate network with its own identity. A telephone for enquiries about IRRO has been provided by the MSDN for the time being. Funds are being sought for the employment of an information officer to develop and maintain databases. A number of databases have been identified to start the network's information capability, and the Steering Committee has been enlarged to extend the expertise and geographical spread.

BIODIVERSITY: The intense political interest in biodiversity and its conservation have led to a number of meetings and conferences on the topic. The World Federation for Culture Collections (which sponsors MSDN) is planning a Workshop to be held in Brasil in 1992 to discuss mechanisms to link the different biodiversity networks already in operation or planned. The plant, animal, insect and microorganism information networks must be linked to provide a biodiversity resource if the separate elements are not to remain isolated and under-used. The IUBS and IUMS have agreed to co-sponsor the Workshop and the Tropical Database and the MSDN are organising the event. A computer conference will be established on the MSDN system to allow persons unable to attend in person to contribute to the discussions.

OTHER CONTRACTS: The MSDN continues to collaborate with the EPA and the NIDR (NIH) in the development of specific databases and services. Additionally, the DGXIII IMPACT project continues and the MSDN knowledge base will be incorporated into the DISNET system and a gateway established between MSDN and DISNET.

USAGE: Overall, 69 new mailboxes have been requested and usage is steadily increasing. It is anticipated that the developments described above will lead to a year of increased activity as more databases and services are incorporated in the network.

Usage of the system in the US has not been increasing as well as in the rest of the world and steps have been taken to encourage awareness by demonstrating at IBEX91 and by appointing a US support person. Towards the end of the year a number of new mailboxes from the US have been requested and the usage trend appears to have been reversed.

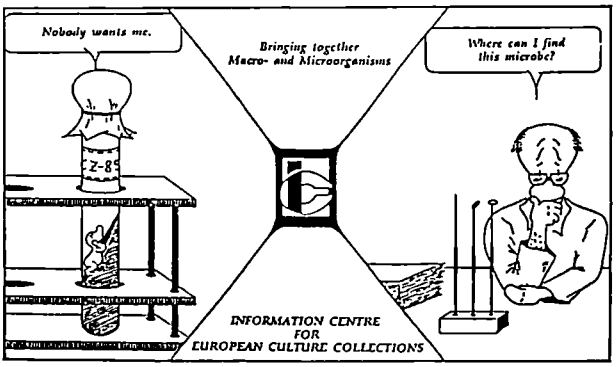
WIDER CONSIDERATIONS: The measures taken in 1991 to develop the MSDN system will lead to a sharp increase in the amount of data linked to the network. This, combined with the incorporation of flexible and easy-to-use software will be attractive to both database providers and users alike. The outcome will be increased information exchange and increased usage of MSDN. Additionally, the improved hosting/searching facility will encourage use of the MSDN database storage service and limit the need for increased gateways to remote hosts. This in turn will simplify both the system for the user and training.

COOPERATIVE ACTIVITIES: No partners, so nothing to report. But please note large numbers of meetings, visits and training activities with European scientists listed above.

PUBLICATIONS:

* 'Needs and Specifications for an Information Resource for the Release of Organisms into the environment', Eds. B E Kirsop, M I Krichevsky, UNEP, Nairobi, 1991.
* Newsletters: February 1991, June 1991, January 1992.

TITLE:	Information Centre for European Culture Collections (ICECC)
CONTRACT NUMBER:	BIOT-CT90-0162
OFFICIAL STARTING DATE:	01.01.1991
COORDINATOR:	Dr. D. Claus, DSM, Braunschweig, DE
PARTICIPANTS:	DSM, Braunschweig, DE
OBJECTIVES SET FOR THE REPORTING PERIOD:	Information on European culture collections; advice on cultures and databases



MAJOR PROBLEMS ENCOUNTERED:	Nothing to report

RESULTS

Advice and information on cultures and culture collections

The Information Centre for European Culture Collections (ICECC) is a follow up project of the CEC-Culture Collection Information Centre supported by the Commission under its Biotechnology Action Programme during 1989 and 1990. It reflects the demand from academic and industrial institutions for a central European contact point for information on different types of biological material, on culture collections and their services and holdings.

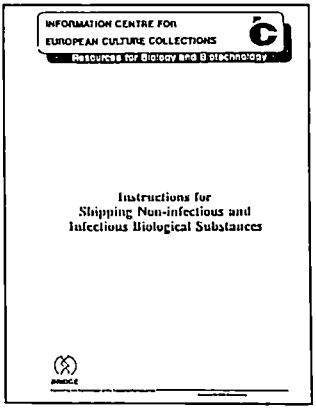
Within the reporting period the ICECC has extended its stock of catalogues so that copies from all European culture collections are now available. Catalogues from the most important collections outside Europe have been also obtained. Thus, the Information Centre is in a position to give detailed information on the availability of specific strains and cell lines from collections in Europe or world-wide and on strains with known production or degradation capabilities.

On-line access to the World Data Centre for Microorganisms (WDC) in Japan and to different national culture collections in Europe have been established in 1991. The database of the UK culture collections, the Microbial Culture Information System (MiCIS),is still held at the ICECC. Searching of information during 1991 has been considerably improved.

The ICECC, together with the DSM-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, has compiled a list of names of bacteria which are validly published as defined in the International Code of Nomenclature of Bacteria. The list is based on the Approved Lists of Bacterial Names (1980) and subsequent additions and alterations published in the International Journal of Systematic Bacteriology in the form of original articles or in the Validations Lists. The list (at present 38 pages) will be updated four times a year and is available for a nominal fee. It is also available on floppy disk and on-line.

A database with the current assignment of all bacterial species to one of the generally accepted risk groups is also available. It is based on a list published by the German Occupational Safety and Benefit Authority of the Chemical Industry.

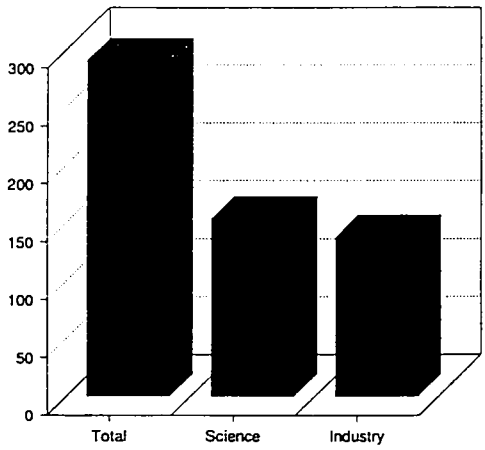
During 1990 and 1991 several requests on the safe shipment of cultures have arrived at the ICECC. Therefore, a booklet on "Instructions for Shipping Non-infectious and Infectious Biological Substances" was prepared and published by the ICECC in 1991. On 31 pages the booklet gives details on the international regulations (postal services and air freight). In addition, necessary stickers for sending such material can be obtained from the ICECC. The brochure will now be supplemented by appropriate national regulations. For information customers a list of prices for cultures and other services offered European culture collections has been published in May 1991.



For discussions on prices within the European Culture Collections' Organization (ECCO) a special list was prepared for culture collections only. In this list all prices of the collections have been converted to Deutsche Mark in order to make prices comparable.

From the many requests arriving at the ICECC it can be concluded that the Information Centre has now acquired a certain reputation. The following graph summarizes part of the daily routine activities of the ICECC.

Enquiries 1991 from Science and Industry
(via letter, fax, e-mail, telephone)



Public relation activities

The ICECC has continued its activities as a promotion centre of the European culture collections. It has participated in the BIOEXPO Paris (9-12 March, 1991) on a common stand with the French national node of the Microbial Information Network Europe (MINE France) and the Microbial Stain Data Network (MSDN, Cambridge) and has presented the services provided by the European culture collections. The numerous catalogues of the European collections have found considerably interest as well as the services offered by the different collections for the identification of bacteria and fungi.

For the BIOTECHNICA Hannover (22-24 October, 1991) the Information Centre was invited by the Commission of the European Communities to share the CEC stand. The whole spectrum of services and activities of the European culture collections, including the MINE database, was again presented. Emphasis was laid mainly on the presentation of European culture collections as depositories for patent strains and on culture collection services for the identification of microorganisms.

The Centraalbureau voor Schimmelcultures (CBS), Baarn, and the ICECC joint together the BIOINFORMATICS IN THE 90's, which was held on 20-22 November 1991 in Maastricht. CBS took the opportunity to demonstrate the Microbial Information Network Europe (MINE), whereas the Information Centre demonstrated the services offered by the main European culture collections.

During the fairs and meetings promotion material supplied by the different culture collections or prepared by the ICECC itself was distributed. Folders for more than 10 collections were prepared by the ICECC and leaflets on "Biotechnology Resources in Europe" and "Patent Depositories in Europe" have been updated. This material was also send to prospective customers for general information.

The third number of the NEWS of the Information Centre for European Culture Collections was published in March 1991.

Contact with European research and biotechnology

The Information Centre has started a study on the deposit of microorganisms in culture collections. Although some scientific journals ask to make available microorganisms described in scientific papers, the total number of microorganisms described and deposited in culture collections is rather low. The study will be published during 1992.

During a visit at the Microbial Strain Data Network the ICECC discussed further cooperation within Europe.

On request of a European company working in the field of biotechnology the ICECC has performed an extensive search on the ecology and physiology of different bacterial species. The incoming money was used for public relations activities of the Information Centre.

Liaison with international bodies

During the reporting period the ICECC has contacted the International Air Transport Association (IATA) in Montreal, Canada, and the Postal Union in Geneva, Switzerland for instructions on the shipping of biological material. The information given was very useful in the preparation of the shipping brochure.

MINE database

The Microbial Information Network Europe (MINE) have been demonstrated during fairs and to visitors of the information centre. The head of ICECC is actively engaged in the project as a member of the MINE Responsible Committee for Bacteria.

Training Centre

Within the scope of the International Training Program 1991 of the GBF Braunschweig the participants were introduced to the work of the ICECC and the MINE database.

HIGHLIGHTS/MILESTONES:

The ICECC Newsletter is distributed world-wide. The Shipping list has found great interest.

WIDER CONSIDERATIONS:

Are you interested in microbes, their use, shipping or risks? Do you need information on European culture collections, their holdings and services? Would you like to know more on MINE, the Microbial Information Network Europe? Contact the ICECC-Information Centre for European Culture Collections at Braunschweig.

COOPERATIVE ACTIVITIES:

The cooperation between ICECC and MINE as well as MSDN has been strengthen. The ICECC has sent out a lot of information on MINE and will continue to do so. Naturally, the ICECC is very active in the cooperation with the European culture collections.

The Information Centre was engaded in the preparation of the Xth Annual Meeting of the European Culture Collections' Organization held in Valencia, Spain, from June 5 to 8, 1991. A proposal that Ms Katja Fröhlich, secretary of the ICECC, should take over the task of the Secretary of ECCO was unanimously agreed.

During the Valencia meeting the Advisory Board of the ICECC held its annual meeting. Dr. Dieter Claus reported on the activities of the ICECC. In the following discussion proposals on further activities of the ICECC have been given. The proposals will improve ICECC's work.

LIST OF JOINT PUBLICATIONS/ PATENTS WITH TRANSNATIONAL AUTHORSHIP:

Nothing to report

OTHER PUBLICATIONS/PATENTS:

Information Centre for European Culture Collections NEWS, No. 3, March 1991

Instructions for Shipping Non-Infectious and Infectious Biological Substances. ICECC Publcation No. 1, May and October 1991

Price List of the ECCO culture collections. ICECC Publication No. 2, April 1991

List of Bacterial Names. ICECC Publication No. 3, December 1991

TITLE: Microbial Information Network Europe (MINE)

CONTRACT NUMBER: BIOT-CT 91-0280 (SMA)

OFFICIAL STARTING DATE: 1 June 1991

COORDINATOR: Dr. D. van der Mei, CBS, Baarn, NL

PARTICIPANTS:

B	-BCCM	Brussels	National Node	**Mr. J. de Brabandere, pm**
	-LMG	Ghent	DIN	Bacteria
	-MUCL	Louvain-la-Neuve		Fungi/Yeasts
	-IHEM	Brussels		Fungi/Yeasts
FR	-BRG	Paris	National Node	**Dr. M. Chauvet, pm**
	-LCP	Paris		Fungi
	-CFBP	Angers		Bacteria
	-CNRZ	Jouy-en-Josas		Bacteria
	-ADRIA	Villiers-Bocage		Bacteria
I	-DBVPG	Perugia	National Node	**Prof. A. Martini, pm** Yeasts
E	-CECT	Burjasot-Valencia	National Node	**Prof. F. Uruburu, pm** Bacteria, Fungi, Yeasts
GR	-ATHU-M	Athens	National Node	**Prof. G. Kalantzopoulos, pm** Fungi
	-ACA-DC	Athens		Bacteria
	-BPIC	Kiphissia, Athens		Bacteria, Fungi
NL	-RNAAS	Amsterdam	Contractor	
	-CBS	Baarn, Delft	DIN, National Node	**Dr. D. van der Mei, pm** Fungi, Yeasts
	-LMD	Delft		Bacteria
	-Phabagen	Utrecht		Plasmids, Bacterial Mutants
	-CDI	Lelystad		Animal Pathogens, Mycoplasmas
	-KIT	Amsterdam		Leptospira
	-PD	Wageningen		Bacteria, Plant Pathogens
	-LMW	Wageningen		Bacteria
	-RIVM	Bilthoven		Bacteria, Bacterial Serotypes
GB	-IMI	Kew	National Node	**Prof. D.L. Hawksworth, pm** Fungi
	-ECAAC	Porton Down		Animal Cell Lines
	-CCAP	Ambleside/Porton Down		Algae, Protozoa
	-NCTC	Colindale, London		Bacteria
	-NCIMB	Aberdeen		Bacteria
	-NCFB	Reading		Bacteria
	-CCPF	Colindale, London		Fungi
	-NCWRF	Watford		Fungi
	-NCYC	Norwich		Yeasts
FRG	-DSM	Braunschweig	National Node	**Dr. R.M. Kroppenstedt, pm** Bacteria, Plasmids, Fungi, Yeasts, Plant Virusses, Plant and Animal Cell Cultures
	-IMET	Jena		Bacteria
	-DIMDI	Köln	Central Data Node	**Dr. H.-E. Kurzweily**
P	-IGC	Oeiras	National Node	**Prof.Dr. I. Spencer-Martins, pm** Yeasts

S	-CCUG	Gothenburg	Dr. E. Falsen, pm Bacteria
SF	-VTT	Aspoo	Dr. M.-L. Suihko, pm Bacteria, Fungi, Yeasts

OBJECTIVES SET FOR THE REPORTING PERIOD:

Introduction:

The MINE-project is a continuation of the work started under BAP, during which the format was defined for the storage of data on strains of microorganisms.

The general objective of MINE concerns the integration and harmonization of the data in the participating collections and the subsequent deposition of these data in one Central Data Node (CDN), giving the users of data - scientific, industrial and environmental organizations - an easy entry to the rich variety of microorganisms present in the European collections.

This project, in which many nationalities and institutions cooperate and information on about 130.000 strains has to be processed, is rather complicated and would have been impossible without the support of the EC.

Shaping of the project:

A rather long time was needed to formulate the project in such a way that all participants had a clear idea about what should be done and how, and consensus was reached about the organization and structure. Especially the discussion about the CDN proved to be cumbersome, a decision only being reached in the beginning of 1991 with the unanimous choice of DIMDI, Köln.

In retrospect this delay was absolutely necessary to prevent inbuilt possibilities for conflicts later on. The contract between EC and the head-contractor, the Royal Netherlands Academy of Arts and Sciences, was signed on September 24, 1991, leaving precious little time for any activities in this first year. It was fortunate that the MINE board, in consultation with EC, agreed on an earlier start of the project, by setting the official date on June 1, 1991.

Objectives for 1991:

The objectives to be reached in 1991 were:

a) Updating and first integration of the minimum data sets by the collections and Data Integrating Nodes (DINs).
b) Start of correction and harmonization of supplied data by the Responsible Committees (RCs).
c) Initiation of coordinative discussions between DINs and CDN about database structure and transfer of data.

MAJOR PROBLEMS ENCOUNTERED:

No major problems have been encountered, but some problems with respect to the time schedule are inevitable, partly as consequence of the start of the project far ahead of EC funds becoming available.

Also the fact that especially the work of the RCs consists of "learning by doing", meaning that during the process all kinds of problems, sometimes of a fundamental taxonomic character have to be solved, makes it rather difficult to stick to the schedule as proposed in the project.

Consultative meetings between DINs and RCs and a pragmatic approach should prevent too great a deviation from the timetable. In 1992 3 meetings have already been planned to ensure optimum coordination of the project.

RESULTS:
The collections provided the DINs (Fungi/Yeasts: CBS, Baarn; Bacteria: LMG, Ghent) with the most recent data. After processing and integration, the DINs prepared the Report Files (Fungi/Yeasts: 1700 pages; Bacteria: 1500 pages) for the RCs (Fungi/Yeasts - chairman Prof.Dr. G.L. Hennebert; Bacteria - chairwoman Dr. Danielle Janssens).
The chairpersons, in close cooperation with the DINs (Baarn: Gerrit Stegehuis; Ghent: Danielle Janssens) established the guidelines for correction and harmonization by RC members.
In a number of meetings between DINs and DIMDI, the format of the central data base and coordination of supply of data from the DINs to the CDN were discussed.
After that testfiles were sent to DIMDI.
Dates were fixed for demonstrations of the central data base to the MINE member collections.
MINE collections participate in the establishment of an European data base on plasmids, virusses and transposable elements; a common format was established along the lines of the MINE formats for fungi/yeasts and bacteria.

HIGHLIGHTS/MILESTONES:
Nothing to report for 1991.

WIDER CONSIDERATIONS:
Biodiversity is becoming a central theme for scientific, political and economical consideration.
In the Global Change and Biodiversity Programmes of IUCN and IUBS worldwide attention is demanded for the importance of biodiversity research for conservation of endangered habitats and nature and for assessment of processes in Global Change. In these programmes the crucial function of collections and of microorganisms is rightfully emphasized. The same holds for the ever increasing role and potential of micro-organisms in biotechnological research and in handling of waste.
The MINE programme is the first international, albeit still regional approach to get good insight in and easy access to the wealth of microorganisms present in collections. Undoubtedly MINE will have an impact on the establishment of a truly world wide system, essential for the effort to watch and monitor the still far underexploited gene pools on our globe.

COOPERATIVE ACTIVITIES:
Within the BRIDGE programme:
Apart from, but closely related to MINE, the Information Centre on European Culture Collections (ICECC) as part of the BRIDGE programme was established at DSM, Braunschweig. In cooperation with national nodes this centre disseminates, through publications and presentations the MINE programme to all interested groups.

Demonstrations:

Bioexpo	Paris	April	9-12	(ICECC + French node)
Biotechnica '91	Hannover	October	22-24	(ICECC)
Bioinformatics in the 90's	Maastricht	November	20-22	(ICECC + Dutch node)

Other organizations:
Within Europe: European Culture Collections' Organization (ECCO);
1991: Combined meeting MINE-ECCO Valencia Spain.
1992: Combined meeting MINE-ECCO Gothenburg Sweden.

Global organizations:
World Federation for Culture Collections (WFCC);
1992: Beijing China.

SOME FIGURES ON THE INTEGRATION:

Number of strains received by DINs:	Bacteria	32.749
	Fungi/Yeasts	56.232
Retained after integration: Unique strains:	Bacteria	26.286
	Fungi/Yeast	42.481
Strains present in more than one collection:	Bacteria	3.824
	Fungi/Yeasts	5.615

AREA : B

ENABLING TECHNOLOGIES

TITLE: "Engineering of microbial peptide lantibiotics for use in agro-food and biomedical industry"

CONTRACT NUMBER: BIOT-CT91-0265 (MNLA)

STARTING DATE: 1 May 1991

COORDINATOR: C.W. Hilbers
Lab. Biophys. Chem., KUN, Nijmegen, NL

PARTICIPANTS: H.-G. Sahl, Inst. Med. Microbiol., Fr. Wilhelms Univ. Bonn, FRG
R. Siezen, Ned. Inst. Zuivel Onderzoek, Ede, NL
G. Jung, Inst. Org. Chemie, Eberhardt-Karls Univ. Tübingen, FRG

OBJECTIVES SET FOR REPORTING PERIOD:

1) Screening for novel lantibiotics and large scale production.
2) 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.
3) Chemical synthesis of leader peptides, pro- and prenisin, chemically modified lantibiotics, and mutant/variant lantibiotics.
4) Characterization of processes involved in:
   a) biosynthesis: genes, prepeptides and enzymes involved,
   b) cell killing and immunity of producing strains.

MAJOR PROBLEMS ENCOUNTERED:

1) No problems.
2) The structure elucidation of novel lantibiotics will require the isolation and purification of additional amounts of material.
3) No problems.
4) Identification of the staphylococcal immunity peptide and putative processing protease are problematic, due to difficulties with production of antibodies.

RESULTS:

ad 1).

A total of 800 staphylococcal strains were screened for production of lantibiotics. Nine strains produced active substances, two of which did not produce sufficient activity for characterization. Seven strains produced lantibiotics; one strain produced three active lanthionine containing peptides, so that a total of nine lantibiotics was obtained. All peptides were purified and analyzed as to molecular mass, amino acid composition and partial

sequence. Two peptides proved to be epidermin, seven others represent novel lantibiotics. So far, one peptide (called K7) was produced on a large scale (20 mg) for primary and spatial structure elucidation.

Of the twenty six nisin-producing *Lactococcus lactis* strains tested, twelve were found to possess the nisA gene for the natural variant nisin A, and fourteen contained the nisZ gene for the variant nisin Z. The gene for a second lantibiotic from *L.lactis*, lactacin 481, was identified and characterized (collaboration with J.C. Picard, INRA, Jouy-en-Josas, France).

ad 2)

A main goal was the structure elucidation of intermediates in the biosynthesis of lantibiotics such as Pep5 (Bonn group) which were found to arise from a precursor protein consisting of a leader sequence followed by the pro-Pep5 part in different stages of dehydrated serine and threonine residues. Using sequence analysis and electrospray mass spectrometry the Tübingen group could unequivocally analyse the non-modified ribosomally synthesized pre-Pep5 as well as all intermediates which are derived by dehydration of Ser and Thr residues.

Furthermore the α-helical conformation of the complete natural pre-Pep5 and of all leader segments of prelantibiotics has been determined by circular dichroism in lipophilic solvents. These findings point to a common principle of lantibiotic biosynthesis. This α-helical character is most likely induced by the lipophilic environment: the nisin leader peptide appears a random coil in aqueous solution as indicated by NMR. Its structure determination in alcoholic solution is planned.

The first structural characterizations of the novel lantibiotics isolated by the Bonn group are presently carried out in Tübingen using sequencing and MS. Two dimensional NMR studies of K7 have been carried out in Nijmegen. The results show that K7 is a novel lantibiotic indeed: it contains two dehydroalanines, two dehydrobutyrines, two methyllanthionines and possibly also a lysinoalanine; this combination has not been found in any of the other known lantibiotics. The typical ^{1}H NMR patterns of 31 residues were identified, most of which could be positioned relative to each other in the amino acid sequence. For a complete structure determination a complete resonance assignment is required; this can be obtained once the amino acid sequence has been established/confirmed by the Tübingen group. An attempt will be made to accomplish this also using ^{13}C NMR but this will require more material.

Analysis of the structure of purified mutant nisins and nisin degradation products by 2D NMR techniques is currently in progress. Initial results indicate that it is possible to exchange or introduce additional dehydrated amino acid residues. Activity measurements against various indicator strains show that mutants may have altered activity and/or selectivity, and that removal of the leader peptide is not a prerequisite for activity. The dehydroalanine residue at position 5 is found to be important for activity. Physical properties such as pH-dependence of solubility and chemical stability of native and mutant (pre)nisins are being analysed.

The three dimensional structures of nisin in aqueous solution (Nijmegen), and of gallidermin and Pep5 in alcoholic solvents (Tübingen) have been determined by means of 2D NMR. Structural studies in more membrane-like environments, such as detergent micelles, are in progress. Although the structures appear rather flexible, some common characteristics could be discerned. They possess a screw-like shape, with a clearly amphiphilic character, i.e. along the long axis of the molecule there is one typically hydrophobic face and one less hydrophobic. A high dipole moment was found for gallidermin and Pep5. These findings are in agreement with a model in which lantibiotic molecules penetrate the lipid bilayer, a process which probably requires a trans membrane voltage, and aggregate to form transmembrane pores.

ad 3)

The chemical synthesis of the leader peptide (23 amino acids) and the prolantibiotic part (34 amino acids) of prenisin have been carried out in Tübingen. The leader peptide has been obtained in highly purified form and is being studied by the Nijmegen group using 2D NMR. The five cysteine residues containing pronisin has been purified and characterized in its cysteine Acm-protected form, and optimized cleavage conditions are being worked out presently. The synthetic peptides are analyzed by modern peptide analytic methods including HPLC on-line electrospray mass spectrometry, automatic Edman sequencing and cicular dichroism.

The success of total synthesis of the 57 amino acids long prenisin depends largely on the optimized couplings of the C-terminal pronisin part. Attempts for coupling the side-chain protected leader part to the amine terminus of the pronisin part are being made.

According to the workplan, chemical modifications of natural nisin are to be carried out in the forthcoming period. However, the Tübingen group has already prepared chromophore labeled substrates for the leader peptidase of prenisin. Screening for this enzyme and related activities are carried out by the Ede group.

Three different systems have been developed in Ede for the expression of mutant nisin genes, one of which also produces nisin A in addition to mutant nisin. By site-directed matagenesis more than 20 different mutant (pre)nisins were produced, many of which were secreted. Several mutant (pre)nisins were subsequently purified in sufficient quantities for structural and functional analysis. Two mutants were obtained with a leader sequence still attached to the nisin molecule.

ad 4).

A 2 kb fragment covering the Pep5 structural gene (*pepA*) was sequenced. Two genes adjacent to *pepA* were identified. The upstream gene is necessary for producer self-protection ("immunity") and codes for a 69 amino acid, probably membrane-associated, peptide. The downstream gene codes for a 285 amino acid protein, with sequence homology to serine proteases, which could act as a processing protease. For all genes specific mRNAs were detected corresponding in size to the structural genes (i.e. there is no polycistronic mRNA in the case of Pep5). A relevant 5kb *Hind* III fragment upstream of *pepA* was cloned into pBR 322 and is currently being sequenced. Further, the Bonn group is constructing an expression system for production of mutant Pep5 in a *S. epidermin* 5 variant which is devoid of pED 503.

The Pep5 prepeptides have been isolated and characterized by ion spry MS, CD, amino acid analysis and sequencing. All biosynthetic intermediates (unmodified, partially and completely dehydrated, with and without thioethers) were found in the cytoplasmic cell fraction. This implies that all steps of the biosynthesis of Pep5 take place in the cytoplasm.

Epitope prediction plots were used to synthesize two peptides from the immunity gene, and six peptides from the protease gene. Both immunity gene peptides and four protease peptides were not immunogeic in rabbits; two protease peptides raised antibodies, bot both sera did not stain a protein in the 32 kDa range (the expected size of the protease). Estimation of the amount of protease mRNA in cells suggests that the protein concentration in crude cell extracts may be too low to be detected in Western blots.

Analysis of the nisin operon on transposon Tn5276 has shown that several genes are located directly downstream of the the structural nisin A gene. These are presently being sequenced. Homology analysis suggests that products of these genes may be involved in translocation of nisin precursor(s) across the cell membrane, and in cleavage of the leader peptide.

HIGHLIGHTS

- Isolation of seven novel lantibiotics.
- Detection of the first lantibiotic immunity gene.
- Isolation of Pep5 prepeptides in all stages of modification that had been postulated.
- Identification of a natural nisin variant: nisin Z.
- Overproduction of nisin by *L.lactis* strains.
- First protein engineering of nisin, resulting in various mutants with altered structure and activity.
- Sequencing of genes of nisin operon.
- Synthesis and conformational characterization of all known leader peptides of prelantibiotics.
- 3D structure determinations of nisin and Pep5.

WIDER CONSIDERATIONS

Excellent progress has been made in all facets towards achieving our original goal: understanding of the interrelations between biosynthesis, producer immunity, structure and function, as a basis for the rational design of new lantibiotics. The first novel lantibiotics with altered properties have been developped.

COOPERATIVE ACTIVITIES

- Meetings:
Meeting during "The first international workshop on lantibiotics" in Bad Honnef (FRG) on April 15-18, 1991

- Materials exchanged:

Bonn - Tübingen:	9 isolated lantibiotics for MS and sequencing; 10 mg of K7, Pep5 for 3D structure elucidation
Bonn - Nijmegen:	10 mg of K7 for NMR
Bonn - Ede	DNA sequences, purified Pep5
Ede - Tübingen:	nisin analogs and degradation products for MS
Tübingen - Bonn:	synthetic immunogens for preparation of antibodies, chromophor labeled substrate for processing protease of Pep5
Tübingen - Ede:	chromophor labeled substrate for processing enzyme of prenisin, nisin leader peptide
Tübingen-Nijmegen:	10 mg of synthetic leader peptide of prenisin

- Staff exchanges:

Tübingen-Nijmegen:	coworkers NMR discussion
Tübingen-Bonn:	for precursor peptide work
Ede-Tübingen:	for MS work
Bonn-Tübingen:	for structure elucidations novel lantibiotics
Ede-Nijmegen:	regular visits for NMR analysis of mutants etc.

JOINT PUBLICATIONS

- G. Jung and H.-G. Sahl (eds) "Nisin and novel lantibiotics" ESCOM, Leiden (1991) therein six joint papers:
  - Freund et al. p. 103-112
  - Kellner et al. p. 141-158
  - Frey et al. p. 180-188
  - Sahl et al. p. 332-346
  - Benz et al. p. 359-372
  - De Vos et al. p. 457-463

OTHER PUBLICATIONS

- in: G. Jung and H.-G. Sahl (eds) "Nisin and novel lantibiotics" ESCOM, Leiden (1991)
  - F.J.M. van de Ven et al., p. 35-42
  - M. Reis and H.-G. Sahl, p. 320-331
  - H.-G. Sahl, p. 347-358
  - G. Bierbaum and H.-G. Sahl, p. 386-396
  - E. Molitor and H.-G. Sahl, p. 434-439
  - G. Jung, p. 1-34
  - S. Freund et al., p. 91-102
  - A. Beck-Sickinger et al., p. 218-230
  - O.P. Kuipers et al., p. 250-259
  - P.J.G. Rauch et al., p. 243-249
  - H.S. Rollema et al., p. 123-130

- in: R. James, C Lazdunski, F. Plattus (eds) "Bacterial plasmid-coded toxins: bacteriocins, micrococci and lantibiotics" Springer, Heidelberg (in press 1992)
  - H.-G. Sahl "Lantibiotics, an introduction"
  - H.-G. Sahl "Biosynthesis of the lantibiotic Pep5 and mode of action of type A lantibiotics"
  - S. Freund et al. "NMR study of Pep5"
  - R.N.H. Konings et al. "NMR studies of lantibiotics. The 3D structure of nisin"

- G. Jung, Angew. Chem. Int. ed. Engl. 30, 1051-1192 (1991)
- J. Hugenholtz, O.P. Kuipers, H.S. Rollema, Voedingsmiddelentechnologie, 21, 19-22 (1991)
- O.P. Kuipers, H.J. Boot, W.M. de Vos, Nucl. Ac. Res. 19, 4558 (1991)
- J.W.M. Mulders, I.J. Boerrigter, H.S. Rollema, H.S. Siezen, R.J. de Vos, Eur. J. Biochem. 201, 581-584 (1991)
- P.J.G. Rauch, W.M. de Vos, J. Bacteriology 174, 1280-1287 (1992)
- F.J.M. van de Ven, H.W. van den Hooven, R.N.H. Konings, C.W. Hilbers, Eur. J. Biochem. 202, 1181-1188 (1991)

TITLE:	MUTIDISCIPLINARY APPROACH TO THE ANALYSIS OF ENZYME CATALYSIS, PROTEIN STABILITY AND FOLDING
CONTRACT NUMBER:	BIOT CT91 0270
OFFICIAL STARTING DATE:	01.06.91
COORDINATOR:	Dr. O. Misset, Gist-brocades, Delft, NL
PARTICIPANTS:	Prof. Dr. A.R. Fersht, MRS, Cambridge, GB Prof. Dr. S. Wodak, Université Libre de Bruxelles, Brussels, B Prof. Dr. P.L. Mateo, University of Granada, Granada, ES

OBJECTIVES SET FOR THE REPORTING PERIOD

In the Working Programme (as attended to the Contract and the Consortium Agreement) it was recognized that the project objective ("Providing more understanding of the mechanisms related to protein stability and folding on the one hand and enzyme kinetics on the other hand") was a difficult one to plan and measure in quantitative terms. It was therefore suggested to relate the "provided understanding" to the number of mutant enzymes that are designed, made and tested for this purpose.
However, the group of Fersht (Cambridge) is working already for many years on Barnase and has generated some 50 mutants all in relation to stability and activity.
Therefore, the number of mutants generated will not be used further to measure the progress made. Rather, the latter will be evaluated using the number of studies on the various mutants and wild type enzyme.

MAJOR PROBLEMS ENCOUNTERED

1. Project management problems

The major problem which was encountered during the execution of the project was the fact that the "Advance Payment" by the EC was made much too late! It prohibited that the project could start efficiently.

All together this has led to rather unbalanced activities by the four participants groups which is intended to be corrected in the coming time.

2. Scientific problems

The modelling activities which were going to be carried out by Wodak and Aehle were delayed since it was found that the earlier 3D-model for the complex of barnase with the penta-ribonucleotide 5'3' (ApAp-cyc 2'3'GMP-ApAp) transition state, derived using mainly manual computer graphic operations, followed by limited energy minimization procedures, displayed some unusual dihedral angle values in the nucleotide portion, probably as a result of applying specific inter-molecular distance constraints. The model was therefore rebuilt, using a systematic and automatic survey of all relevant dihedral angles of the bound nucleotide without imposing distance constraints.

RESULTS

The results which have been obtained in the reporting period can be divided into 3 categories.
1. Development of the research tool box.
2. Results aiming at the understanding of the mechanism of folding and stability.
3. Results aiming at the understanding of the mechanism of enzyme kinetics.

The results will now be described accordingly.

1. Development of the research tool box

1.1 Structure determination (Cambridge)
Apart from the various X-ray crystal structures which are already available for Barnase (in various complexes), Bycroft, in Fersht's laboratory, has succeeded in determining the solution structure of Barnase by NMR.

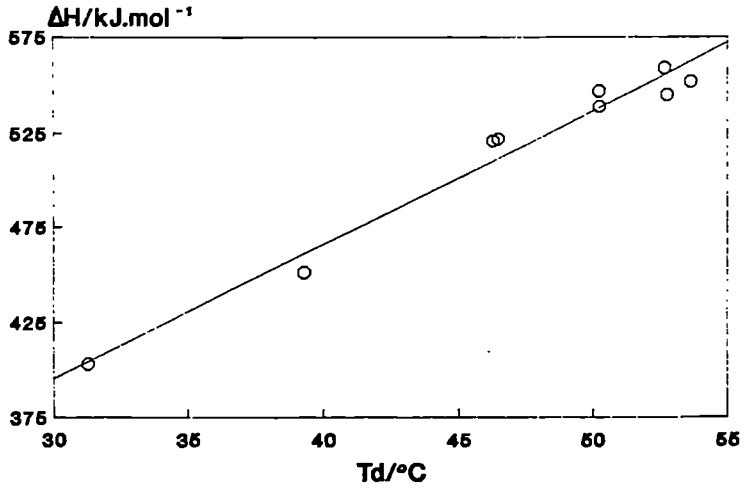
1.2 Modelling tools (Brussels, Delft)
- In setting up the simulations of the free energy change upon histidine deprotonation, problems concerning energy parameters (those for protonated histidines are routinely available) had to be solved. It was also necessary to investigate simulations conditons that provide adequate sampling and convergence.
- Set-up of MD simulations of barnase in presence of 5 phosphate ions ($CH_3PO_4^{(2-)}$) required incorporating the appropriate energy parameters into the BRUGEL and BIOSYM package, and providing initial positions for the phosphate ions relative to the enzyme surface. The latter task was performed on the basis of the analysis of the electrostatic potential on the enzyme accessible surface (computed in earlier studies by the Delphi program).

* The BIOSYM software package (Insight II and Discover) was installed on the new Silicon Graphics IRIS workstation of Gist-brocades and slightly modified for the protein modelling purposes.

1.3 Synthesis of substrates (Cambridge)
In order to carry out the kinetic studies on Barnase, the following substrates were synthesized by solid state synthesis:
* GpApA, GpApC, CpUpC, CpGpApC (completed)
* GpUp, GpAp and GpGp (in development)
* GpNp-methyl esters (in development)

2. Results aming at the understanding of the mechanism of folding and stability

2.1 Thermal analysis (Granada)
The thermal stability of wild type Barnase (WT) and the T16S-mutant was investigated using high-sensitivity Differential Scanning Calorimetry (DSC). From the observation that the ratio of the calorimetric to the Van 't Hoff enthalpy was 0.99 ± 0.02 for WT and 0.97 ± 0.02 for T16S (in the pH range 2.5 - 5.0) was concluded that the protein denaturation is an all-or-none, two-state transition. The calorimetric enthalpy values were also found to be linear functions of the denaturation temperature, T_d (see the figure for WT) with a ΔC_p (= $\delta\Delta H/\delta T$) value of 6.2 ± 0.7 and of 6.0 ± 0.3 kJ/K.mol for WT and TS16 respectively. Since these values agree well with the corresponding average ΔC_p values obtained directly from the individual thermograms, ΔH appears to depend only on T_d. From the above ΔH and ΔC_p values, entropy (ΔS) and Gibbs energy changes (ΔG) for the unfolding of both proteins were obtained as a function of temperature. Extrapolation of the ΔG functions shows a maximum in stability for both proteins around 250 K.



Note: Only a limited amount of work could be carried out as was explained under the heading MAJOR PROBLEMS ENCOUNTERED.

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

3.1 A study on the subsite specificity of the transesterification reaction catalysed by Barnase (Cambridge)

Barnase has a strong preference for guanosine immediately 5' to the phosphodiester bond that is broken during the transesterification reaction. Earlier studies have shown that dinucleotide monophosphates (GpN) are poor substrates for barnase relative to RNA. This led us to believe that there must be subsites on barnase. Wodak's laboratory has carried out a number of theoretical studies on barnase subsites and the present work is designed to both test this theoretical work, and also to provide empirical results which will allow the theoretical work to be extended.

In order to analyse barnase for subsites, the following substrates were purchased (GpA, GpC, GpG, GpU, GpUp) or made by solid state synthesis (GpApA, GpApC, GpUpC, CpGpApC). The kinetics of catalysis of transesterification of these substrates by barnase was measured. Increasing the length of the dinucleotide monophosphate substrate, GpU, by one phosphate to give GpUp gave rise to a 1000 fold increase in k_{cat}/K_m. This increase is spread unevenly between ground state and transition state binding, with a decrease of 10-fold in K_m and an increase of 100 fold in k_{cat}, indicating that this subsite contributes to binding of both the ground and transition state. A further increase in length to GpUpC leads to a further 20-fold increase in k_{cat}/K_m. In this case K_m is nearly doubled relative to GpUp; this increase is more than compensated for by the 30-fold increase in k_{cat}. The parameter, k_{cat}/K_m approaches diffusion control for the best trimer substrate, GpApA. There appears to be no subsite 5' to the guanosine, i.e. GpApC and CpGpApC have identical kinetics of transesterification.

In summary for substrates of type $Zp_0Gp_1Xp_2Y$, where p is phosphate, X, Y and Z are nucleotides and G is guanosine: G occupies the primary specificity site. The most important subsite if for p_2, followed by that for Y. There appears to be no subsite for the Z or p_0 positions. There are differences in base specificity at the two subsites for X and Y downstream of the scissile bond. The binding energies of different substrates have been analysed using thermodynamic cycles. These show that the contributions of the X and Y sites are non-additive.

3.2 Modelling and analysis of the Barnase: pentanucleotide transition state model (Brussels)

The amended barnase-penta-nucleotide transiton state model differs from the initial proposal in the details of the interactions made between the enzyme and the nucleotide. Dihedral angles value of the bound nucleotide, except for the cyclic 2'3' pentacovalent sugar phospate, now correspond to conformations most commonly observed in nucleotides. Salient features of the model include: interactions associated with specific guanine recognition and observed in complexes of homologous microbial ribnucleases are maintained. The O2' and O5' atoms of the central phosphate closely interact with the catalytic residues Glu73 and His102 respectively. This phosphate also forms H bonds with the active site Arg83 and Arg87. The next phosphate in the 3' direction also binds close to the catalytic site of barnase forming additional H bonds with Arg83 and Arg87, thereby completely sealing these residues from water. Subsites are identified for binding the two bases following the guanidine 3' direction. They involve interactions with Trp35 (+1 base) and Phe82 (+2 base). Using the detailed three dimensional picture of the barnase-transition state complex provided by our modelling study, we were then able to rationalize many of the experimental observations made in A. Fersht's laboratory on the kinetics of oligo-nucleotide catalysis in barnase (Day et al, 1992). Our model also seems to agree very well with data on changes in Nuclear Magnetic Resonance (NMR) chemical shifts measured for residues in barnase upon di- and oligo-nucleotide binding.

3.3 Molecular dynamic simulation of barnase in the presence of 5 phosphate ions ($CH_3OPO_3^{2-}$) and water (Brussels and Delft)
This study is carried out with the purpose of getting more information about substrate recognition and binding. The structure obtained by averaging the structures collected during the simulation will be the basis for calculations with the software package Delphi.
The simulation is presently carried out both in Delft (Discover software) and Brussels (Brugel software) which allows a direct comparison of the two force fields. The system consists in total of one barnase, five methylphosphate and 2359 water molecules. A rectangular box of 82681 $Å^3$ (a = 48.12Å, b = 35.7Å, c = 48.13Å) is used for the simulation under periodic boundary conditions. It is performed at 300 K with constant box volume.

3.4 Simulation of Histidine (de)protonation
Preliminary tests determining optimal simulation conditions for convergence and sampling of the perturbation pathway have been completed and the final trajectories required to compute the free energy changes accompanying de-protonation of a histidine dipeptide unit in water and the gas phase have been generated. Computations of the free energy change from these trajectories are now in progress. Equivalent trajectories have been generated for native barnase.

4. Summary of research activities executed during the first reporting period

subject	enzyme/compound	activities	group
general	Barnase WT	3D-structure by NMR	Fersht
general	substrates	organic synthesis	Fersht
stability	Barnase WT	thermal analysis (DSC)	Mateo
stability	Mutant T16S	thermal analysis (DSC)	Mateo
kinetics	Barnase WT	substrate specificity	Fersht
kinetics	Barnase WT + pentanucleotide	remodelling complex	Wodak
kinetics	Barnase WT + phosphate	MD-simulation	Wodak/Aehle
kinetics	Dihistidinepeptide	Simulation (de)protonation	Wodak

HIGHLIGHTS/MILESTONES

* Solution structure of Barnase by NMR.

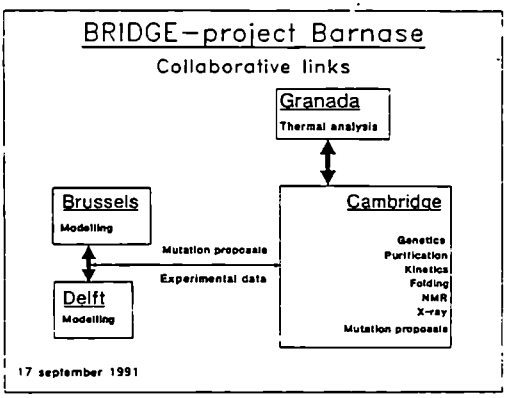
* A detailed 3D model for the barnase-pentanucleotide transition state complex which is consistent with the experimental data on barnase catalysis and with experimental NMR measures has helped gain insight into the factors that govern catalysis.

WIDER CONSIDERATIONS

One of our aims is to extend our analysis of enzyme-nucleotide interactions, to the general case of protein-ligand interactions. This would require efficiently incorporating dynamic aspects and explicit solvent contributions. Methods to achieve this are being devised.

COOPERATIVE ACTIVITIES

The collaborative links between the groups are based on the respective tasks of each group in the project and can be visualised as follows:



The group of Fersht in Cambridge is very much involved in the protein engineering of Barnase (approximately 8-10 people) and, consequently, their contribution to the BRIDGE project targets is small compared with their own activities. Almost all off the many techniques and disciplines of the protein engineering cycle are carried out in Cambridge. Since Mateo in Granada is responsible for the thermal analysis of Barnase and its mutants, there exists a collaborative link with Fersht who supplies the protein material. The modelling group in Brussels (Wodak) and Delft (Aehle/Misset) interact intensively with respect to Molecular Dynamics Simulations of Barnase. Furthermore, there is a fruitfull collaboration and information exchange with Fersht's group concerning in particular barnase oligonuleotide catalysis.

Project meetings

1. 05/09/91 in Delft (minutes, d.d. 18/09/91)
   During this first meeting of the project team, specific goals and cooperations have been set up.
2. 22 and 23/01/92 in the chemical laboratory of Cambridge (minutes, d.d. 10/02/92).
   During this particularly useful meeting, the wide scope of the ongoing work in Fersht's group was presented to the other BRIDGE members and extensively discussed.

Visits

3. 19/11/91 W. Aehle (Delft) visited Wodak and was introduced to the Brussels group and the field of molecular modelling and MD-simulations.
4. 26/02/92 W. Aehle (Delft) brought a second visit to Wodak in which the MD-simulation work was initiated.

Lectures

5. 06/09/91 Prof. S. Wodak gave a lecture on Molecular Modelling for the Research and Development organisation of Gist-brocades (Delft).
6. 06/09/92 Prof. P.L. Mateo gave a lecture on Differential Scanning Calorimetry for the Research and Development organisation of Gist-brocades (Delft).

Materials exchanged

* Coordinates of Barnase and Barnase:d(GpC) complex were obtained by Aehle from Fersht and Wodak respectively.
* 20 mgs of Barnase and the T16S mutant were obtained by Mateo from Fersht.

LIST OF PUBLICATIONS/PATENTS WITH TRANS-NATIONAL AUTHORSHIP

Nothing to report.

OTHER PUBLICATIONS/PATENTS

1. Gordon-Beresford, R.M.H., Coulonbeau, C. and Wodak, S. (1992) Biochemistry, submitted for publication.
2. Serrano, L., Bycroft, M. and Fersht, A.R., Aromatic-aromatic interactions and protein stability: Investigation by double-mutant cycles, Pure and Applied Chemistry **63**, 187-194 (1991).
3. Horovitz, A., Serrano, L and Fersht, A.R., Cosmic analysis of the major α-helix of barnase during folding, J. Mol. Bio. **219**, 5-9 (1991).
4. Sali, D., Bycroft, M. and Fersht, A.R., Stabilization of Barnase by an Interaction between two oppositely charged site chains, Techniques in Protein Chemistry II **28**, 295-303 (1991).
5. Ferhst, A.R., Bycroft, M., Horowitz, A., Kellis Jr, J.T., Matouschek, A and Serrano L., Pathway and stability of protein folding, Phil. Trans, R. Sco. Lond B **332**, 171-176 (1991).
6. Loewenthal, R., Sancho J. and Fersht, A.R., Fluorescence spectrum of barnase: contribuions of three tryptophan residues and a histidine-related pH dependence, Biochemistry **30**, 6775-6779 (1991).
8. Sali, D., Bycroft, M. and Fersht, A.R., Surface electrostatic interactions contribute little to stability of barnase, J. Mol. Biol. **220**, 779-788 (1991).
9. Sancho, J., Meiering, E.M. and Fersht, A.R., Mapping transition states of protein unfolding by protein engineering of ligand-binding sites, J. Mol. Biol. **221**, 1007-1014 (1991).
10. Day, A.G., Parsonage, D., Abel, S., Brown, T. and Fersht, A.R., Barnase has subsites that give rise to large rate enhancements, (1992) submitted for publication.

TITLE:
Stability studies and protein design studies with triosephosphate isomerases

CONTRACT NUMBER:
BIOT-CT90-0182

OFFICIAL STARTING DATE:
01/01/1991

COORDINATOR:
R.K. Wierenga, EMBL, Heidelberg, Germany

PARTICIPANTS:
Opperdoes, ICP, Brussels, Belgium
Wierenga, EMBL, Heidelberg, Germany
Martial, University of Liège, Liège, Belgium
Hol, University of Groningen, Groningen, The Netherlands
Kessler, Technical University of Munich, Munich, Germany
Renard, Eurogentec, Liège, Belgium

OBJECTIVES SET FOR THE REPORTING PERIOD:
(as written in the original workplan)

Trypanosomal TIM
Some mutants of TIM should have been overexpressed and purified, for example Cys14Gly (tip of loop-1), Val46Ala, Val46Gly (tip of loop-2), His47Ser (tip of loop-2), Thr75Gly (tip of loop-3). These mutations will destabilise the dimer to varying extents. The purified mutant TIMs will be enzymologically characterised (ICP) and crystallisation experiments will be carried out with and without class-1 cyclohexapeptides (EMBL).
Cocrystallisation experiments of wild type TIM plus the class-2 cyclopeptides (EMBL) will have been done.
Computer graphics analysis of the subunit-1/subunit-2 interactions across the interface of trypanosomal TIM, yeast TIM and chicken TIM will have been completed (EMBL).

Human TIM
Overexpression of human TIM will have been carried out (Liège). Crystallisation experiments will have been initiated (Groningen). The Glu104Asp mutation experiments, causing thermolability of human TIM will have been initiated.

***E. coli* TIM**
Several more mutants of *E. coli* TIM will become available (Liège). The mutations will be selected such as to destabilise the dimer. The *E. coli* mutants will have been enzymologically characterised (ICP). Crystallisation experiments will have been initiated (EMBL).

Octarellin
Model building studies for the second round of octarellin design will have been completed (Liège, EMBL). The mutagenesis for octarellin-2 will be underway.

Psychrophilic TIM and thermophilic TIM
Overexpression of at least one of these TIMs will have been done (Liège). Crystallisation experiments will have been initiated (Groningen).

Cyclopeptides
Considerable amounts of class-1 and class-2 cyclopeptides will become available (Munich). The binding properties of these cyclopeptides to wild-type TIMs and mutant TIMs will have been tested (ICP). Cocrystallisation experiments of class-1 and class-2 cyclopeptides and trypanosomal TIM will have been done (EMBL).

Loop transplantation modelling studies
Model building studies on the basis of the structures of trypanosomal TIM, chicken TIM and yeast TIM will have given some clues as to which kind of loop transplantation experiments should be considered (Groningen).

At the end of the first year, large quantities (>100 mg) of wild type trypanosomal TIM, mutant trypanosomal TIM, wild type *E. coli* TIM, mutant *E. coli* TIM, wild type human TIM, and wild type psychrophilic TIM (or thermophilic TIM) should be available. Also, large quantities of class-1 and class-2 cyclopeptides will be available for testing. The enzymological tests carried out with the trypanosomal TIM mutants and the *E. coli* TIM mutants will already indicate some of the properties of the TIM mutants.

MAJOR PROBLEMS ENCOUNTERED: Nothing to report

RESULTS:

Due to the good collaborations (see also section "cooperative activities") between the various groups, significant progress has been made. Most of the results are still being evaluated and several papers are in preparation. Here we will only briefly present the most important efforts of the different groups. The major results are summarised in the next section ("highlights/milestones").

I. ICP (Opperdoes, Callens)
The work concerns the kinetic studies of the cyclohexapeptides and the enzymological characterisation of TIM-variants.

T. brucei TIM is more labile than its homologous counterparts from other organisms. This lability could be related to a number of amino-acid substitutions in the dimer interface area. The availability of recombinant *T. brucei* TIM now allows us to test this hypothesis. As a

first approach, a mutation has been introduced in one of the 32 interface residues. Histidine 47 in loop 2, which is involved in a water-mediated hydrogen bond with aspartate 85 in helix 3, was replaced by an aspargine. This mutant H47N indeed turns out to be much more labile. The mutant enzyme has similar affinities for the substrates, but exhibits a lower kcat with these substrates. Activity is rapidly lost upon dilution, probably due to dissociation into the individual subunits. The subunits associate again upon concentration with regain of activity. In gel filtration experiments, two major protein peaks can be discerned, one representing the inactive monomer and the other the active dimer. Contrary to the native enzyme, the pH optimum of the H47N mutant is very narrow with an optimum at pH 7, suggesting that the dimer is only stable at neutral pH.

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

The efforts are focussed on four topics:

1. Overexpression and mutagenesis of trypanosomal TIM;
2. Characterisation of mutant trypanosomal TIM;
3. Crystallographic binding studies with class II cyclohexapeptides;
4. Crystal structure determination of *E. coli* TIM.

Trypanosomal TIM can now be routinely overexpressed in *E. coli* and purified in large quantities.

A number of TIM variants have been made in which the dimer is expected to be less stable. The following interface residues have been changed by site-directed mutagenesis: C14G (at tip of loop-1), and V46D, V46G, H47N (at the tip of loop 2). So far only the H47N-variant has been characterised to some extent in collaboration with Opperdoes (Brussels, Belgium). The H47N-variant is less active than the wild-type and the remaining activity depends on the concentration of the variant. The H47N-variant is also less stable, as is suggested by circular dichroism (CD) measurements and temperature gradient gel electrophoresis (TGGE) measurements. Wild-type TIM denatures irreversibly at a temperature near 45°C under these conditions. This transition temperature for the H47N-variant is approximately 33°C. The H47N-variant could not yet be crystallised.

Cocrystallisation experiments with trypanosomal TIM have been carried out in the presence of cyclopeptides synthesised by the Kessler-group or by Dr. Nalis (EMBL). New crystal forms were obtained (space group is triclinic). These crystals diffract to 1.7Å resolution, but no binding of cyclopeptides was observed.

A large number of crystallisation experiments have been carried out with *E. coli* TIM. A few good crystals were obtained. Two datasets (at 2.8Å and at 2.6Å of two different crystals) have been collected. The structure has been solved by molecular replacement.

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

This contribution concerns mainly:

- gene screening and cloning
- site-directed mutagenesis
- optimisation of high level production and purification of several TIMs studied in the research project
- studies of *de novo* designed TIM-barrels

(a) The first objective is the characterisation of a range of different bacterial TIMs which differ widely in stability. The TIM genes from the thermophilic bacterium *Bacillus stearothermophilus* and the psychrotrophic *Moraxella spp. TA137* have been determined. These proteins have been produced in large amounts via the T7 expression plasmid and have been purified. Genes encoding TIMs will be isolated from other extremophilic bacteria, such as the hyperthermophilic *Thermatoga maritima* eubacterial strain and specific archaebacteria as well as the hyperpsychrophilic *Vibrio marinus*.

b) *Escherichia coli* TIM. Large quantities of purified overexpressed *E.coli* TIM have been used for crystallisation and the crystal structure has been solved at EMBL. Several mutants, in which Thr75 (interface loop 3) has been mutated into Val, Asp, and Trp (as suggested by Wierenga group), have already been produced. A "domain-directed" mutagenesis experiment has been done with the *E. coli* TIM barrel, leading to an *E. coli*/chicken hybrid TIM. The eighth structural motif "turn/β-strand/turn/α-helix" was replaced by the equivalent motif present in chicken TIM. The hybrid enzyme was successfully produced in *E. coli* and purified.

c) Human TIM. The cloning of the human TIM gene has been performed. Using the *E. coli* expression system based on the T7 promoter, large quantities of soluble protein were produced.

d) A project concerning the *de novo* protein design of a TIM-barrel protein with an 8 times repeated artificial peptide (with a "turn/β-strand/turn/α-helix" motif) is being continued. Concluding that the attempted α/β-barrel conformation in Octarellin I may be loosely packed, the criteria used to design Octarellin I have been reviewed and two new types of polypeptide sequences were designed, based on either an 8-fold symmetry (Octarellin II) or on a 4-fold symmetry (Octarellin III and IV).

IV. University of Groningen (Hol, Terpstra, Pijning, Mande)

Crystallography, model building and sequence studies concerning TIM from human, *B. stearothermophilus* and *Moraxella*.

The following studies were carried out: (i) using interactive computer

graphics techniques a model of human triosephosphate isomerase (TIM) was constructed using the coordinates of chicken TIM as a starting point. (ii) On the basis of this model suggestions were made for site-directed mutagenesis experiments of human TIM to be carried out in Liège. The first set of suggestions was aimed at arriving at monomeric human TIMs. The second set was meant as a way to improve the stability of TIM by "alanizing" α-helices. (iii) Crystallization studies of human TIM have been initiated, so far yielding only millions of useless needles. (iv) Comparison of TIM barrel proteins has resulted in ideas about transplanting a loop from tryptophan synthase to human TIM since the active site of the former enzyme looked most similar to TIM among the ten TIM-barrel enzymes with coordinates at our disposal. (v) An analysis of the structure-thermostability relationship has been carried out for TIMs from *Moraxella*, and *B. stearothermophilus*, a psychrotrophic and a thermostable member of the TIM family. A joint paper with the Liège group on this subject is nearing completion.

V. Technical University of Munich (Kessler, Kurz)
The research is focussed on the design, synthesis and conformational analysis of cyclic hexapeptides as inhibitors for trypanosomal TIM.

A number of cyclic hexapeptides have been synthesized and tested in Brussels for inhibition of trypanosomal TIM. All structures were totally analyzed and their conformations in solution were determined by modern 2D NMR spectroscopy in combination with molecular dynamic calculations.
The following results have been observed so far:
- All peptides adopt a conformation with two β-turns in which a D-amino acid residue occupies the position i+1 of a βII' turn.
- Activity of better than 10 μmolar inhibition of trypanosomal TIM has been found in several cyclic hexapeptides. In addition, these compounds are selective for trypanosomal TIM and do not inhibit TIM of rabbit, dog and *E. coli* as have been tested.
- The structural change from an oxopeptide to the thiopeptide cyclo(PheΨ[CSNH]-Phe-Gly-Pro-Phe-Val-) results in a drastic increase in biological activity (IC_{50} = 150 μM vs. 6 μM).
- Molecular modelling studies in collaboration with Prof. K. Müller, Hoffman-La Roche, Basle, indicate a possible binding cleft by comparing the three-dimensional structure of the cyclic hexapeptides in solution and using the X-ray structure of trypanosomal TIM. From these studies new compounds were designed which have to be synthesized.
- A mutant of TIM (A100W) was designed to prove the binding region. Although the above-mentioned cleft is more or less closed, the mutant is still active and its activity is still inhibited by the cyclic hexapeptides. However, dynamic modelling via MD exhibits that a fitting is still

possible in the mutant which could not be foreseen with hand-sphere modelling (the work in this respect is still in progress).
- Peptides were synthesized for soaking and cocrystallization experiments.

HIGHLIGHTS/MILESTONES:

1) TIM-variants of trypanosomal TIM and *E. coli* TIM have been made and partly characterised (papers in preparation by Wierenga, Opperdoes (trypanosomal TIM) and Martial, Wierenga (*E. coli* TIM).
2) The sequences of TIM from thermophilic *B. stearothermophilus* and psychrotrophic *Moraxella spp TA137* have been determined (paper in preparation by Martial, Hol et al.).
3) The structural change from an oxopeptide to the thiopeptide cyclo(PheΨ[CSNH]-Phe-Gly-Pro-Phe-Val-) results in a drastic increase in biological activity (IC_{50} = 150 μM vs. 6 μM) (paper in press, Angewandte Chemie by Kessler, Opperdoes, Wierenga et al.).
4) The crystal structure of wild type *E. coli* TIM has been determined at 2.6Å resolution. Refinement is in progress (paper in preparation by Wierenga, Martial).

WIDER CONSIDERATIONS: Nothing to report

COOPERATIVE ACTIVITIES:

Two major joint meetings have been organised so far.
1. Liège, 25 January 1991 (host: Martial)
2. Heidelberg, 23 January 1992 (host: Wierenga)

In addition, many meetings and visits between individual groups have taken place. Protein samples, cyclopeptide samples, sequences and various experimental data are exchanged regularly.

LIST OF JOINT PUBLICATIONS / PATENTS WITH TRANSNATIONAL AUTHORSHIP:

H. Kessler, H. Matter, A. Geyer, H.J. Diehl, M. Köck, G. Kurz, F.R. Opperdoes, M. Callens, R.K. Wierenga, Angew. Chem. 1992, in press.

OTHER PUBLICATIONS/PATENTS: Nothing to report

TITLE	**Design and engineering of alpha-helical bundle proteins: modified structures and novel functions**
CONTRACT NUMBER	BIOT-CT91-0262 (LNBE)
OFFICIAL STARTING DATE	30 April 1991
COORDINATOR	C. Sander, EMBL, Heidelberg, D
PARTICIPANTS	P. Arosio, U of Milano, I P.J. Artymiuk, U of Sheffield, UK H. Blöcker, GBF, Braunschweig, D G. Cesareni, U of Roma, I H.-J. Hinz, U of Münster, D M. Kokkinidis, IMMB, Heraklion, GR C. Sander, EMBL, Heidelberg, D
OBJECTIVES	The immediate goals are to understand, by a combination of theory and experiment, the structural principles of the 4-alpha-helix bundle proteins *rop* and *ferritin.* as a basis for engineering novel functions. Variations to be engineered in the first year included core and loop changes, transfer of a ferroxidase site and introduction of an immunogenic epitope. Where possible, three-dimensional structures were to be determined by crystallography.

RESULTS

Milano

The H and L variants of human ferritins are the models for alpha-helix bundle proteins studied in Milano. They have 55% sequence identity, and coassemble in natural ferritins into 24-mer shells. H ferritin differs from L ferritin in that is has a ferroxidase site and is less stable with respect to chemical denaturation. The ferroxidase site is located within the bundle of H ferritin and can be inactivated by the substitutions Glu62->Lys + His65->Gly.

Construction of ferroxidase site in L ferritin.

The two ferritins have very similar 3D structure, and the construction of a ferroxidase site in L ferritin chain appears feasible. The mutagenesis program was planned by computer modelling in collaboration with our partner group in Crete. Residues involved in the H ferritin metal binding site were introduced into L ferritin by sequential mutageneses of Y27E +E57H +E60H +K62E +G65H +F137Y +E141Q +L144A. The substitution K62E abolishes the expression of a soluble ferritin E.coli. This problem was not overcome by the further substitutions or by modifications of the expression procedures. Thus, in vitro renaturation studies have been performed. Preliminary results indicate that: i) the insoluble variants can be renatured in low yield, under controlled conditions, ii) the substitutions K62E +G65H are not sufficient for ferroxidase activity. The study of more substituted variants is in progress.

Ferritin denaturation / renaturation
H ferritin is significantly less stable to denaturation than L ferritin. Two H ferritin variants with H to L ferritin substitutions (E62K +H65G, and C90E) have increased stability. Crystallographic studies in collaboration with our partners in Sheffield are aimed at identifying the interactions responsible for the stability differences. In addition they may clarify why Cys90 strongly quenches Trp93 fluorescence, and why the quenching is removed by mutational or chemical modification of Cys90. Renaturation studies of purified H and L ferritins and variants have been initiated. Preliminary results (see figure M1) indicate that: i) renaturation and denaturation paths are different; ii) H and L ferritins renature in similar conditions; iii) heteropolymers of the two chains can be produced in vitro.

Subunit monomer
The production of soluble and stable ferritin subunit monomers may allow NMR studies and easier comparson with Rop protein. Monomers that do not assemble appear to be readily degraded by bacteria during expression. We have substituted in H ferritin one of the residues responsable for dimer formation (Leu85) by Cys. When Cys85 is modified by chemical reaction reassembly of the chain is prevented, and the peptide remains soluble. Studies are in progress, in collaboration with the Sheffield's group to study the conformaiton of the peptide.

Ferritin as an epitope carrier
An allergenic peptide of 90 amino acids was genetically fused to the C-terminus of H ferritin. The construct is efficiently expressed in bacteria in a soluble form and is easy to purify. Preliminary data show that the chimeric product is recognized by IgE of allergic subjects.

Roma

Most of the work in the group has concentrated on the characterization of the loop regions of Rop and ferritin in order to learn how to modify them without disturbing the general conformation of the two molecules.

Influence of the amino acid sequence of the ferritin D-E loop on folding.
We have isolated and characterized a number of mutants in which the amino acid sequence of the peptide that connects helix D and helix E in the H chain of human ferritin has been randomized. Our results indicate that, although no single loop residue is absolutely required for ferritin to attain the native conformation, most of the mutants which we have obtained by random regional mutagenesis affect its folding/assembly process. Mutants that have positively charged residues at position 159, 160 or 161 fail to assemble into the native protein shell and form an insoluble aggregate. Interestingly, some loop amino acid sequences cause the E-helix to reverse direction and to expose its COOH terminal, normally hidden inside the protein cavity, to the solvent. This conformation (Flop) is favoured by the absence of Gly at position 159 and 164.

Linking an easily selectable phenotype to the folding of a common structural motif: Selection of rare turn mutations that prevent the folding of Rop
We have asked whether the Rop bend region contains any important folding information. We have extensively mutated three residues that form the connection between the two α-helices of the Rop protein. The characterization of a collection of random mutants indicated that this structure is rather insensitive to amino acid substitutions and that the Rop native structure tolerates most amino acid types in these positions. In order to identify the rare amino acid sequences that would prevent Rop from folding and/or dimerizing, we have exploited the observation that Rop can functionally substitute the dimerization domain of the λ repressor. In fact, plasmids expressing a hybrid protein, formed by the amino terminal domain of the λ repressor covalently linked to Rop, confer immunity to λ infection on their hosts. We have shown that this property depends on the ability of the Rop moiety to fold and dimerize. The

analysis of approximately 1000 Rop mutants containing random amino acid sequences at position 30, 31 and 32 allowed us to identify 3 mutant Rop proteins that are not able to dimerize, probably as a consequence of their inability to fold properly. In these mutants, the two helices are connected by the tripeptides VED, VPD and YPD in place of the wild type DAD (positions 30, 31 and 32).

Engineering a highly immunogenic protein by genetic grafting of the interleukin 1β peptide VQGEESNDK onto the ferritin structure.

The conclusions described above have taught us a small number of rules that have to be followed to modify the loop connecting helix D and E in ferritin without disturbing its the folding and assembly. This expertise allowed us to construct a number of hybrid proteins in which specific peptides are inserted in this region. Particularly interesting is the case of the oligopeptide VQGEESNDK that corresponds to an exposed region of the protein interleukin 1 β.This oligopeptide was chosen because a group at Sclavo (Siena) had previously shown that the isolated peptide maintains the immunostimulatory properties of the native interleukin 1 β molecule. In collaboration with the group in Siena we wanted to test whether it was possible to increase the immunogenicity of any protein antigen by grafting the peptide onto its structure. We have constructed two hybrid proteins in which the VQGEESNDK peptide was inserted either between amino acid Pro161 and Ser162 in the D-E loop or at the carboxy-terminus of the ferritin protein. In the first construction the oligopeptide is inserted in a region exposed to the solvent while the second protein segregates the inserted oligopeptide in the inside of the protein cage. Both hybrid peptides fold and assemble correctly into multimeric proteins whose structure is very similar to wild type. As predicted by the different localizations of the oligopeptide, the first protein is recognized, in the native form, by an antibody specific for the VQGEESNDK peptide, while the second is not. When the hybrid protein containing the peptide inserted into the loop is injected into mice, the anti-ferritin immune response is increased up to levels comparable to what is obtained by coinjection of wild type ferritin and native interleukin 1 β . No comparable results are obtained when injecting the carboxy-terminally extended ferritin, suggesting that the immunostimulatory function of the oligopeptide requires its exposure to the solvent on the exterior surface of the ferritin assembly.

Sheffield

Engineering of crystal contacts and new ferritin structures

Early in 1991 we published, with the groups from Milano and Roma, (Lawson et al., Nature **349**, 541-544 (1991) the account of the engineering of metal sites in human H ferritin in order to achieve crystallization, and the resulting discovery of the ferroxidase site in ferritin. This work was done under the auspices of the EC Biotechnology Action Programme. A number of site-directed variants have now been investigated crystallographically. In addition, we have recently engineered crystal contacts and solved the structure of the ferritin from *Schistosoma mansonii*. The accompanying table (figure S1) summarizes the ferritin structures and variants so far solved in Sheffield.

Heraklion

A thorough understanding of the structural principles of 4-α-helical bundle proteins is required, before novel proteins which use this tertiary motif as a structural scaffold can be designed and engineered in a rational fashion. The investigation of the relationship between sequence, structure and stability by a combination of crystallographic and computational approaches is the goal of our part in the collaborative project. The specific aims this year were:

1) Crystallographic analysis of specifically designed ROP mutants and attempts to elucidate relationships between the observed changes in structure and stability.

2) Analysis of sequence/topology relationships for the 4-α-helix bundle motif in proteins using all available sequence and structural data.

Refinement of crystal structures of ROP cavity mutants

A number of ROP mutants with altered hydrophobic cores are now at the final stage of crystallographic refinement at very high resolution. Further (non-isomorphous) mutants with altered loop regions are being analysed. The major results of the crystallographic analysis are:

1) The folding of the ROP bundle is determined mainly by hydrophobic core side chain interactions and is relatively insensitive to point mutations in the core, or to even drastic changes in the loop region: the topology of the bundle and the basic features of core packing remain basically unaltered.

2) The loss of stability of the ROP mutants is usually associated with an increased accessibility of the hydrophobic core. When a cavity is generated in the core by a mutation, the decrease of stability is correlated with the size of the cavity. For equally sized cavities, the loss of activity is correlated with the loss of packing efficiency in the core.

Sequence/topology relationships of 4-α-helical bundle proteins

Using sequence and structural information from seven families of 4-α-helical bundle proteins we derived relationships between the architecture of the 4-α-helix bundle and the sequences which are compatible with it. Three types of analyses were carried out:

1) Analysis in terms of amino acid distributions in the basic building unit of the 4-α-helix bundle, a repeat of seven residues *a,b,c,d,e,f,g* (heptad).

2) Analysis of the amino acid distributions along the axis of the bundle.

3) Analysis of short loop topologies.

The analysis of the amino acid distributions in the heptad positions yielded a highly motif-specific pattern. The results have been incorporated in a program for the evaluation of protein sequences of unknown structures (Figure H1) and can be used for structure prediction. The axial distribution of amino acids in the bundle (Figure H2) also exhibits a characteristic and motif-specific pattern. The statistics from both analyses can be used in the design and engineering of 4-α-helical bundle proteins. The conformations of the short interconnecting loops in 4-α-helix bundles have been also analysed and have been found to be atypical, different from those of classic β-turns. The determination of specific structure/sequence relationships has predictive power and can be used in the rational design of proteins.

Muenster

Quantification of the forces responsible for protein stability and folding of ROP and some of its mutants. ROP is predominantly stabilized by inter-subunit interactions.

Differential scanning calorimetry (DSC) studies in the presence and absence of the strong denaturing agent guanidinium hydrochloride (GuHCl) showed that the intrahelical forces are of minor significance for the overall stability of the protein. The four-helix bundle structure is mainly stabilized by the short range van der Waals interactions resulting from optimal packing of the hydrophobic core residues. Both the highly cooperative, mono-exponential unfolding and the occurrence of hysteresis in equilibrium refolding of native ROP can be rationalized on the basis of the assumption that maximal intersubunit interaction must occur. This interpretation is corroborated by the results obtained with two cavity mutations Leu^{41}Val and Leu^{48}Val. In both mutations the exchange of leucine by the smaller residue valine leads to a decrease in stability indicated in the reduced Standard Gibbs free energy change (ΔG^{o}). More interestingly, the energetic interactions reflected in the enthalpy change (ΔH) are drastically perturbed by this seemingly minor exchange.

The present studies reveal another significant aspect of exchange strategies. The proper location of exchanges is of utmost importance due to the delicate balance of stabilizing and destabilizing forces. Cavity sizes and their relative position play a decisive role by influencing the interactions existing in the native state. This conclusion is illustrated by a comparison of unfolding isotherms of Leu^{41}Val and Leu^{48}Val mutants shown in Fig. MU1. The Leu^{41}Val

mutant exhibits higher resistance to GuHCl unfolding than the $Leu^{48}Val$ mutant, in agreement with the results of the DSC studies. These findings are completely consistent with accessible surface area calculations by M. Kokkinidis.

Braunschweig

Gene planning, synthesis, expression and protein purification of redesigned Rop variants

The group has improved several techniques in the protein engineering cycle of Rop protein. Complete gene synthesis was performed for a new topologically redesigned Rop monomer, using overlapping cassettes designed using the software GENMON developed by the group. Several Rop mutants were produced, including:

- LmRop1 (left handed topologically reconnected Rop monomer): expression in pEX70, improvement of direct purification by optimized FPLC protocol, reducing production time from three weeks to 4 days. About 100mg of pure protein (>98%) were produced for structural studies
- LmRop2 (point mutant of LmRop1): expression as fusion protein with maltose binding protein. Fusion construct is produced as 50-70% of total cell protein. Efficient purification on affinity column, followed by sequence specific protease cleavage. Production cycle reduced to 3 days for 5-10 mg of pure protein (>98%). Total of about 15-20 mg protein has led to crystals suitable for crystallography.
- DcRop (designed to bind copper): fusion constructs have led to the first purifcation in significant amounts of this somewhat unstable variant.

Constructs of Rop with inserted functional loops have been sequenced and protein expression is in progress.

The improvements in expression and purification methods at the GBF Braunschweig were a crucial element in the successful solution in Heidelberg of the first crystal structure of a topologically reegineered protein.

Heidelberg

Reengineering protein topology: LmRop1 and LmRop2

Wild type Rop protein is a homodimeric four-helix bundle, both in the crystal and in solution, with two antiparallel helices in each subunit. In a cut-and-link protein design experiment, we have rearranged the topology of loops connecting the four helices. One loop was deleted, and two new loops and a C-terminal extension were added. The amino acid sequence in α-helical segments was kept, except for one point mutation. The designed LmRop gene codes for 120 residues, almost twice the length of the wild type. The synthetic gene was overexpressed in *E. coli* and the protein purified. Interhelical distance constraints from 2-D nuclear magnetic resonance (NMR) spectroscopy and unit cell dimensions of crystals prove that the packing of the four helices of LmRop protein is the same as in the wild type protein, in spite of the loop rearrangements. This result is consistent with the 'core hypothesis' which asserts that the dominant factor determining the native protein fold is the interaction of amino acid residues in the structural core. The recognition that topological reconnections can be tolerated without affecting the core structure of a globular protein may open up new degrees of freedom in the design of useful proteins. Solution of the crystal structure of LmRop2, the more crystallizable of two variants, is in progress.

Design of core and loop mutants of Rop dimer and monomer

Several designs were made which (1) compensate for holes in the core of Rop (2) add functionally interesting loops. Synthesis and protein purification is currently in progress at the GBF Braunschweig.

Software development
Several software tools were improved for the design of new protein variants. (1) WHATIF (G. Vriend), a comprehensive protein modelling and drug design system. (2) MaxSprout and TORSO (L.Holm) for automatic model building and model optimization. (3) MaxHom (R.Schneider) for more selective and improved database searches for sequence similarities taking into account evolutionary information.

HIGHLIGHTS/MILESTONES

Engineering crystal contacts: the first successful design of a metal binding site that helps proteins (ferritin) crystallize. The technique holds some promise of removing one of the major bottlenecks of protein structure determination in some cases.
Reengineering protein topology: first successful topological reengineering of a protein (Rop) that goes beyond cyclic shift of chain ends. If this can be generalized, protein engineers have considerably more latitude in designing protein structures than previously thought.
Ferritin as an epitope carrier: engineering of a highly immunogenic protein by genetic grafting of a peptide, i.e. a protein fragment, from the medically important cytokine interleukin 1β onto a protein (ferritin) that assembles into higher order structures. This type of construction may be useful in the development of new vaccines.
Invention of a genetic selection system for folding mutants: engineering of a hybrid between Rop protein and the DNA binding domain of a genetic switch element (the λ repressor), such that ROP acts like the activating principle (dimerization domain) of the genetic switch. Cells that have a well folded Rop grow better than those which do not. In this way, the immense power of evolutionary selection is put at the disposal of the protein engineer.

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. New functional pieces were introduced into the natural protein frameworks. One of them helps the proteins form regular crystalline arrays. Another is a medically important activator of the immune system which can thus be repackaged in possibly useful ways in the attempt to develop new vaccines. A third design is an unexpected rearrangement of the threading of the protein chain which nonetheless spontaneously forms the correct three-dimensional shape. A fourth design demonstrates how the immense amplification power of natural selection can be put at the disposal of the protein engineer: a genetic switch element was coupled to a protein fragment such that only correctly assembled protein fragments will allow cells to grow, amplifying and selecting out the 'best' molecules. In this way, basic contributions have been made to the emerging discipline of biomolecular engineering - engineering at a scale 100 time smaller than the smallest elements of microchips - that will have both medical and technological impact in the years to come.

COOPERATIVE ACTIVITIES

Dr Rovida from Milano visited Iraklion for an ECE course on protein modelling in September 1991.The Milano group visited Sheffield in March 1992. Milano received plasmids from Roma. Milano provided protein samples and plasmids to Sheffield.Sheffield received from Roma cells of ferritins with an interleukin peptide inserted in the DE loop, other DE changes and deletions at the N and C termini. Amyra Treffry's group in Sheffield is using an expression system from Roam to engineer changes in the ferroxidase centre. Heraklion exchanged with

Roma and Regensburg/Münster purified protein, structural and sequence data. Heraklion exchanged with Sheffield coordinates used in the analysis of sequence/topology relationships. Heidelberg supplied computer models and structural redesign specifications to Braunschweig. Braunschweig supplied purified protein to Heidelberg. Heidelberg supplied purified protein to Regensburg/Muenster. All groups exchanged information and results and had an intensive discussion and planning meeting in Braunschweig in September of 1991.

LIST OF SELECTED JOINT PUBLICATIONS WITH TRANS-NATIONAL AUTHORSHIP

Lawson, D.M., Artymiuk, P.J., Yewdall, S.J., Livingstone, J.C., Treffry, A., Luzzago, A., Levi, S., Arosio, P., Cesareni, G., Thomas, C.D., Shaw, W. and Harrison, P.M. (1991) Genetic engineering of crystal contacts leads to the structure solution of human H ferritin. Nature, 349:541-544.

Wade, V.J., Levi, S., Arosio, P., Treffry, A., Harrison, P.M. and Mann, S. (1991) Influence of site-directed modifications on the formation of iron cores in ferritin. J. Mol. Biol. 221: 1443-1452.

Santambrogio, P., Levi, S., Arosio, P., Palagi, L., Vecchio, G., Lawson, D.M., Yewdall, S.J., Artymiuk, P.J., Harrison, P.M., Jappelli, R. and Cesareni, G. (--) Evidence that a salt bridge in the L chain contributes to the physical stability difference between H and L human ferritins. Submitted.

Levi, S., Santambrogio, P., Cozzi, A., Rovida, E., Alvertini, A., Yewdall, S.J., Harrison, P.M., Arosio, P. (1992) Evidence that H and L ferritins have co-operative roles in the iron uptake mechanism of human ferritin. Submitted.

Paliakasis, C.D. and Kokkinidis, M. (1991). The stability of the four-α-helix bundle motif. Prot. Engineering 4: 849-850.

Paliakasis, C.D. and Kokkinidis, M. (1992) Sequence/topology relationships for the 4-α-helix bundle motif in proteins. Submitted.

S. C. Emery, M. Sagermann, W. Eberle, W. Klaus, H. Blöcker, P. Roesch, D.Tsernoglou & C. Sander (1992) Reengineering protein topology. Submitted.

C.Sander and G.Vriend eds. (1992) *Protein Design on Computers. Five new proteins: Shpilka, Grendel, Fingerclasp, Leather and Aida.* Proteins 12, 105-110.

Figures

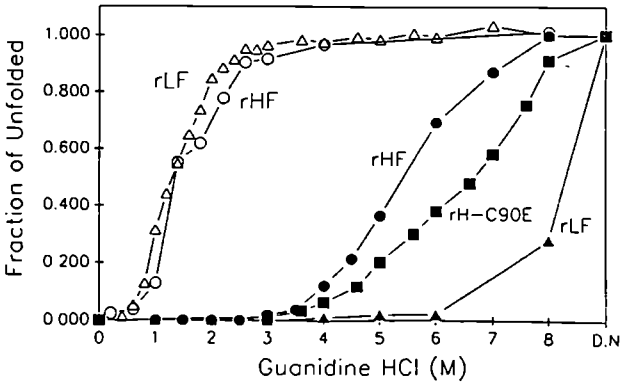


Figure M:Ferritin denaturation/renaturation - Milano
Denaturation (solid symbols) and renaturation (empty symbols) of ferritin homopolymers of L ferritin (rLF) H ferritin (rHF) and of the H ferritin variant Cys90->Glu (C90E). The proteins differ in their denaturation profiles, while renaturation is similar

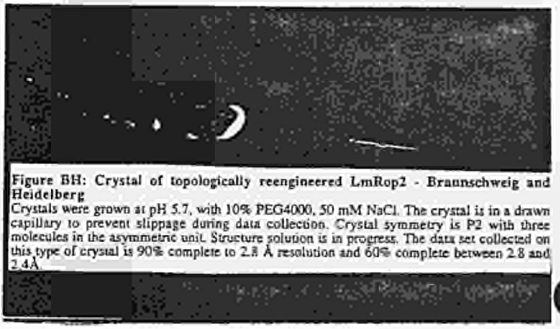


Figure BH: Crystal of topologically reengineered LmRop2 - Braunschweig and Heidelberg
Crystals were grown at pH 5.7, with 10% PEG4000, 50 mM NaCl. The crystal is in a drawn capillary to prevent slippage during data collection. Crystal symmetry is P2 with three molecules in the asymmetric unit. Structure solution is in progress. The data set collected on this type of crystal is 90% complete to 2.8 Å resolution and 60% complete between 2.8 and 2.4Å

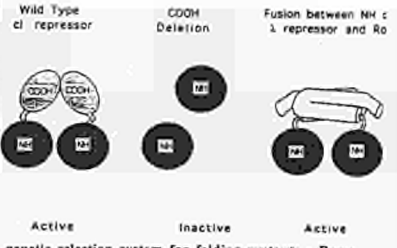


Figure C: Design of a genetic selection system for folding mutants - Roma
Schematic representation of the experiment that demonstrates that ROp can functionally replac the C-terminus of the lambda repressor, a genetic switch element. On this basis, a selectio system was constructed that rejects incorrectly folded proteins.

Figure S: Progress of X-ray analyses of ferritin - Sheffield

Ferritin class	Ferritin type	Mutation & Comments (H-chain numbers)	Crystal size/mm	Resolution of data (Å)	Refined R-factor (%)
Native (heteropolymers)	Rat liver	Wildtype (66%L,34%H)	0.40	2.3	21.0
	Horse spleen	Wildtype (85%L,15%H)	0.50	2.6	23.1
Recombinant H	Human CdM	CdM = K86Q	0.50	1.9	18.9
	" 173	CdM + E134H	0.25	2.4	17.7
	" 174	CdM + D131H	0.30	2.2	17.9
	" 175	CdM + D131H+E134H	0.30	2.7	19.2
	" 203	CdM + ATDKN(121-5) GSART	<0.10	Crystals too small at present	
	" 204	CdM + E134A	0.35	2.3	18.2
	" 206/GC	CdM D131A+E131A	0.42	1.9	In Progress
	" 222	CdM + E62R+H65G	~0.05	Crystals too small at present	
	" 223	CdM + V110I,	0.30	Poor diffraction	
Recombinant L	Rat rLF	R5G (increased solubility)	0.40	2.3	19.5
	Rat rLF + del	-Bres.loop: Q162PAQTGVA	0.05	Crystals too small at present	
Schistosoma	Sch.mansoni	S86Q (crystal contact)	0.40	2.9	32.0

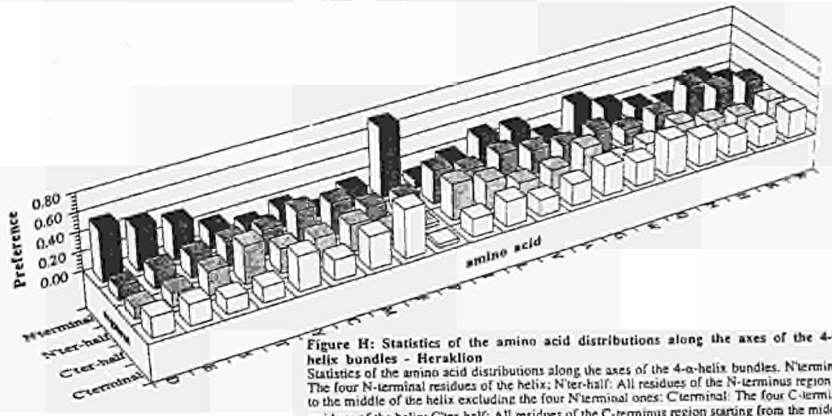


Figure H: Statistics of the amino acid distributions along the axes of the 4-α-helix bundles - Heraklion
Statistics of the amino acid distributions along the axes of the 4-α-helix bundles. N'terminal: The four N-terminal residues of the helix; N'ter-half: All residues of the N-terminus region up to the middle of the helix excluding the four N'terminal ones; C'terminal: The four C-terminal residues of the helix; C'ter-half: All residues of the C-terminus region starting from the middle of the helix excluding the four C'terminal ones. The statistics were obtained from different families of 4-α-helix bundle proteins (ROP not included).

Figure HD: Crystallographic electron density of LmRop2 - Heidelberg
Stereo view of preliminary electron density of one of the three LmRop2 helical bundles in the asymmetric unit. Residues 52-56 at one end of a helix. Map is $2f_O$-f_C, contoured at 1.2 σ, with data to 2.5Å resolution. At the left middle density for a Tyrosine side chain is clearly visible. THe electron density is truncated at the right for display purposes.

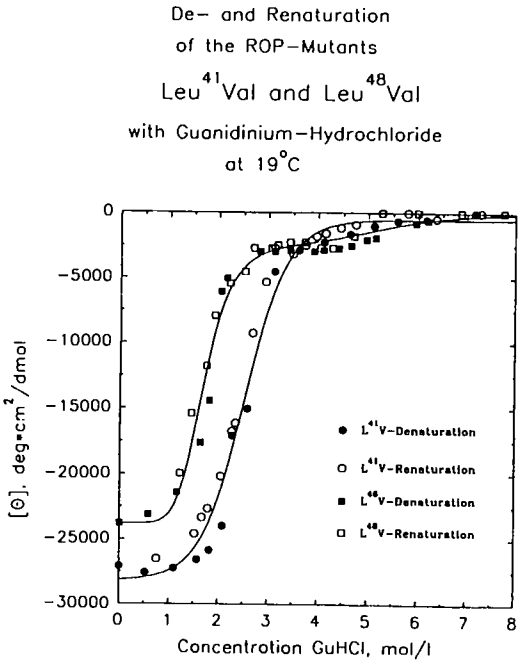


Figure MU: Comparison of unfolding isotherms of Leu41Val and Leu48Val mutants - Münster

TITLE:

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

CONTRACT NUMBER: BIOT - CT90 - 0176 (TSTS)

OFFICIAL STARTING DATE: 1 / 2 / 1991

COORDINATOR: F.N. Kolisis, National Hellenic Research Foundation, Athens, GREECE.

PARTICIPANTS:

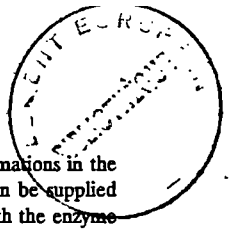
F.N. Kolisis, Biosystems Technology Lab, Chem. Engineer.Dept., NTUA and Institute of Biological Research and Biotechnology, NHRF, Athens, GREECE.
T. Scheper, Institut fuer Technische Chemie, Hannover Univ., Hannover, GERMANY
A. Ballesteros, Instituto de Catalysis, CSIC, Madrid, SPAIN.
D. Combes, Dep. de Genie Biochimique et Alimentaire, INSA, Toulouse, FRANCE
U. Menge, Gesellschaft fur Biotechnologische Forschung mbh, Braunscweig, GERMANY

OBJECTIVES SET FOR THE REPORTING PERIOD:

Our objectives for the reporting period are (a) Studies on the enzymatic acylation of natural glycosides in organic solvents and on the behavior of enzymes given by different participants in microemulsions, (b) Installation of on-line analysis and comparative studies of various esterification reactions in aqueous systems, liquid membranes, organic solvents and supercritical CO_2, (c) Study of native enzymes in hydrolytic and synthetic reactions of sugars and lipids and preparation of "new" enzymes by immobilization and chemical modification, (d) Study of model reactions in different solvents and screening of reactions and enzymes, and (e) Development of screening methods, production of raw materials and development of purification processes for new enzymes.

MAJOR PROBLEMS ENCOUNTERED:

Problems arised with the experimental setup of the supercritical CO_2 reaction unit. The knowledge/equipment of European industries in this area is low.



RESULTS:

Lipases, oxidases and dehydrogenases, enzymes which can be useful for biotransformations in the new media are prepared by the GBF group. At the moment the other partners can be supplied with lipases from two bacteria and three fungi: *Staphylococcus carnosus* (cloned with the enzyme from *Staphylococcus hyicus*), *Pseudomonas spec.*, *Penicillium simplicissimum* and *notatum*, *Rhizopus arrhizus*, partially purified or even in an homogeneous state. The purification of lipases to homogeneity suffers from their unusual strong hydrophobic interaction, which reduce the overall yield to 10-20%. Significant amounts of lipases are, e.g., lost using ultrafiltration necessary for final concentration and buffer exchange. A *Geotrichum candidum* lipase has been purified by ion-exchange, hydrophobic interaction and gel filtration chromatography. 42 mg of pure lipase have been produced. Another lipase produced by GBF lab and distributed to the other partners was from *Penicillium simplicissimum*. This lipase was concentrated from crude fermentation broth by ultrafiltration and precipitation with ammonium sulfate. The redissolved precipitated was bound to Phenyl Sepharose and eluted with octyl-β-D-glucopyranoside. 40 mg of pure lipase have been obtained with this procedure. Other methods used for the purification of this lipase was of aqueous two-phase system as well as the solubilization of the enzyme with various organic additives. A third lipase purified was from *Pseudomonas cepacia*. A screening for new lipases has also been started by GBF lab. 23 strains of bacteria, 5 from yeast and 18 from filamentous fungi showed hydrolytic activity. A considerably high activity of 12 units/ml was produced by filamentous fungi. Some of these strains have been identified and a preliminary characterization of these new presumably new lipases has been started.

A number of the above mentioned lipases have been tested in synthetic reactions in microemulsions (Athens lab). Esterifications of various aliphatic alcohols (hexanol, cycloexanol, cholesterol, etc) with natural fatty acids (oleic, lauric, myristic, caprylic, butyric, etc) have been performed and different parameters affecting the enzyme activity were studied. Preliminary results shows that 1,3 regio specific lipases better catalyzes the esterification of long chain fatty acids, while non-specific lipases can catalyze the esterification of secondary alcohols with fatty acids. Transesterification between ethyl butyrate and glycerol using native, chemical modified and immobilized lipase in a two phase system was also investigated (Madrid lab). Optimal conversion was obtained in the presence of 5% water. The selective hydrolysis of peracylated sucrose by several lipases in water, two-phases system and AOT-microemulsions has been studied by the same lab. The synthetic activity of lipase was further studied in supercritical CO_2 (Toulouse lab). The enzyme (Lypozyme from *Mucor miehei*) appears quite stable in a range of pressure from 13 to 18 MPa, showing a similar behavior as in n-hexane. In addition the first complete kinetic study of enzyme reaction in $SCCO_2$ in comparison with an organic solvent has been undertaken. This study was based on the determination of the kinetic constants of the reaction, related to a mechanism (Bi Bi ping pong), suggested by a very good correlation of calculated kinetics curves with experimental data. A comparative study of hydrolysis, esterification and transesterification by lipase in organic solvents and supercritical CO_2 was carried out (Hannover lab) in an attempt to present the best method for enantiomerically pure substances taking into account the advantages of the system used. Also a biosensor for determining enantiomeric excesses and an enantiomeric analysis via gas chromatography for different chiral compounds have been developed by this laboratory.

Apart of lipases, proteases have also been tested in the non-conventional media systems. Esterification of various natural glycosides by subtilisin in organic solvents have been performed (Athens lab). The various parameters affecting the system as well as the reaction mechanism have been studied. It was found that enzymatic acylation of rutin with fatty acids in organic solvents

resulted in a significant change of its cytological properties.

HIGHLIGHTS/MILESTONES:

- Influence of surfactant nature and organic solvent hydrophobicity on lipase specificity
- Influence of reaction phase composition (additives, cosolvents) on enantioselectivity.
- Influence of reaction conditions (temperature, stirrer speed, reactor design) on productivity.
- enantiomeric separation strategies for a GC process monitoring.
- on-line determination of enantiomeric excesses of amino acids in different solvent systems with the use of an enzyme thermistor as biosensor.
- Enzyme stability in supercritical CO_2.
- Influence of water on enzyme activity.
- Enzyme reaction mechanism in supercritical CO_2.
- Production and purification of new lipases.

WIDER CONSIDERATIONS:

It has been noticed that a better transfer to industrial partners is necessary . Thus, the participation in the COMETT-program was initiated.

COOPERATIVE ACTIVITIES:

Exchange of materials: Purified microbial lipases have been send from GBF lab to NHRF lab and to Hannover lab.

Immobilized lipase has been send from SCIC lab to NHRF lab for studies of various esterification reactions.

Exchange of staff: One member of Hannover lab visited GBF lab for 6 weeks for the purification of a microbial lipase.

One member of Hannover lab visited NHRF lab for 2 weeks as assistance in the experimental set up of their supercritical CO_2 reaction unit.

Joint experiments: INSA lab with Hannover lab for the set up of the supercritical CO_2 unit of the latter.

Hannover lab with Prof. W.A Koning (Hamburg Univ.), Prof. H.H. Meyer (Hannover Univ.) and Prof. F. Schubert (Berlin Univ.) for the developing of the analysis of chiral compounds and chiral biosensor.

Hannover lab with Merck for exchange of know-how and chemicals (about 2000 DM).

Joint meetings: Meeting of all partners in Athens, 25 October 1991.

JOINT PUBLICATIONS:

1. A. Capewell, U. Bornscheuer, T. Scheper, H.H. Meyer and F.N. Kolisis (1992). A comparison of enzymatic reactions in aqueous, organic and supercritical phases. *Ann. N.Y. Acad. Sci.* in press

2. T. Scheper, U. Bornscheuer, A. Capewell, A. Herar, H.G. Hundeck, H.H. Meyer, F.N. Kolisis and F. Schubert (1992). Studien zu enzymatischen reaktionen in wassrigen, organischen und uberrkritischen phasen. DECHEMA - Conference, Karlsruhe, 1 - 3 June.

3. T. Scheper, U. Bornscheuer, A. Capewell, A. Herar, H.H. Meyer, H.G. Hundeck, F.N. Kolisis and F. Schubert (1992). Studies of enzymatic reactions in aqueous, organic and supercritical media. Fundamentals of Biocatalysis in Non-conventional media, Noordwijkerhout, 26-29 April.

4. H. Stamatis, A. Xenakis, F. N. Kolisis, H. Sctajer and U. Menge (1992).Studies on the specificity of lipases catalyzed esterification reactions in microemulsions. Fundamentals of Biocatalysis in Non-conventional media, Noordwijkerhout, 26-29 April.

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

OTHER PUBLICATIONS:

1. T. Valis, A.Xenakis nn F.N. Kolisis, (1991). Comparative studies of Lipase from *Rhizopus delemar* in various microemulsion systems, *Biocatalysis*, in press

2. A. Xenakis, T. Valis and F.N. Kolisis, (1991). Microemulsions as a tool for enzymatic studies.The case of Lipase. *Progr. Colloid Polym. Sci.*,84, 508 - 511.

3. G. Kodelis and F.N. Kolisis (1992). Studies on the reaction catalysed by subtilisin for the acylation of flavonoids with fatty acids in organic solvents. *Ann. N.Y. Acad. Sci.* in press

4. U. Bornscheuer, S. Schapohler, T. Scheper and K. Schugerl (1991). Influences of reaction conditions on the enantioselective transesterification using *Pseudomonas cepacia* lipase. *Tetrahedron Asymmetry* 2(10), 1011-4.

5. U. Bornscheuer, S. Schapohler, T. Scheper, W.A. Konig and K. Schugerl. Application of enantioselective capillary gas chromatography in lipase catalyzed transesterification reactions in organic media. *J. of Chromatography*, in publication process.

6. M.A Cruces, C. Otero, M. Bernabe, M. Martin-Lomas and A. Ballesteros (1992). Enzymatic preparation of acylated sucroses. *Annals N.Y. Acad. Sci.*, in press.

7. M. L. Rua, T. Diaz-Maurino, C. Otero and A. Ballesteros (1992). Isoenzymes of lipase from *Candida cylindracea*. Structural studies on the carbohydrate chains. *Annals N.Y. Acad. Sci.*, in press.

8. E. Pastor, C. Otero and A. Ballesteros (1992). Hidrolisis de grasas catalizada por lipasa immobilizada sobre soportes siliceos. Proc. XIII Simposio Iberoamericano de Catalisis, S. Mendioroso (ed), in press.

9. F.J. Plou, C. Otero and A. Ballesteros (1992). Preparacion de mejores biocatalizadores por modificacion quimica. Proc. XIII Simposio Iberoamericano de Catalisis, S. Mendioroz (ed), in press.

10. A. Marty, W. Chulalaksananukul, R.M. Willemot and J.S. Condoret (1992). Kinetics of the lipase-catalyzed esterification in supercritical CO_2. *Biotechnol. Bioeng.*,39, 273-279.

11. W. Chulalaksananukul, J.S. Condoret and D. Combes (1992). Kinetics of geranyl acetate synthesis by lipase catalyzed transesterification in n-hexane. *Enzyme Microb. Tech.*, in press.

12. J.S. Condoret, A. Marty and D. Combes (1991). Etude cinetique de catalyse enzymatique en fluide supercritique. Colloque Bioconversion et Synthese Organique, Grasse. France, 22-24 Mai.

13. D. Combes, W. Chulalaksananukul, A. Marty and J.S. Condoret (1991). Comparison of immobilized lipases behavior in supercritical CO_2 and n-hexane. RSC conference: Lipases: structure function and application in biotransformations. University of Warwick, Coventry, U.K., July 16-18.

14. A. Marty, V. Moreau, D. Combes and J.S Condoret (1991). Production en continu d'esters d'acide gras par biocatalyse dans du dioxyde de carbone supercritique. 2eme Colloque sur les Fluides Supercritiques, Paris, France, 16-17 October.

TITLE:	**Characterization and surface properties of semi-synthetic redox enzymes for application in biosensor devices**
CONTRACT NUMBER:	**BIOT-CT91-0279 (RZJE)**
OFFICIAL STARTING DATE:	**1-October 1991 (effectivily 1 January 1992)**
COORDINATOR:	**Dipl.-Ing. A.F. Bückmann, Gesellschaft für Biotechnologische Forschung mbH (GBF),Braunschweig, Germany**
PARTICIPANTS:	**Prof. J.V. Bannister, Cranfield Institute of Technology (CIT), Bedfordshire, England** **Dr. C. van Dijk, Agrotechnological Research Institute (ATO), Wageningen, The Netherlands** **Dr. S.G. Mayhew, University College Dublin (UCD), Dublin, Ireland** **Prof. D. Cocco, University of Cagliari (UC), Cagliari, Italy** **Dr. J.E. Prenosil, Eidgenossische Technische Hochschule Zürich (ETH), Zürich, Switzerland** **Prof. G. Gottschalk, Wiss-Trans Umwelt GmbH (WT) Göttingen, Germany** **Dr. J. Higgings, Cranfield Biotechnology LTD (CBL), Bedfordshire, England** **Dr. S. Salden, Euro Diagnostics B.V. (ED), Apeldoorn, The Netherlands**
OBJECTIVES SET FOR THE REPORTING PERIOD:	**Working out of a method to purify FAD analogues, for example, N^6-(2-aminoethyl)-FAD by HPLC. Isolation, characterization, and amino acid sequencing of NADH oxidase from *Thermus Aquaticus*.**
MAJOR PROBLEMS ENCOUNTERED:	**Nothing to report**

RESULTS:	**(GBF): From a reaction mixture partially purified N^6-(2-aminoethyl)-FAD in mg amounts could be obtained. By working with a new HPLC column better results may be feasible.**
	(CIT): A new purification procedure giving a higher yield for NADH oxidase has been divised.Preliminary amino acid sequence of the first 8 amino acids has been obtained.
	(UC): Confirmation that the physico-chemical properties are identical to the previously purified NADH oxidase. Effects of pH, temperature, substrate specificity and kinetic parameters were all identical.
HIGHLIGHTS/MILESTONES:	**Nothing to report yet**
WIDER CONSIDERATIONS:	**Nothing to report yet**
COOPERATIVE ACTIVITIES:	**Nothing to report yet**
LIST OF JOINT PUBLICATIONS/PATENTS WITH TRANSNATIONAL AUTHORSHIP:	**Nothing to report yet**
OTHER PUBLICATIONS /PATENTS:	**Nothing to report yet**

New types of redox enzymes for the production of chiral synthons: basic research, functionalisation and application.

Contract Number: BIOT-CT90-0157

Official starting date: March 1st, 1991

Coordinator: H. Günther, Org. Chemie + Biochemie, TU München, Garching, FRG
Participants:
H. Simon/H. Günther, Org. Chemie + Biochemie, TU München, Garching, FRG
W. Somers, Netherlands Organization for Applied Sci. Res., Zeist, NL
J.A. Duine, Delft University of Technol., Fac. Chem. Technol. Delft, NL
I. Moura, Centro di Tecnologia Quimica e Biológica, Oeiras, PT

Objectives set for the working period: March 1st, 1991 to Febr. 29th, 1992

1. Synthesis of commercially not available mediators
3. Chemical stability test of new mediators
4. Supply of new viologen dependent enzymes and/or cells
5. Large scale production of anaerobes
6. Purification of viologen accepting oxidoreductases
7. Screen for new PQQ enzymes
8. PQQ enzyme purification
9. PQQ modification
10. Enzymology of PQQ holoenzymes
11. Electroactivity tests
13. Quantitative electrochemical measurements
17. EPR spectroscopy
18. NMR, EPR spectroscopy of modified PQQ enzymes
20. NMR spectroscopy
23. Mathematical modelling

Major problems encountered: nothing to report

Results:

For the reduction of prochiral compounds to chiral synthons or the selective dehydrogenation of one chiral form of a racemic mixture in a preparative scale, redox enzymes are useful catalysts if a low cost regeneration of pyridine nucleotides, if NAD(P) dependent enzymes are involved, is simply arrangeable. In a wide variety of aerobic and anaerobic microorganisms enzyme activities exist, which reversibly transfer electrons between artificial 1-electron mediators and pyridine nucleotides. The group in Munich coined for these enzymes the name VAPOR (Viologen accepting pyridine nucleotide oxido reductases). They are interesting in twice a respect:

i) As catalysts for pyridine nucleotide regeneration and
ii) because of mechanistic aspects, especially with the change between 1-electron and 2-electron transfer steps.

The group in Munich detected in crude extracts of *C. thermoaceticum* rather stable VAPORs of a specific activity between 3.5-8 $U*mg^{-1}$ protein for the

dehydrogenation or reduction of pyridine nucleotides. No significant decrease of activity in the crude extracts was detectable during aerobic storage for 7 days at -18° C.

The suitability of these VAPOR activities was tested with respect to pyridine nucleotide regeneration and preparative formation of chiral compounds. Experiments performed to separate the proteins carrying VAPOR activity and formate dehydrogenase (=FDH)-activity resulted in indistinguishable R_F-values for VAPOR- and FDH activity on two different types of gel-electrophoresis. Since FDH of *C. thermoaceticum* was already characterised by others, this may shed light on structure-function relationships, structures which accept single electrons and reduce substrates in a two electron transfer step.

Three commercially not available viologens were synthesised:

i) 1,1'-2-(1,3-dioxolane-2''-yl)ethyl-4,4'-bipyridinium salt embodies easily available aldehyde groups in the dioxolane moiety, possibly useful in immobilisation experiments,

ii) 1,1'-(p-phenylsulfonato)methyl-4,4'-bipyridinium salt, neutral at physiological pH in oxidised and negatively charged in reduced state.

iii) 1-CH_3-1'-[$HOOCCH_2$-(O-CH_2-CH_2)$_n$-O-CH_2CO-NH-$(CH_2)_3$]-bipyridinium salt, a "mono methylviologen" with a functionalised polyethylene glycol spacer.

Three ß-ketoester oxidoreductases, one from *C. kluyveri* and two from *C. tyrobutyricum* reducing ß-ketoesters to 3-hydroxy esters were purified to homogeneity and characterised, also an enzyme from *C. tyrobutyricum*, which reduces ß-keto acids. All reduce an acetoacetate moiety to [S]-3-hydroxy butyrates with ee-values better than 98%. The two enzymes of *C. tyrobutyricum* reducing the esters are NAD- the other enzymes NADP dependent.

In electrochemical cells the regeneration of NAD(P)(H) by VAPORs of *C. thermoaceticum* was demonstrated using the above mentioned ß-ketoacid-, ß-ketoester oxidoreductases, isocitrate dehydrogenase or glutamate dehydrogenase respectively. [R]- as well as [S]-isocitrate, [S]-glutamate and [R]-4-chloro-3-hydroxybutyrate, a synthon for [S]-carnitin synthesis were formed with productivity numbers all better than 10 000 with respect to cell suspensions of *C. thermoaceticum*.

The Delft Enzymology group worked on the characterisation of a quinohaemoprotein alcohol dehydrogenase (QH-EDH) from *Comamonas testosteroni* and further novel alcohol- and aldehyde oxidoreductases:

1.a) Purification of apo- and holo-QH-EDH.

Preparations of apo-QH-EDH that appear homogeneous on native gel-electrophoresis, show additional bands when subjected to SDS-PAGE. Analysis of the polypeptides was carried out. By making use of the finding that recombination of apo-QH-EDH with PQQ leads to reduced holo-QH-EDH, separation of active and inactive holoenzyme was possible. The purity and stability of apo- and holo-QH-EDH obtained by different procedures was investigated.

1.b) Cloning of the gene of QH-EDH from *Comamonas testosteroni*.

Partial amino acid-sequences were obtained for the 70 kD QH-EDH, as well as for the two major polypeptides that were found to accompany QH-EDH. Edman

degradation showed that the N-terminus of the major, 40 kD, polypeptide was identical to that of the QH-EDH. Synthetic oligo nucleotides complementary to the amino acid codons of the available polypeptide sequences were designed as probes to locate the QH-EDH gene. To reduce the degeneracy of the probes, use was made of the codon-usage established for a protein from *C. testosteroni* with known nucleotide sequence. Specific hybridisation has been observed for two restriction fragments of the *C. testosteroni* chromosome.

1.c) Enantioselective properties of QH-EDH.

Investigations of the enantioselective oxidation of racemic isopropylidenglycerol, solketal, have been conducted. Analysis of the enantioselective properties of QH-EDH by novel methods, showed that the selectivity is especially high (enantiomeric ratio 100) for the first step in which solketal is oxidised to the corresponding aldehyde. A low (enantiomeric ratio 3) and opposite specificity was measured for the second step, in which the aldehyde is oxidised to the carboxylic acid. Preliminary attempts at "cofactor engineering", by recombining apo-QH-EDH with PQQ-analogues- and derivatives, have shown that both the over-all activity as well as the enantioselective properties of the enzyme can be changed.

2.) Novel alcohol- and aldehyde oxidoreductases.

Gram-positive methanol-utilisers appear to have a unique set of enzymes when compared to their gram-negative counterparts. Thus, *Amylolatopis methanolica* appears to contain: two novel types of alcohol dehydrogenase with bound NAD and NADP, respectively, functioning as a cofactor; a formaldehyde dehydrogenase and a formate ester hydrolase.

The mechanistic aspects of these enzymes and their enantioselectivity are unknown. Work aimed at the complete characterisation of these enzymes is in progress.

The CTQB-group has studied enoate reductase and an alcohol dehydrogenase from Munich and Delft, respectively. UV/visible redox titrations of enoate reductase do not reveal an extensive formation of a blue semiquinone (absorbance at 580 nm very low). Redox potentials obtained from the absorbance at 450 nm (where all cofactors contribute) are presented in Table 1.

EPR spectra of the reduced enzyme show rhombic spectra with g-values at 2.013, 1.943 and 1.860. These spectral features are not observable at temperatures higher than 60 K. A flavin semiquinone radical is observable by EPR up to 100 K. EPR redox titrations were performed using dithionite or NADH as reductants. Samples poised at different redox potentials were observed at high and low temperatures. The intensity of the rhombic signal was measured as well as the intensity of the radical species in function of redox potential. Redox potentials of the flavin are presented in Table 1. The EPR characteristics can not be used to unambiguously conclude the type of iron-sulphur clusters of the protein (1 [4Fe-4S] or 2 [2Fe-2S]). The spin integration value obtained by EPR suggests the presence of a [4Fe-4S] cluster. The unusual low g-values of the iron-sulphur centre and the positive redox potential may indicate non-sulphur coordination.

Table 1.

	Fl_{ox}/Fl_{sq}	Fl_{sq}/Fl_{hq}	[Fe-S]	(*) redox process detected at 450 nm (contribution of all redox centers).
EPR-Dithionite	- 190 mV	- 350 mV	- 180 mV	
EPR-NADH	- 177 mV	n.d.	- 167 mV	
UV/Visible-Dithionite		- 178 mV(*)		
UV/Visible-NADH		- 167 mV(*)		

EPR spectra of apo- and holo- alcohol dehydrogenase from *C. testosteroni* obtained at low temperature showed a rhombic signal with g-values at 3.01, 2.28 and 1.37 typical of a low-spin heme. This signal observed from both forms of protein indicates a conserved coordination structure when PQQ is bound. A small broadening is observed on the apo-form. UV/visible redox titrations of apo- and holo- enzyme and the position of the Soret peak indicate non identical redox potentials (Table 2).

Table 2.

	Heme Redox Potential	Soret wavelength	
		oxidised	reduced
Apoenzyme	+ 80 mV	409.4 nm	416.0 nm
Holoenzyme	+ 140 mV	408.6 nm	414.4 nm

In ^{1}H-NMR spectra of the apoform of the enzyme four heme methyl groups and the methyl group from the axial methionine were identified proving the heme *c* as an Met-His axial type coordination.

By adding PQQ to the system (to generate the holoform) a large modification was observed especially in one of the heme methyl groups that felt a change of = 7 ppm. This, combined with the information of the shift of the Soret band and the difference of redox potentials in the heme, suggests that these two cofactors must be very close and in interaction.

Aim of the project at TNO/Zeist is the development of a preparative electrochemical cell. The bio-electrochemical conversion of glucose to gluconic acid by glucose oxidase was studied as a model system. Direct adsorption of the enzyme on carbon felts and carbon particles was achieved. Almost no biocatalytic activity was obtained when glucose oxidase was immobilised on porous carbon or carbon felts in phosphate buffer pH 7.0 (Table 3).

The immobilised enzyme activity could be enhanced by the addition of glucose (300 mM) to the immobilisation medium (see Table 3). On porous carbon the enzyme activity was retained to up to 20 $U*mg^{-1}$ protein.

Electrochemical cells with immobilised glucose oxidase were build to study the long term performance. Benzoquinone was used as the electron mediator. Typical turnover numbers of 2-3 μmol $glucose*min^{-1}*mg^{-1}$ protein were achieved, being only 1-2% of the total enzyme activity used for immobilisation. Furthermore a fast reduction in biocatalytic activity, reflected in a sharp reduction of the catalytic current in the electrochemical cell, is found in the initial phase of the conversion.

Another approach is now under development. Glucose oxidase is able to com-

Table 3. Immobilisation of glucose oxidase on carbon carriers (ca. 20 mg enzyme/g carrier).

type of carrier	addition of glucose during immob. (300 mM)	Activity (U/mg)
free enzyme	-	130
carbon felt	-	< 2
(carbon Lorraine)	+	< 2
porous carbon	-	< 2
(Norit Rox 0.8)	+	15-20
passive carbon	-	< 2
	+	< 2

municate directly with an electrode via a conducting polymer. The enzyme is adsorbed to electrodes consisting of a platinum coated membrane on which a layer, consisting of polpyrrole is applied. Thus the enzyme is in direct communication with the electrode via the conducting polymer. An advantage of this system is that there is no need for a mediator in the reaction medium. Experimental results show that 0.05 $U*cm^{-2}$ of enzyme activity is immobilised. Calculations reveal that the specific activity of the immobilised enzyme fraction is at least 10 $U*mg^{-1}$ but probably higher. At this moment a PQQ dependent alcohol dehydrogenase from *Pseudomonas testosteroni* is studied with respect to the immobilisation on and the communication with the conducting polymer matrix.

Highlights/Milestones:

The suitability of new redox enzymes for the enantioselective synthesis by enzymatically catalysed reduction or oxidation are demonstrated for a variety of compounds. One is a synthon for [S]-carnitin synthesis. Enzyme immobilisation experiments for mediator free electrochemical cells are on the way. Mechanistic aspects of the catalysed reactions were investigated.

Wider Considerations:

NAD(P) dependent oxidoreductases, which represent approx. 2/3 of the known oxidoreductases, can be used in preparative organic synthesis only if the regeneration of the pyridine nucleotides can be achieved because of their high prices. For reductive regeneration suitable processes already exist. Here the practical feasability of oxidative processes are demonstrated.

Cooperative activities:

Meeting of the group at TUM with J. Moura on the occasion of his lecture on March 8^{th} 1991 at Martinsried (MPI).
Visit of I. and J. Moura and J. Caldeira at Garching April $12^{th}/13^{th}$ 1991.
Meeting of all groups on June $21^{th}/22^{th}$ 1991 at Garching.
Visit of W. Somers and Mr. Eijsma from TNO in the 30^{th} week at Garching.
Visit of J. Caldeira from CTBQ in Delft from Sept. 22^{th} to Sept. 25^{th}.
Supply of *P. vulgaris* by the group at TUM to TNO (July 1991).
Supply of pure alcohol dehydrogenase by the group at Delft to CTQB.
Supply of pure enoate reductase by the group at TUM to CTQB.
Prescription for the synthesis of 4 viologens by the group at TUM to ATO.

TITLE:
Enantioselective biotechnical resolution of racemic epoxides in the production of optically pure epoxides.

CONTRACT NUMBER:
BIOT-CT91-0269 (TSTS)

OFFICIAL STARTING DATE:
01.06.1991

COORDINATOR:
Prof. J.A.M. de Bont Agricult. Univ. Wageningen The Netherlands

PARTICIPANTS:

Dr D.J. Leak	Imp. College	London	U.K.
Dr M.M.R. Fonseca	Inst. Sup. Técn.	Lisbon	Portugal
Dr V.H.M. Elferink	Andeno B.V.	Venlo	The Netherlands

OBJECTIVES SET FOR THE REPORTING PERIOD:
A stereoselective enzyme has been discovered in a Xanthobacter species which resolves cheap racemic epoxide mixtures to very valuable optically pure epoxides to be used in subsequent industrial chemical synthesis of optically pure bio-active 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 on basis of which the industrial partner or eventually others can decide on industrial application of the method.
For the reporting period the following specific objectives were set:
At the Agricultural University Wageningen optimization studies of Xanthobacter Py2 as well as screening for other epoxide-degrading organisms.
At the Imperial College purification and study also by ^{13}C-NMR of the epoxide-degrading enzyme from Xanthobacter Py2.
At the Instituto Superior Técnico development of analytical techniques, immobilization and aqueous phase work, and solvent screening.
Andeno B.V. was to advise on analytical techniques.

MAJOR PROBLEMS ENCOUNTERED:
The work at Imperial College started on 01.10.1991 rather than on 01.06.1991. For the three other partners the scheduled programme was followed. An alternative strategy for the Agricultural University has been worked out for the near future since the epoxide-degrading enzyme will also be studied via a molecular-genetics approach.

RESULTS:
Aspects 1-4 given below have been dealt with at the Agricultural University, aspects 1 and 3 have also been dealt with at Imperial College and aspects 5-7 have been completed at Instituto Superior Técnico.

(1) Screening programmes
Screening programmes for the isolation of other and possibly new epoxide-degrading organisms were set up in Wageningen and London and have now been completed. More than twenty organisms were isolated from water, soil and mud samples. Interestingly, from preliminary inspections it seemed all organisms contained an epoxide-degrading enzyme similar to the enzyme in Xanthobacter Py2. However, the screening programme set up at the Imperial College apart from the usual organisms also resulted in the isolation of

a Corynebacterium which was able to degrade cyclic epoxides.

(2) Induction of the epoxide-degrading enzyme

The expression of the epoxide-degrading enzyme in Xanthobacter Py2 was studied in chemostat culture. The organism was grown under propene-limited conditions and steady states were obtained at various dilution rates. The data obtained indicate the expression of the enzyme does not react to variations in growth rate. Subsequently, limitations other than propene will be tested.

(3) Cell-free extracts

The enzymology of epoxide degradation was studied in extracts of Xanthobacter Py2 grown on propene. It was demonstrated epoxides were degraded to ketones. The exact mechanism, however, remains not clear and two alternative routes were considered. Until now it has not been possible to purify the enzyme. It was recently observed the enzyme could be fractionated in a low molecular fraction and an inactive protein which, when combined, yielded an active epoxide-degrading preparation. This part of the work will be further pursued at Imperial College.

(4) Mutants of Xanthobacter Py2

Initial experiments were undertaken to obtain mutants of Xanthobacter Py2 unable to grow on epoxides. Such mutants are valuable in transformation experiments using a gene bank derived from the wild type Xanthobacter Py2 in order to eventually obtain the gene encoding for the epoxidedegrading enzyme.

(5) Cell growth

Xanthobacter Py2 cells were cultivated batchwise in mineral medium (from a pre-inoculum grown on yeast extract and glucose) to which 0,1% v/v of 1-pentene was added as an inducer of the epoxide degrading enzymatic system. Due to the low solubility of 1-pentene in the aqueous phase, two approaches were used, namely to inject 1-pentene through the rubber bungs of the conical flasks (i) as such and (ii) dissolved in a preselected biocompatible organic solvent.

(6) Reactional system

1,2-Epoxyhexane was chosen as a test average molecular weight substrate for the degradation studies because it is a commercially available epoxyalkane, even though the strain is only enantioselective towards the 2,3-epoxyalkanes. However, it has been established in Wageningen that if cells degrade non-selectively 1,2 epoxyalkanes, they will degrade 2,3-epoxyalkanes enantioselectively. Free cell activity was measured in aqueous-organic systems where the second phase was introduced to serve as reservoir for the epoxyalkane. Solvents used include n-hexane, n-heptane, n-octane, iso-octane and n-dodecane.

Although initial rates were solvent independent the highest final conversions were obtained in n-dodecane. In order to check whether the reaction was mass transfer limited, experiments were carried out at 200 rpm and 400 rpm. The results indicate that no liquid-liquid mass transfer problems exist at the lower shaking speed.

Cell re-utilization was tested in successive degradation experiments. Oxygenation of the degradation medium prolongs the degrading activity of cells.

No difference in cell activity could be detected in cells grown according to the two different protocols mentioned under (5).

(7) Cell immobilization

Cell immobilization was carried out during growth (in situ) and using pre-grown cells. In the first case cells were cultivated in the presence of pre-formed polyurethane foam cuboids. In the second case cells were harvested 48h after inoculation, separated from the fermentation broth by

centrifugation, resuspended and subsequently immobilized in k-carrageenan or added to polyurethane pre-polymer, so that they became entrapped in the resulting polyurethane foam.
The matrix which presented the best performance was by far the pre-formed polyurethane foam. The specific activity of these immobilized cells was in fact higher by a factor of 3 than that of the free cells. This is an indication that the physiological state of the cell at the time of immobilization may play an important role in the cell degrading capacity.

HIGHLIGHTS/MILESTONES:
Original achievements obtained were the demonstration of epoxide-degrading activity in cell extracts and the observation that activity of cells immobilized *in situ* was three times as high as of cells in suspension.

WIDER CONSIDERATIONS:
Nothing to report.

COOPERATIVE ACTIVITIES:
A researcher from the Instituto Superior Técnico, Lisbon worked during one month in Wageningen to get acquainted with specific aspects of *Xanthobacter* Py2, as for instance its cultivation on the gaseous compound propene.

A joint meeting was held on December 20th in Wageningen with the participation of Prof. M.M.R. Da Fonseca (Lisbon), Dr D.J. Leak (London), Dr V.H.M. Elferink (Venlo), Prof.Dr J.A.M. de Bont, Drs J. Swaving, M.M.J.A.-C.M. de Vaan and C.A.G.M. Weijers (Wageningen). During the meeting the ongoing activities were evaluated and the following future research programme was agreed upon.

Lisbon:
Continuation of the ongoing activities with an emphasis on:
- Methods to obtain higher cell densities.
- Re-utilization of epoxide-degrading cells.
- Immobilization techniques and optimization of the activity of immobilized cells.

London:
- Enzyme purification (follow-up of work at Wageningen).
- Characterization of the epoxide-degrading enzyme.
- Purification and determination of the low molecular fraction (together with Wageningen).
- ^{13}C-NMR work.
- Possibly extension of the work to other organisms.

Wageningen:
- Continuation of the work on optimization of *Xanthobacter* Py2 in chemostat culture.
- Purification of the low molecular weight fraction (together with London).
- Isolation of mutants.
- Genetics of epoxide degradation in *Xanthobacter* Py2.

LIST OF JOINT PUBLICATIONS/PATENTS WITH TRANS-NATIONAL AUTHORSHIP:
Nothing to report.

OTHER PUBLICATIONS/PATENTS:
Weijers, C.A.G.M. and Bont, J.A.M. de (1991).
Enantioselective degradation of 1,2-epoxyalkanes by *Nocardia* H8.
Enzyme Microb. Technol. 13, 306-308.

TITLE: *Glycosyltransferases from Streptomycetes as tools in biotransformations*

CONTRACT NUMBER: *BIOT - CT90 - 0155*

OFFICIAL STARTING DATE: *01. 3. 1991*

COORDINATOR: *W. Piepersberg, BUGHW FB9, Wuppertal, FRG*

PARTICIPANTS:

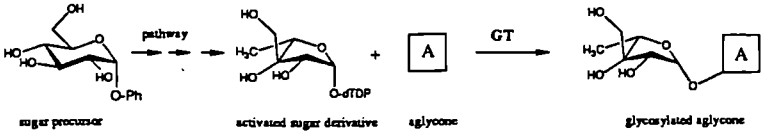
(1) A. Jiménez, Universidad Autonoma, Madrid, Spain

(2) E. Cundliffe, ULEIC, Leicester, GB

(3) S. Grabley, B. Bräu, Hoechst AG, Frankfurt a.M., FRG

OBJECTIVES SET FOR THE REPORTING PERIOD:

General goal of this project is to identify and develop microbial glycosyltransferases (GT) as tools for the modification of various aglycons via biotransformation:



Planed for 1991:

- *cloning and identification of GT genes*
- *setting-up of GT assays*
- *preparation of aglycons*

MAJOR PROBLEMS ENCOUNTERED: *none*

RESULTS:

DNA probes were designed from the streptomycin (SM) production genes *strDELM and H* of *Streptomyces griseus*. The genes *strD* (dTDP-glucose synthase), *strE* (dTDP-glucose dehydratase), *strM* (dTDP-4-keto-6-deoxyglucose 3,5-epimerase) *strL* (dTDP-dihydrostreptose synthase) are by various evidence involved in the 4 steps of dTDP-dihydrostreptose formation (Distler et al.

1987, Pissowotzki et al. 1991). By mutant phenotype *strH* seemed to code for a dihydrostreptosyltransferase (Mansouri and Piepersberg 1991). The probes for *strD*, *strE*, *strLM* and *strH* were hybridized against *Bam*HI cleaved genomic DNA extracted from 43 different actinomycete strains 27 (63%) of which were known to produce 6-deoxyhexose (6DOH) moieties in at least one of their known secondary metabolites. 30 strains (70%; 11 were not known to produce any 6DOH compounds) gave a positive signal at least with one of the probes (Stockmann and Piepersberg 1992).

The gene *strH* also seemed to have relatives in 15 of the strains tested.

More intensive genomic mapping by a combination of restriction and hybridization was carried out in *S. purpurascens* R44 (produces cytorhodines; 6DOHs: rhodosamine, 2-deoxy-L-fucose, rhodinose), *S. noursei* DSM40635 (produces nystatin; 6DOH: mycosamine), and *S. nodosus* DSM40109 (produces amphotericin A, B; 6DOH: mycosamine; coordinator; Stockmann and Piepersberg 1992).

In the DNA of the A201A producer *S. capreolus* also *strDE* hybridizing genomic segments could be mapped. The rhamnosyl-transferase gene is currently searched for in neighborhood to these genes by cloning and analysis (participant 1).In the tylosin (*tyl*) gene cluster of *S. fradiae strD* and *strE* - related genes were detected and sequenced; 6DH - transferase gene(s) are expected to be located in the same subcluster (participant 2)

To establish a dihydrostreptosyltransferase test we have started to establish an enzymatic preparation for the substrate, dTDP-dihydrostreptose. For this purpose we have started to express the genes *strDELM* in *S. lividans* 66 TK23. Till now the genes *strD* and *strE* have been cloned downstream the strong promotor *ermE*-up in *S. lividans*.(coordinator). For the clone bearing the *strE* gene (pKMWE5) preliminary enzyme tests have shown an increased dehydratase activity (collaboration with A. Stein and L. Elling, Forschungszentrum Jülich, FRG).

Amphotericin B, produced by *S. nodosus* DSM40109 and NRLL B-2371, consists of a polyenic polyhydroxylated ring and the 6DH residue D-mycosamine. Since the first steps in the 6DH biosynthetic pathways are common, it was tried to isolate the genes corresponding to *strD* and *strE* by heterologous hybridization.

Genomic hybridizations with total DNA from both *S. nodosus* strains cut with *Bam*HI showed hybridizing fragments of 2.4kb (*strD*-probe) and 2.3 + 4.4kb (*strE*-probe). The 2.4kb *Bam*HI-fragment from strain DSM40109 (hybridazing with the *strD*-probe) has been cloned. Further subcloning and sequence analysis is in progress (participant 3).

As an initial step for the cloning of glycosyltransferase genes implicated in the biosynthesis of A201A from *S. capreolus*, genes for the selfresistance has been cloned. From a gene library, constructed in the *E. coli - Streptomyces* shuttle cosmid pJAR4, 5 *S. lividans* transformants resistant to >1000µg A201A / ml were isolated. Comparison of restriction maps and successive hybridization experiments showed that the clones constitute three groups with apparently different determinants for A201A-resistance.

The possible inactivation of A201A by cell-free extracts from *S. capreolus* was examined. No modifications by either phosphorylation, adenylation or acetylation was found. The resistance, therefore, could be due to a permeability barrier, ribosome modification or drug inactivation by a mechanism different to those already examined (participant 1).

From *S. lividans* 66 TK21 a chromosomal coded macrolide resistance gene was cloned and shown to be a macrolide glycosyl transferase (*mgt*, Jenkins and Cundliffe 1991). Further examinations concerning the specificity of the enzyme revealed that (i) the MGT used UDP-glucose to inactivate erythromycin or tylosin *in vitro*, (ii) the target was located within mycaminose, the amino sugar attached to C-5 of the lactone ring of tylosin, (iii) the MGT preferes a monosaccharide substituent at C-5, ideally lacking a 4´-OH and (iv) the 2´-OH group of the macrolide antibiotics is a prime

site for their modification and inactivation (participant 2; Cundliffe 1992).

citations:

Cundliffe E (1992) Antimicrob. Agents Chemother. 36:348-352.
Distler J., Ebert A., Mansouri K, Pissowotzki K, Stockmann M, Piepersberg W (1987) Nucleic Acids Res. 15:8041-8056.
Jenkins G, and Cundliffe E (1991) Gene 108:55-62.
Mansouri K, Piepersberg W (1991) Mol. Gen. Genet. 228:459-469.
Pissowotzki K, Mansouri K, Piepersberg W (1991) Mol. Gen. Genet 231:113-123.
Stockmann M, Piepersberg W (1992) FEMS Microbiol. Lett. 90:185-190.

HIGHLIGHTS/MILESTONES:

1. One GT and its gene was identified and characterized by specific assays.
2. Many gene clusters for glycosylation of secondary metabolites were detected and partially characterized.
3. First steps in enzymatic substrate preparation were achieved.
4. Aglycones of the macrolide and polyene classes were prepared or found under natural fermentation endproducts.

WIDER CONSIDERATIONS:

The basics for developement of biotransformations via glycosylation of natural and synthetic aglycones have been founded. This will largely extend the tools in modulation of the specific functionality of natural pharmaceuticals.

COOPERATIVE ACTIVITIES:

2 meetings for all contractors:

- 4.february 1991, Hoechst AG Frankfurt a.M., FRG.
- 12.october 1991, Universidad Autonoma Madrid, Spain.

During ISBA-congress 1991 in Madison, Wisconsin, USA: coordinator with participants 1 and 2.

Exchange of material: see "results" (genetic and biochemical tools)

LIST OF JOINT PUBLICATIONS/PATENTS WITH TRANSNATIONAL AUTHORSHIP: none

OTHER PUBLICATIONS/PATENT:

Cundliffe E (1992): Glycosylation of macrolide antibiotics in extracts of *Streptomyces lividans*. Antimicrob. Agents Chemother. 36:348-352.

Jenkins G, and Cundliffe E (1991): Cloning and characterization of two genes from *Streptomyces lividans* that confer inducible resistance to lincomycin and macrolide antibiotics. Gene 108:55-62.

Mansouri K, Piepersberg W (1991): Genetics of streptomycin production in *Streptomyces griseus*: nucleotide sequence of five genes, *strFGHIK*, including a phosphatase gene. Mol. Gen. Genet. 228:459-469.

Pissowotzki K, Mansouri K, Piepersberg W (1991): Genetics of streptomycin production in *Streptomyces griseus*: molecular structure and putative function of genes *strELMB2N*. Mol. Gen. Genet. 231:113-123.

Stockmann M, Piepersberg W (1992): Generally applicable gene probes for the detection of 6-deoxyhexose metabolism in secondary metabolite-producing streptomycetes. FEMS Microbiol. Lett. 90:185-190.

German patent 41 30 967 (HOE 91/F 300): Verfahren zur Isolierung von Sekundärmetabolit-Biosynthesegenen aus Aktinomyceten sowie deren Verwendung.

Title: **Alternative methods of DNA-sequencing.**

Contract No: BIOT-CT90-0252 (SMA)
Official Start Date: Feb 91 (Actual start date delayed until Dec 91)

Coordinator: Robert J.G. Carr, CAMR, Porton Down, Wiltshire, UK

Participants:
R. Carr, **CAMR**, Porton Down, Wiltshire, (UK)
C. Christiansen, **Biotechnology Institute**, Lyngby, Copenhagen, (DK)
G. Charalambos, **Optimum Limited**, Athens, (GR)
O. Buchardt, **University of Copenhagen**, Copenhagen, (DK)

Reporting Period Objectives:
Initial use of High Energy Electron Beam Lithography for fabrication of test sub-micron apertures in opaque screens. Initial design and construction of optical launch and detection system. Immobilisation of DNA binding proteins (e.g. DNA polymerase [ex *T.aquaticus*]) onto model substrate matrices (simulating their future use in apertures on solid substrates to permit monitoring of sequential addition of fluorescently labelled bases to single stranded DNA and hence determine sequence). (CAMR).

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. (Biotechnology Institute).

Determine specification and classification of the functional Hardware and Software requirements both for the new sequencing gel reader systems as well as for the near field aperture devices. (Optimum Ltd.)

Major Problems Encountered:
A change in participant structure (withdrawal of 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 for a method to enable rapid, one-step **mapping** of DNA (both physical and genetic mapping of large (10sKb) DNA fragments).

Results:
CAMR and Biotechnology Institute have, during the three months since the actual start date of this project, determined the major design features (optical characteristics, DNA manipulation and labelling, electrophoretic cell design, and model test system) of the aperture mapping system and model systems for future study identified as an interim step to starting the development of the sequencing system.
Prior to the actual start date of the project, CAMR carried out a study on the immobilisation of Taq polymerase on carbohydrate and glass matrices and substrates to demonstrate retention of enzymatic activity following immobilisation. Whilst there was evidence to show a small degree of activity, the low concentrations obtained were considered to be impractical and further attempts were abandoned.

Optimum Ltd. have initiated a software simulation of the expected data from the aperture devices in order to predict sample volumes required and analysis times necessary for sufficient statistical accuracy in sequencing and mapping applications.

Highlights/Milestones:
CAMR is now completing installation and commisioning of a complete Electron Beam Lithography facility for use in this project. (Facilities include thin film [thermal and spin] coating plants, electron beam pattern generation, plasma and chemical etching systems, SEM, clean room, etc..). This facility will enable the rapid and dedicated fabrication of sub-micron structures for use throughout this single-DNA-molecule characterisation project.

Wider Considerations:
The proposed mapping application has generated a significant amount of interest from a major European biotechnology instrumentation manufacturer. Accordingly, they will now be actively supporting (both materially, with significant expertise and knowhow and at no cost to the participants or the EC) this aspect of the project with a view to commercially exploiting the proposed DNA mapping technique.

Cooperative activities:
Up to and during this early, but crucial, definition stage of the project, personnel from all the participants have met regularly. A three day meeting was held between all the participants (Optimum 1, CAMR 2, Biotechnology Institute 3, Danish sub-contractors 2) in Denmark [April 91]) and subsequent shorter meeting between CAMR and Biotechnology Institute have taken place in both the UK [Mar 92]) and Brugge [Oct 91].
Similarly, staff from CAMR have visited Optimum Ltd. in Athens [May 91] to define simulation requirements for s/w development.

Meetings have been held (and are planned) between CAMR and Biotechnology Institute and the Instrumentation company on future collaborative work on mapping.

The participants were also collectively invited to present their work at
i) the EC meeting on Sequencing the Yeast Genomes II and XI (Brugge, Belgium - Oct 91) and at
ii) the EC meeting on Genome Analysis in the EC (Elounda, Crete - May 91).

Published Work:
Carr, R. (1991) "A possible Technique for Solution phase Sequencing of DNA", Abs. EC Int. Meet. on Genome Analysis in the EC, Crete, May 1991.

Carr, R., Holmstrom, K., Buchardt, O. and Georgiou, C. (1991)
"Alternative Methods to DNA Sequencing", Abs. EC BRIDGE Meeting on Sequencing the Yeast Chromosomes II and XI, Brugge Sept., 1991, p 90-91.

AREA : C

CELLULAR BIOLOGY

TITLE: Integration of primary metabolism, secondary metabolism and differentiation in *Streptomyces coelicolor*: A biochemical, physiological and genetical approach

CONTRACT NUMBER: BIOT-CT91-0255 (SMA)
OFFICIAL STARTING DATE: 01/04/91
CO-ORDINATOR: D.A. Hopwood, John Innes Institute, Norwich, GB
PARTICIPANTS: L. Dijkhuizen, University of Groningen, NL
P. Postma, University of Amsterdam, NL
C. Hardisson and J. Salas, University of Oviedo, E
C.J. Thompson, Institut Pasteur, Paris, F
F. Malpartida, C.S.I.C., Madrid, E
D.A. Hodgson, University of Warwick, UK

OBJECTIVES SET FOR THE REPORTING PERIOD: Establish unified conditions for *S. coelicolor* cultivation for physiological studies - Assay glucose metabolic enzymes in relation to growth and move towards cloning the genes - Measure glucose uptake in relation to growth, glucose kinase activity, glucose repression and stringent response - Isolate CR-relieved mutants and move towards characterising the mutants - Characterise and isolate adenyl cyclase - Measure enzymes of glycogen and trehalose metabolism in relation to growth, glucose flux, CR and stringent response and start to purify enzymes - Identify ribosomal proteins that change during growth and development; start to purify and partially sequence selected proteins - By 2D gel analysis, start to identify *act* proteins and effects on them of *act*II-ORF4 over-expression, of regulatory mutations, and physiological changes (glucose flux, CR and stringent response) - Express *act*II-ORF4 (in *E. coli*) to isolate protein, leading to antibody production and analysis of *in vitro* activity by DNA binding, etc.

MAJOR PROBLEMS ENCOUNTERED:
1. **Choice of strains and growth conditions:** Realisation that there is no strain ideal for both physiological and genetic studies has delayed choice of a single strain and of unified conditions for physiological work. Nevertheless, there is now an urgent need for unification. Towards this end, the Norwich group has started to compare growth and antibiotic production of M145, D132 and MT1109 and to define "minimal" nutritional levels needed for growth and antibiotic production.
2. **Assay of Actinorhodin:** It has become clear that, in many A3(2) strains, only a minority of blue pigment is actinorhodin itself. Strenuous efforts are being made at the University of Bristol (T.J. Simpson) to identify the other pigment(s). Since they represent part of the flux of carbon into the actinorhodin pathway, it is imperative to be able to assay each compound.
3. **Glycogen biosynthesis:** Very low activities of glycogen biosynthetic enzymes (especially absence of detectable glycogen synthase), and instability during purification, have limited biochemical progress. Meagre information about sequences of homologous enzymes from other organisms is also disappointing. To overcome these problems: (1) E. Miguélez (Oviedo) will visit A. Smith (Norwich), an expert on the plant enzymes, to modify the biochemical approach and to use antibodies against heterologous enzymes. (2) Mutants defective in glycogen synthesis will be obtained and characterised. (3) The availability at Norwich, from plant scientists, of a growing unpublished database on glycogen and starch biosynthetic genes is allowing the design of better oligonucleotide probes.

RESULTS:
1. **Glucose metabolic pathways**

University of Groningen (L.V. Bystrykh, A. Alves and L. Dijkhuizen). All three prototrophic *S. coelicolor* strains checked (D132, M145 and MT1109) sporulated equally and MT1109 was chosen for the experiments. Initial attempts to grow it in batch flasks, and in a fermentor under steady state conditions, were successful, with production of blue pigment in phosphate-limited conditions. In batch flasks, a temporal acidification of the culture medium occurred, probably caused by accumulation of pyruvate and/or α-ketoglutarate. Glucose was utilized continuously and independently of growth rate (i.e. even after the culture entered idiophase). FPLC, HPLC and TLC were used to assay actinorhodin. TLC analysis revealed at least three blue pigments. This will be highly significant in future work: direct spectrophotometric measurements of actinorhodin production will need to be interpreted with great care.

Optimization of the following enzyme assays has been achieved: phosphofructokinase (PFK), KDPG (2-keto-3-deoxy-6-phosphogluconate) aldolase, glucose-6-phosphate dehydrogenase (NAD- and NADP-dependent), 6-phosphogluconate dehydrogenase (NAD- and NADP-dependent), pyruvate dehydrogenase, pyruvate kinase (PK) and citrate synthase. No KDPG aldolase was found in cell samples from any stage of batch growth, yielding no evidence for the Entner-Doudoroff pathway of glucose utilization. The ratio of the two pentosephosphate cycle dehydrogenases, specific either to NAD or NADP as electron acceptor, varied greatly depending on growth conditions.

We are focussing on the glycolytic enzymes for detailed enzymological analysis, especially in view of the possible accumulation of pyruvate in the medium. PFK and PK could be partially purified using dye-affinity chromatography. The main problem is their instability. Preliminary kinetic studies of PFK revealed that this enzyme is regulated in the classical way, namely inhibition by ATP, PEP, and citrate, and stimulation by ADP. PK also appears to be regulated at the activity level. Therefore it becomes increasingly unlikely that glycolysis is unregulated. Analysis of cell samples from different growth phases revealed that PK activity increased proportionately with growth rate, whereas pyruvate dehydrogenase was most active at the stage of growth limitation and pigment production. This could cause temporal accumulation of pyruvate in the culture medium, but gives no information about the onset of blue pigment formation.

2. Glucose transport and catabolite repression

Glucose Repression (a): John Innes Institute (S. Angell and M.J. Bibb). Mutants of *S. coelicolor* A3(2) resistant to 2-deoxyglucose (2-DGlc) cannot use glucose (Glc), are deficient in glucose kinase (GK) activity, and are defective in glucose repression. A 2.9 kb DNA fragment that complements a 2-DGlc^R deletion mutant for all four phenotypes carries two co-transcribed open reading frames for proteins of 20 kD (ORF2) and 33 kD (ORF3), with ORF3 also transcribed separately. ORF3 alone appears to complement all the mutant phenotypes. It is homologous to two repressor proteins, XylR of *Bacillus subtilis*, and NagC of *E. coli*. Although this might suggest that ORF3 encodes an activator for GK rather than the enzyme itself, the ORF3 protein clearly has GK activity. Spontaneous Glc$^+$ derivatives of an *S. coelicolor* ORF3 deletion mutant are 2-DGlc-sensitive, presumably reflecting the activation or modification of another hexokinase. The "revertants" have almost wild type levels of "GK" activity, have a hexokinase that migrates differently from that of the wild type GK on non-denaturing gels, and appear to confer Glc-repression on agarase production.

Glucose Repression (b): E.C. Slater Institute, University of Amsterdam (J. Kwakman and P. Postma). While in Norwich, J.K. isolated UV mutants of *S. coelicolor* M145 that formed zones of agarase activity in the presence of Glc. Six mutants were apparently also Glc-derepressed for galactose (Gal) uptake. The activities of galactokinase and glycerol kinase, enzymes which are also sensitive to glucose repression, as well as GK, are being determined. When the mutants are characterized better, the agarase Glc-derepressed phenotype will be complemented by a genomic bank of *S. coelicolor* cloned in a øC31 derivative (available from K.F. Chater). We are also using Tn*5096* to isolate genes involved in Glc repression. Several mutants showing Glc derepression of the agarase phenotype are being tested for the enzyme activities mentioned above.

To isolate mutants defective in Glc transport and metabolism, the Tn*5096* bank was screened for 2-DGlc-resistant colonies, yielding three classes of mutants: Glc$^+$ Gal$^+$, Glc$^+$ Gal$^-$ and Glc$^-$ Gal$^+$. The first (and possibly the second) is interesting, since most existing Glc repression-resistant mutants are Glc$^-$ because of a defective GK. The mutants will be characterized further with respect to Glc and 2-DGlc uptake, GK activity, and Glc repression-sensitive enzymes. Mutated genes will be cloned either by using Tn*5096* or by complementation. We shall concentrate on mutations in genes affecting several different (Glc-repressible) processes.

Transcription of genes involved in antibiotic production in S. coelicolor A3(2) and the possible role of ppGpp: John Innes Institute (E. Takano, H.C. Gramajo, J. White and M.J. Bibb). Using our conditions for reproducible exponential growth and antibiotic production by batch cultures of *S. coelicolor* M145, transcription of structural and putative regulatory genes for actinorhodin and undecylprodigiosin synthesis was analysed. Although transcripts for the biosynthetic enzymes appear only at the onset of stationary phase, correlating with the appearance of ppGpp, those corresponding to the pathway-specific activator genes *act*II-ORF4 and *redD* are seen (at low levels) during exponential growth, and increase sharply during transition from exponential to stationary phase. Nutritional shiftdown of an exponential culture results in production of ppGpp, a rapid decline in GTP, an increase in the *act*II-ORF4 transcript, and appearance of actinorhodin structural gene transcripts; addition of the GMP synthetase inhibitor decoynine, which produces a similar reduction in GTP and growth rate but no detectable ppGpp, does not elicit antibiotic

production. Transcription of *bldA*, whose product ($tRNA^{Leu}_{UUA}$) is required for translation of *act*II-ORF4 mRNA, occurs during exponential growth, suggesting other limitations on actinorhodin production during rapid growth in these conditions. Moreover, translational fusions of the N-terminus of *act*II-ORF4 (containing either the wild-type TTA codon or a replacement by TTG) fused to *ermE* (lincomycin resistance) as reporter suggest no limitation in expression at the translational level in exponential liquid cultures, or in young agar-grown cultures. Thus, although *bldA* is *required* for expression of the *act* genes, it seems not to be responsible for the *temporal regulation* of expression *in these conditions*.

To assess whether the correlation between ppGpp synthesis and transcription of antibiotic biosynthetic genes is causal we are trying to clone the ppGpp synthetase gene (*relA*) of *S. coelicolor* to create a null mutant. Attempts to use the *E. coli relA* gene, and antibody raised to its product, to isolate the *S. coelicolor* ppGpp synthetase gene have failed. We are now trying to isolate enough of the enzyme for N-terminal sequencing to allow a reverse genetic approach.

Role of cAMP in the S. coelicolor A3(2) life cycle: University of Warwick (F. Amini and D.A. Hodgson). *S. coelicolor* produces cAMP throughout its growth cycle, with 10% in the cells and 90% in the medium, as in other bacteria. cAMP levels did not vary with carbon source. Adenyl cyclase activity is present in cell-free extracts, in contrast to the situation in enteric bacteria, in which loss of cell integrity leads to loss of most of the adenyl cyclase activity. The activity of the enzyme was as high in cells grown in complex medium as those in minimal medium and so the former have been used to study some of the properties of the enzyme. It prefers Mn^{2+} to Mg^{2+}, but in the presence of Mg^{2+} is stimulated by pyruvate. It is present in the cytoplasmic fraction, at most only loosely bound to the membrane. The pH buffer used, affects enzyme activity, with PIPES having the most positive effect. We are characterizing the enzyme further in order to purify it as a step towards cloning the gene. We have tried cloning by complementation of an *E. coli cya*$^-$ strain, but so far without success. We shall now use as a heterologous probe the *cya* gene of *Brevibacterium liquefaciens*, an actinomycete with a very similar adenyl cyclase to that of *S. coelicolor*.

3. Storage compound synthesis and degradation

University of Oviedo (C. Hardisson, and E. Miguelez) and John Innes Institute (K.F. Chater, C.J. Bruton and K.A. Plaskitt). Work has started on the enzymes of *Streptomyces antibioticus*, because glycogen is more abundant than in *S. coelicolor* in liquid-grown cultures. ADP-glucose pyrophosphorylase, glycogen branching enzyme and glycogen phosphorylase activities were all detectable, albeit at low levels, but so far not glycogen synthase.

Several probes have been designed to identify sequences specifying conserved regions of branching enzyme and ADP-glucose pyrophosphorylase. Cloning and sequencing of hybridising fragments has failed to identify relevant sequences. We have constructed a gene library of *S. coelicolor* J1501 in the bifunctional cosmid pKC505. We hybridized total *S. coelicolor* chromosomal DNA with two different probes: the gene encoding the branching enzyme of *Synecoccocus*; and part of the *strD* gene from *S. griseus* which has homology with glycogen pyrophosphorylases from several bacteria. Neither probe hybridised with *S. coelicolor* DNA under the conditions used. We have therefore tried to make a glycogen pyrophosphorylase probe by PCR and are characterising three candidate clones.

When cloned glycogen genes are available, their developmental expression will be studied, correlating the observed pattern of glycogen accumulation in the wild-type and in suitable mutants. A *bldA* mutant showed no glycogen in hyphae near the colony surface, unlike the wild-type. The same deficiency has now been observed for *bldC,D,G* and *H* mutants, whereas a *bldF* mutant makes abundant glycogen. The absence of glycogen from so many *bld* mutants is consistent with a model implicating glycogen metabolism in aerial mycelium development. The *bldF* mutant also differs from most other *bld* mutants in other respects: (1) in producing abundant undecylprodigiosin; (2) in having a bald phenotype on all media tested (*bldA, C, D, G* and *H* mutants can sporulate when mannitol replaces glucose as carbon source); and (3) in being able to express a test gene containing TTA codons (*bldA, D, G* and *H* mutants all lacked this ability).

4. Regulatory cascades that lead to actinorhodin production

Centre for Biotechnology, Madrid (F. Malpartida, R.P. Mellado and M.A. Fernandez-Moreno). Transcription of the actinorhodin biosynthetic genes depends on the *act*II-ORF4 product, a polypeptide of 28.7 kD, a target for the *bldA* $tRNA^{Leu}_{UUA}$. Our main goal is to understand the factor(s) (apart from the *bldA* product) affecting expression of *act*II-ORF4, and the mechanism by which the ActII-ORF4 protein switches on the *act* biosynthetic genes, including identification of its target sequences.

S. lividans usually fails to produce actinorhodin even when carrying the whole *act* cluster, including *act*II-ORF4, on a plasmid vector: perhaps expression of some gene(s) in a presumptive cascade, whose final target is the *act* genes, is a limiting step in antibiotic production. We screened libraries from *S.*

antibioticus and *S. fradiae* (species not known to contain *act* genes) for clones able to cause actinorhodin production in *S. lividans*. Both libraries yielded such clones. The sequence of one from *S. antibioticus* shows one ORF as the actinorhodin-activating sequence. Its deduced product, a polypeptide of nearly 300 aminoacids, contains a typical "helix-turn-helix" domain at its N-terminus: the protein is highly homologous to other transcriptional activators of the LysR family. Experiments are in progress to identify its possible target sequences within the *act* cluster, and to seek and analyse a homologous DNA fragment from *S. coelicolor*.

The ActII-ORF4 gene has been expressed in *E. coli* to yield 25% of total protein as ActII-ORF4; the expressed protein is produced as inclusion bodies, from which it can be recovered in up to 95% purity. Conditions for solubilizing the ActII-ORF4 protein and an "in vitro" assay for its activity are in progress.

5. **Changes in ribosome structure during development**

University of Oviedo (J. Salas). Ribosomal proteins of different stages of development on solid and in liquid medium were compared by 2D-PAGE and HPLC. Only one differential protein - present in substrate mycelium ribosomes and absent from those of aerial mycelium and sporulating cultures - has been clearly detected on solid medium. We are proceeding to identify this differential protein by amino terminal sequencing as a prelude to cloning the gene. We have also cloned the L7/L12 ribosomal protein gene of *S. coelicolor* by screening a cosmid library with a probe corresponding to the homologous protein from *S. antibioticus*. This gene has been sequenced to determine the organisation of the cluster containing it and other ribosomal proteins. We are also making fusions between the gene and *lacZ* to allow purification of the hybrid protein to raise antibodies for further protein purification and analysis of changes in this protein during development.

6. **Variations in proteins regulated by global metabolic switches.**

Institut Pasteur, Paris (P. Kaiser and C.J. Thompson). We have initiated studies of global changes in gene expression leading to antibiotic (actinorhodin) biosynthesis in *S. coelicolor* J1501 using computer-aided analyses of two-dimensional electropherograms. Various media and culture conditions were evaluated for well-defined and reproducible culture conditions that give uniform mycelial growth and a sharp boundary between vegetative growth and actinorhodin biosynthesis. A defined minimal growth medium (MG), and a complex production medium (SY) were chosen, with standardized shaking conditions, giving reproducible growth curves with a characteristic decrease in growth rate 28 h and 24 h post-inoculum in MG and SY media respectively. Actinorhodin production started shortly thereafter (30 hours and 26 hours p.i. respectively).

We have documented dramatic changes in *S. coelicolor* J1501 gene expression as a function of growth. Since each 2D gel resolves more than 700 gene products and at least ten gels must be compared, critical analysis by visual inspection is impossible; however, at least 30% of the gene products are switched on or off as cells approach stationary phase. Preliminary data suggest that this problem is soluble by cluster and principal component analysis. During a six month visit, J. Vohradsky (Institute of Microbiology, Czechoslovak Academy of Sciences) has analysed 200 spots on six autoradiograms representing the growth cycle of *S. coelicolor* in liquid. These spots have been grouped into families having similar patterns of synthesis.

The next phase is to test the validity of the statistical methods. We are running a series of gels to identify heat shock proteins (with A.M. Puglia during a 1 year sabbatical visit from the University of Palermo), ribosomal proteins, and co-regulated genes for the actinorhodin biosynthetic pathway. This will show whether the statistical methods can identify spots representing gene products known, from independent biological studies, to be co-regulated. If the validity of these methods is thus established, we shall be confident in their value as tools to predict regulons and families of developmentally regulated gene products.

HIGHLIGHTS/MILESTONES: Methods successfully developed for reproducible assay of key enzymes of glycolysis and oxidative part of pentose phosphate pathway - Evidence for *regulation* of PFK and PK at activity level - Isolation of presumptive CR$^-$ mutants - Recognition of the GK ORF - Temporal analysis of antibiotic gene transcripts and *correlation* with ppGpp - Successful analysis of adenyl cyclase - Isolation of heterologous genes that activate actinorhodin production - Purification of ActII-ORF4 protein - Identification of a putative differential ribosomal protein - Cloning of the L7/L12 gene - Recognition of families of putatively co-regulated proteins on 2D gels.

WIDER CONSIDERATIONS: Establishment of the consortium has brought together impressive multidisciplinary skills in biochemistry, physiology, genetics and molecular biology. Two groups new to

Streptomyces - but with key experience of other bacteria - and three groups with skills in other streptomycetes have been integrated with three leading *S. coelicolor* laboratories. Progress has been made in most sub-objectives of the project, even though some technical problems have inevitably limited progress in others; the latter have been targeted for action. It is already clear that *S. coelicolor* will indeed be suitable for the multi-disciplinary approach that made it, *a priori*, the most suitable model antibiotic-producing streptomycete to generate new insights into metabolic switching from primary to secondary metabolism.

CO-OPERATIVE ACTIVITIES:

1. **Annual co-ordinating meeting.** On the Great Burgh premises of SmithKline Beecham, by kind invitation of Drs Janet Dewdney and John Hodgson (18-19/6/91). All participating laboratories, two "associated" (non-BRIDGE-funded) teams (I S Hunter, Robertson Institute of Biotechnology, University of Glasgow and C P Smith, Department of Biochemistry and Manchester Biotechnology Centre, UMIST), and four of the seven companies who offered support to the BRIDGE programme (SmithKline Beecham Pharmaceuticals, Glaxo Group Research, Rhône-Poulenc Rorer Recherche-Développement and Lepetit Research Center) were represented.

2. **Staff exchanges.** (i) Working visits: J. Kwakman (Amsterdam): 3 months in Norwich (29.4.91-26.7.91) to acquire expertise in *Streptomyces* handling and mutagenesis; A. Alves (Groningen) one week in Warwick to learn techniques for reproducible rapid growth of *S. coelicolor* in minimal medium. (ii) Co-ordinating visits: L. Bystrykh and L. Dijkhuizen (Groningen) to Glasgow (11.11.91), Manchester (12-13.11.91) and Norwich (14-15.11.91); J. Kwakman and P. Postma (Amsterdam) to Norwich (20-21.7.91); P. Postma (Amsterdam) to Paris (24-25.2.92); F. Malpartida (Madrid) to Norwich (8-12.2.92); K.F. Chater (Norwich) to Oviedo (15-18.1.92); D.A. Hodgson (Warwick) to Norwich (19.3.91; 14.11.91; 28.2.92); D.A. Hopwood (Norwich) to Paris (30.11.91-5.12.91).

3. **Exchange of materials.** Many specialised *Streptomyces* strains between all participating groups. Specific *E. coli* strains (glucose kinase) from Amsterdam to Norwich for mutant studies. *Streptomyces* ribosomal proteins from Oviedo to Paris for analysis.

LIST OF JOINT PUBLICATIONS WITH TRANS-NATIONAL AUTHORSHIP*:

Parro, V., Hopwood, D.A., Malpartida, F., Mellado, R.P. 1991. Transcription of genes involved in the earliest steps of actinorhodin biosynthesis in *Streptomyces coelicolor*. Nucleic Acids Research *19*, 2623-2627.

Fernández-Moreno, M.A., Caballero, J.L., Hopwood, D.A., Malpartida, F. 1991. The *act* cluster contains regulatory and antibiotic export genes, direct targets for translational control by the *bldA* tRNA gene of *Streptomyces*. Cell *66*, 769-780.

Caballero, J.L., Malpartida, F., Hopwood, D.A. 1991. Transcriptional organization and regulation of an antibotic export complex in the producing *Streptomyces* culture. Molecular and General Genetics, *228*, 372-380.

Caballero, J.L., Martinez, E., Malpartida, F., Hopwood, D.A. 1991. Organisation and functions of the *act*VA region of the actinorhodin biosynthetic gene cluster of *Streptomyces coelicolor*. Mol. Gen. Genet. *230*, 401-412.

OTHER PUBLICATIONS*:

Bruton, C J, Guthrie, E P, Chater, K F (1991) Phage vectors that allow monitoring of secondary metabolism genes in *Streptomyces*. Bio/Technology *9*, 652-656.

Holt, T G, Chang C, Laurent-Winter, C, Murakami, T, Garrels, J I, Davies, J E, Thompson, C J (1992) Global changes in gene expression related to antibiotic biosynthesis in *Streptomyces hygroscopicus*. Molecular Microbiology (In press).

Leskiw, B K, Lawlor, E J, Fernandez-Abalos, J M, Chater, K F (1991) TTA codons in some genes prevent their expression in a class of developmental, antibiotic-negative *Streptomyces* mutants. Proceedings of the National Academy of Sciences, USA *88*, 2461-2465.

Strauch, E, Takano, E, Baylis, H A, Bibb, M J (1991) The stringent response in *Streptomyces coelicolor* A3(2). Molecular Microbiology *5*, 289-298.

* * * * * * * * *

*It is too early for papers directly stemming from the BRIDGE-funded work. These papers on prior studies are, however, of particular relevance to the project results.

TITLE: **The production and recovery of biotechnologically important proteins from the yeast *Saccharomyces cerevisiae***

CONTRACT NUMBER: **BIOT-CT90-0165**

STARTING DATE: 01 January 1991

COORDINATOR: Dr. C. Hadfield, University of Leicester, *Leicester*, UK.

PARTICIPANTS: Dr. D. Pioli, ICI Pharmaceuticals, *Macclesfield*, UK.
Dr. W.E. Lancashire, Whitbread & Company, *Luton*, UK.
Dr. P.A. Meacock, University of Leicester, *Leicester*, UK.
Prof. C. Nombela, Universidad Complutense, *Madrid*, ES.
Dr. F. del Rey, Universidad de Salamanca, *Salamanca*, ES.
Dr. J. Dommes, Eurogentec SA, *Liège*, B.

OBJECTIVES FOR THE REPORTING PERIOD:

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

1-1. The isolation and initial characterization of the fermentation-stage-specific promoters of genes *ESP65* and *MOL2*.

1-2. Analysis of the performance of fermentation-stage-specific expression directed by the *HSP12* promoter for the production of heterologous protein (recombinant trout growth hormone) in various fermenter conditions.

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

2-1. Synthesis of a heterologous secretion reporter gene with optimal codon usage for expression in yeast, and sequence verification.

2-2. Incorporation of the newly synthesized high-level expressing secretion reporter gene into a yeast vector.

2-3. Screens for enhanced secretion mutants.

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

3-1. Partial characterization and evaluation of biotechnological interest of thermosensitive autolytic mutants *lyt2*, *slt2*, and *lyt1*.

3-2. Cloning and/or characterization of genes complementing *lyt1* and *lyt2*.

3-3. Detailed analysis of protein kinase gene *SLT2*, including studies on the effect of regulated expression.

3-4. Further isolation of new autolytic mutants.

3-5. Characterization of cloned exo-1,3-β-glucanase genes *EXG1* and *EXG2* and their encoded products.

3-6. Cloning and initial characterization of the sporulation-induced exo-1,3-β-glucanase gene.

MAJOR PROBLEMS ENCOUNTERED:

3-2. There were unexpected difficulties in cloning the *LYT2* gene. Previous attempts to clone this gene led to the isolation of gene *SLT2*, a suppressor of *lyt2* mutations that codes for a protein kinase. New and extensive efforts to clone *LYT2* with the use of centromeric gene banks, led to repeated isolations of the suppressor kinase gene, but not of gene *LYT2*. There could be several explanations for this difficulty, but a new strategy is being implemented by obtaining a genomic library of an *slt2* disrupted strain.

RESULTS:

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

1-1. The isolation and initial characterization of the fermentation-stage-specific promoters of genes *ESP65* and *MOL2*. *(PAM)*

In order to isolate the two genes, *ESP65* and *MOL2*, a library of genomic DNA from strain

S288c was constructed in the *E.coli* bacteriophage vector λEMBL3. Screening of this library with previously isolated cDNA clones of *ESP65* and *MOL2* resulted in the isolation of several clones for each. Previous Southern blot hybridisation analysis of genomic DNA suggested that both *ESP65* and *MOL2* are members of multigene families; there being three members for *ESP65* and four members for *MOL2*. Current research is now focusing on a detailed characterisation of these clones in order to recover the particular DNA fragments that carry the genes into plasmid vectors for further analysis.

Expression of the genes has been investigated, in conjunction with previously isolated genes whose promoters cause transcriptional induction at the end of rapid fermentative growth, as a yeast culture transitions through wholly aerobic growth and into stationary phase. Northern blot analysis was undertaken of RNA samples isolated during the growth of strain S288c on various carbon sources. These studies indicate that two genes, *ESP65* and *HXK1*, are subject to strong glucose repression. Two genes (*MOL1* and *MOL2*) are expressed transiently and only in molasses medium. Both of the heat shock genes, *HSP12* and *HSP26*, show sustained stationary phase induction in all media and are unaffected by carbon source.

These observations were extended by examining expression of the genes in strains carrying mutations in either the glucose/catabolite repression system or the cAMP control system. Results of these studies have confirmed *HXK1* and *ESP65* to be subject to carbon catabolite repression but are independent of cAMP control. Interestingly *HSP26* expression also seems to be influenced by catabolite repression, in contrast to that of *HSP12* which is clearly independent of this regulation but is under cAMP control.

1-2. Analysis of the performance of fermentation-stage-specific expression directed by the *HSP12* promoter for the production of heterologous protein (recombinant trout growth hormone) in various fermenter conditions. *(JD)*

The *HSP12* promoter was fused to a tGHII cDNA devoid of its signal sequence. This expression cassette (containing the *GAPDH* terminator) was cloned into a 2μm-based yeast-*E.coli* shuttle vector pYEGT110, and the resulting recombinant plasmid, pYtGH6, introduced into yeast strains.

The intracellular accumulation of tGHII was monitored by SDS-PAGE and Western blotting. In shake flasks, cells in stationary phase produced about 4 mg of tGHII per litre of rich medium (YPD). In batch fermentation (YPD medium), tGHII accumulation started after complete glucose exhaustion. The level of tGHII production was about 100 mg/l.

The effect of heat shocks before and after glucose depletion was examined. Before glucose depletion, a heat shock induced a transient low level expression of tGHII. After glucose depletion the heat shock had no effect. Pulses of glucose or heat shock given every 5 hours had no effect on the expression by stationary phase cells.

Different culture media were tested. No effect was seen on the production on a per cell basis. However, the final biomass was higher with CCSV medium.

The possibility of *HSP12* promoter induction after depletion of another essential substrate was examined. In a fed-batch fermentation experiment, a sucrose feeding maintained a very low glucose concentration in the medium. The first limiting factor was oxygen level (dropping to zero during the first 2 days). Oxygen level slowly increased during the next 20 h and then rapidly for a few hours. This fluctuating level indicates different metabolic stresses induced by the depletion of a substrate like amino acids, vitamins or trace elements. The level of tGHII did not change during this fermentation.

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

2-1. Synthesis of a heterologous secretion reporter gene with optimal codon usage for expression in yeast, and sequence verification. *(CH)*

2-1. An insulin reporter was selected as the high expression level secretion reporter, as it could be used with a footprint-type assay for colony secretion rather than a halo assay (as, say, with α-amylase), enabling more colonies to be screened per plate following mutagenesis. Insulin also had the advantage of being small and therefore could be synthesized quickly. This choice has been vindicated, as the insulin reporter system has been highly successful and greater progress than envisaged has been achieved.

A DNA sequence encoding a recombinant single chain human insulin precursor was planned that had optimal codon usage for expression in yeast (CBI = 1.0). This was synthesized from overlapping oligonucleotides and cloned into pUC19. Clones were sequenced and one with the correct predicted sequence identified.

2-2. Incorporation into a yeast vector; and expression level. *(CH)*

The insulin precursor encoding sequence was cloned, in frame, downstream of the *MFα1* prepro leader sequence and promoter in pDP314. This was confirmed by sequencing. Insulin product secretion from transformed yeast was confirmed by Western blotting. Shake flask cultures yielded 100 mg/ml of the secreted product. This is equivalent to about 98% of all secreted molecules being insulin, confirming that the desired high expression level had been achieved. Growth rate was reduced in minimal selective medium, but not in rich medium. Plasmid stability (both selective and non-selective) and cell viability were unaffected. Thus, a non-toxic, mild effect of the high expression level was detected.

2-3. Screens for enhanced secretion mutants

2-3.1 With low-level expressing reporters (CH): Using wheat α-amylase (CBI = 0.23) as a secretion reporter, mutants showing 25-30% greater secreted product were obtained. There appeared to be only a very limited bottleneck to the secretion of this product.

Using *Aspergillus niger* glucoamylase (CBI = 3.1) very little secreted product could be detected, despite a similar expression vector construction to the α-amylase and a more favourable codon bias index. Following mutagenesis, mutants were isolated that provided 1.5- to 5-fold increase in secreted product. These would seem to define a bottleneck to the secretion of this product.

2-3.2 With the high-level expressing reporter (CH): The "footprint" secretion assay enabled the isolation of a number of mutants that secrete up to 20-fold more insulin. The *MFα1* promoter is regulated according to the cell cycle, and an increase of 2-fold or so more may result from loss of this regulation. The higher levels of secretion suggest the overcoming of a secretory bottleneck. The isolation of mutants with this reporter is considerably in advance of schedule.

2-3.3 Enhanced secretion from industrial strains (WEL): Mutants have been obtained that give enhanced secretion of glucoamylases from brewing strains. Due to the polyploid nature of the strains, these mutations are thought likely to be dominant characters.

2-3.4 Screens for mutants showing: ability to release periplasmically localized secretory proteins / improved secretion at higher growth temperatures (CH): Use of the unmodified acid phosphatase gene as a periplasmic reporter protein has not proved satisfactory. We have therefore constructed an *MFα1/PHO5* fusion as a more effective reporter.

Investigation of the effect of temperature on growth and secretion revealed that growth, rather than secretion per se, was diminished as culture temperature was increase. Screens for mutants showing increased temperature tolerance have not yet been attempted.

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

3-1. Partial characterization and evaluation of biotechnological interest of thermosensitive autolytic mutants *lyt2*, *slt2*, and *lyt1*. *(CN)*

Strains with *lyt2* or *slt2* mutations lysed when actively growing cultures were switched from 24°C to 37°C; this lysis being prevented by osmotic stabilization with sorbitol, suggesting that it is due to a cell wall alteration. Batch fermenter cultures, carried out with a very precise control of temperature and other environmental factors, led to an expression of the mutant phenotype that could be clearly followed for about 5-6 hours and determined an intense release of protein with a decrease in viability of the cells. This information was used to develop a "two-stage" continuous culture system, which involved the continuous feeding of first stage cells, grown at 24°C, to a second stage at 37°C. This two-stage system provided a continuous flow of a cell population with more than 50% of the cells lysed and a significant release of protein occurring.

lyt1 mutations determined cell wall autolysis that was not prevented by sorbitol,

indicating that the defect was not mainly a cell wall alteration. The mutation was efficiently expressed in fermenter cultures, but the pattern of protein release was significantly different from those determined by *lyt2* and *slt2*.

3-2. Cloning and/or characterization of genes complementing *lyt1* and *lyt2*. *(CN)*
Clones "complementing" these mutations tend to be suppressors rather than the wild-type genes. A strategy to get round this problem has been described above (MAJOR PROBLEMS ENCOUNTERED). A suppressor gene for *lyt2* (*SLT2*) has been isolated and analysis is described below. In addition, a suppressor gene for *lyt1* has also been isolated. The clone carries the *SPO12* gene, described as being critical for meiotic functions. It is interesting that suppression of *lyt1* took place with *SPO12* in a multicopy plasmid, which led to expression of the gene during the mitotic cycle, whereas it is normally expressed only during meiosis.

3-3. Detailed analysis of protein kinase gene *SLT2*, including studies on the effect of regulated expression. *(CN)*
Site-directed mutagenesis of *SLT2*, to change lysine at position 37 (the putative active centre), abolished the function of the gene. The gene was not only critical for cell growth - disruption determining autolysis - but a critical regulation was also necessary. Over-expression of *SLT2*, under the control of a regulated promoter (*GAL10*), or inhibition of expression by anti-messenger plasmid constructions, led to a loss in cell viability due to cell lysis. The usefulness of these observations for the purpose of controlling cell lysis is very clear.

3-4. Further isolation of new autolytic mutants. *(CN)*
The complexity of functions related to cell wall dynamics, which can effect secretion and release of proteins from yeast, has led to the requirement for isolating and characterizing new autolytic mutants. Special attention was paid to those mutants showing an autolytic phenotype that can be complemented by osmotic stabilization with sorbitol. Temperature-sensitive recessive mutations representative of 3 other complementation groups have been isolated, plus a new group of thermosensitive dominant mutations. The phenotypes observed so far are highly suggestive of interesting alterations in cell wall dynamics.

3-5. Characterization of cloned exo-1,3-β-glucanase genes *EXG1* and *EXG2* and their encoded products. *(PR)*
Comparison of the predicted amino acid sequences of the *EXG1* and *EXG2* products revealed 5 highly conserved regions, which are located in the same relative positions in both polypeptides and may be essential for β-glucanase function. Removal of the histidine residues at one of these regions (DHHHY) abolishes β-glucanase activity. The effect of different single substitutions is currently being investigated.

EXG1 has been mapped to chromosome XII, 6.1 cM centromere-proximal to *CDC25*. *EXG2* is located in chromosome IV, between *LYS4* and *GCN2*, at distances of 6.2 and 4.9 cM, respectively.

Regarding the processing of the primary product synthesized from *EXG1*, site directed mutagenesis was carried out to modify the putative KEX2-protease recognition site in order to define the extent of the signal peptide at the amino-terminus of the precursor protein. This would facilitate the design of different constructions in order to investigate the role of the signal sequence, pro-sequence and protease cleavage site in promoting efficient secretion of the exo-1,3-β-glucanase and, eventually, to direct the export of heterologous proteins.

3-6. Cloning and initial characterization of the sporulation-induced exo-1,3-β-glucanase gene, *SSG1*. *(PR)*
Molecular cloning of *SSG1* was achieved by screening a genomic library with a DNA probe obtained by PCR amplification, using synthetic oligos designed according to predicted nucleotide sequence. The nucleotide sequence of *SSG1* shows a 1335 bp open reading frame, encoding a 445 amino acid polypeptide of 51790 Da, with one potential N-glycosylation site. Northern analysis showed a single transcript of 1.7 kb, which is only present in sporulating

diploids (a/α), and does not appear in haploid cells or non-sporulating diploids (α/α) when incubated under sporulating conditions. The meiotic time course of *SSG1* induction indicates a temporal pattern of expression that resembles the "late" class of sporulation-specific transcripts, appearing at about 6 hours after transfer to nitrogen-deficient medium, when meiosis I has been completed. Homozygous diploids are able to complete sporulation, although with a significant delay in appearance of mature asci.

SSG1 has been mapped to the right arm of chromosome XV, 8.2 cM to the centromere-proximal side of *HIS3*.

HIGHLIGHTS / MILESTONES:

(a). The excellent performance of the *HSP12* promoter in producing high yields of a biotech product, recombinant trout growth hormone.

(b). The successful isolation of new mutant yeast strains showing enhanced levels of heterologous protein secretion. These are of biotechnological significance as they increase product yield by many fold, and, in addition, genetically mark bottlenecks in the expression / secretion process.

(c). The possibility of controlling yeast cell wall dynamics by manipulating genes which influence the stability of this structure has been demonstrated; thus extending the usefulness of yeast as a host for biotechnological production. In addition, the isolation of new mutants affecting cell wall dynamics identifies further gene functions that may be manipulated to improve the efficiency of heterologous protein recovery from yeast.

WIDER CONSIDERATIONS:

The baking and brewing yeast, *Saccharomyces cerevisiae*, is a single-celled organism that has the same basic cellular organization and processes as higher eukaryotic organisms, including humans. Furthermore, it is also the most highly characterized eukaryote, and is easy to cultivate and harmless. These features make *S.cerevisiae* an ideal host for the biotechnological production of products normally synthesized by less amenable, or unamenable, higher cells, and many examples now exist that show the huge potential of yeast in this regard (such as hormones, recombinant vaccines and blood proteins, produced from yeast). However, initial levels of production of foreign proteins in yeast have been variable and often disappointing, reflecting gaps in basic knowledge of the processes involved in protein biosynthesis and recovery. This project is aimed at such key areas and results have extended the capability of yeast for the primary production of proteins and their recovery via secretion from the cell, and, in the case of non-secretable proteins, cell autolysis.

COOPERATIVE ACTIVITIES:

(a). Formal meetings.

Regularly scheduled twice-yearly meetings have proved highly successful forums for coordinating and furthering the project as an integrated whole. At the outset, the various participants provided complementary contributions to the overall project. By the second meeting in Avilla, it had become evident that extensive cross-collaboration and the merging of common goals had occurred such that the project had now become fully integrated.

Meeting 1:	June 1991	Leicester, UK.
Meeting 2:	January 1992	Avilla, ES.
Meeting 3:	September 1992 (scheduled)	Liège, B.

(b). Visits.

Pre-programme visits to Leicester University: CN, JD + colleague, DP, WEL.

Pre-programme visit to Salamanca University: CH, PM.

Visit to Departamento de Microbiologia II, Universidad Complutense, Madrid: CH + 2 colleagues (January 1992).

(c). Staff exchanges.

A member of CN's lab (Madrid) spent 3 months (Sept-Nov 1991) in CH's lab (Leicester) isolating a class of enhanced secretion mutants.

A member of CH's lab (Leicester) is to spend 1 week (April 1992) in WEL's lab (Luton) to undertake pilot brewery trials with a lager yeast strain that secretes glucoamylase. The heterologous glucoamylase gene (from *Aspergillus niger*) construct made at Leicester was

incorporated into a Whitbread lager yeast strain and its performance in pilsner-style fermentation compared with Whitbread's experimental strains containing other heterologous glucoamylase genes.

A member of PM's lab (Leicester) is scheduled to spend 1 week (April or May 1992) in WEL's lab (Luton) to determine the effects of growth in wort on *ESP65* and *MOLI* expression.

(d). Materials exchanges.

PM supplied the *HSP12* promoter to JD. CH supplied yeast strains DBY746 and BJ2168 to JD. DP supplied *MFα* promoter/leader sequence vectors pDP314, pDP315 and pDP316 to CH. CH and PM supplied protease mutant yeast strains 20B-12, ABYS1, ABYS66 and BJ2168, and mating-type switching plasmid pGAL-HO to CN and associates. DP supplied plasmids carrying wild-type and disrupted *PMR1*, and *pmr1*- yeast strains, to CH. PR supplied *EXG1* clones to CH. CH supplied α-amylase clone to PR.

JOINT PUBLICATIONS / PATENTS WITH TRANS-NATIONAL ORIGIN:

Joint review article in preparation.

A patent application with respect to one aspect of the project work is under consideration.

OTHER PUBLICATIONS:

Torres, L., Martín, H., García-Saez, M.I., Arroyo, J., Molina, M., Sánchez, M. and Nombela, C. (1991) A protein kinase gene complements the lytic phenotype of *Saccharomyces cerevisiae lyt2* mutants. Mol. Microbiol. 5, 2845-2854.

Praekelt, U. and Meacock, P.M. (1992) *MOL1*, a *Saccharomyces cerevisiae* gene that is highly expressed in early stationary phase during growth on molasses. Yeast. In press.

Hadfield, C., Menon, S., Raina, K.K. and Mount, R.C. (1992) The expression and performance of cloned genes in yeast. Mycol. Res. In press.

TITLE: WIDE DOMAIN CONTROL OF PRIMARY AND SECONDARY METABOLISM IN ASPERGILLI
CONTRACT No.: BIOT-CT90-0169
PERIOD: 1/3/91 - 1/3/92
COORDINATOR: Dr. J. VISSER, DEPT. OF GENETICS, WAGENINGEN AGRIC. UNI., NL.

PARTICIPANTS:
Prof. H. N. ARST (jr.), BACT. DEPT., ROYAL POSTGRAD. MED. SCHOOL, LONDON, G.B.
Prof. C. SCAZZOCCHIO, INST. de GENET. et MICROBIOL., ORSAY, PARIS, FR.
Dr. M. A. PEÑALVA, CENTRO DE INVESTIGACIONES BIOLOGICAS (CSIC), MADRID, ES.
Dr. F. NALIN, LYVEN, CAGNY, FR.

OBJECTIVES SET FOR THE REPORTING PERIOD:
1) Sequencing of *A. nidulans pac*C..(GB).
2) Analysis of IPNS transcription with respect to carbon catabolite repression..(ES).
3) Making constructs to produce regulatory proteins..(FR).
4) Preliminary identification of regulatory proteins on *prn*, *alc* and IPNS genes..(FR & ES).
5) Selection and mapping of *A. niger* mutations affecting carbon catabolite repression and pH regulation..(NL).
6) Metabolic analysis of *A. nidulans* and *A. niger* mutant and wild type strains, including ^{13}C- and ^{31}P-NMR spectroscopy..(NL).

MAJOR PROBLEMS ENCOUNTERED:
A) **Carbon catabolite repression (CCR).**
1) In sexual backcrosses involving the two mutations described in 'RESULTS (1.Aiii)', the parental type is not recovered, although mendelian inheritance of the two copies of the IPNS::*lacZ* fusion is observed (Southern). This fact suggests either (i) close linkage of mutations to *arg*B or (ii) inactivation of repeated (chimaeric) genes by entry into meiosis. Alternative strategy: search of hypofunctional mutations in negative regulators using increased expression of an IPNS::*amd*S fusion (single copy).....(MADRID).
2) The construction of a cDNA clone encoding the transactivator PRNA, necessary for the identification of its targets, has been achieved just recently.....(ORSAY).
B) **pH Regulation.**
1) Considerable difficulty has been experienced in obtaining crosses of the heteroallelic *pac*C_x^- X *pac*C_y^- type because of infertility. It therefore appears that the fine-structure mapping of *pac*C in order to localise mutations for sequence determination is unlikely to be feasible. Dr. David Widdick, the postdoctoral research associate supported by this EC Bridge contract, is therefore currently trying out the use of the carbodiimide reaction (Ganguly and Prockop, (1990) Nucleic Acids Res. 18 p3933; Ganguly *et al* (1991) J. Biol. Chem. 266 p12035) as an alternative method to limit the amount of DNA sequencing necessary to locate mutations.....(LONDON).
2) The delivery and commissioning of a new NMR spectrometer in Wageningen has resulted in a temporary loss of measuring time (over a four month period) and necessitated the investigation of alternative facilities to continue the pH regulation studies. Hence, the emphasis in these twelve months has been put on pH regulation studies with two different methods which have turned out to be complementary and are promising tools for further investigations (see also 'RESULTS (2.B)').....(WAGENINGEN).

RESULTS:
1. MOLECULAR AND GENETIC ANALYSES.
A) CARBON CATABOLITE REPRESSION.
i) **Selection of A. niger mutants**.....(WAGENINGEN).
CCR mutants (*cre*) of *A. niger* have been successfully isolated. The genetic approach to the identification of such mutants has followed the same technique as that applied to *A. nidulans* whereby UV_A induced pseudorevertants of *are*A$^-$ are screened for growth on media containing different N sources in the presence and absence of repressing levels of glucose. *A. niger* pseudorevertants supplied with either glutamate, alanine, histidine, ornithine, proline or asparagine in both the presence and absence of glucose show clear loss of CCR behaviour i.e. no growth on amino acid N- sources in the presence of glucose.

Testing the utilisation of NO^-_2, NO^-_3 and NH^+_3 has confirmed that the pseudorevertants retain the *are*A$^-$ phenotype and therefore constitute CCR mutants. Unlike *A. nidulans* however, in which the *cre*A locus exercises complete control over the pathway for metabolism of alcohol - *cre*A$^-$ mutants are sensitive to allyl and crotyl alcohols in the presence of repressing levels of glucose - the *A. niger* mutants generated do not display total loss of CCR of this pathway. In a similar vein, only partial sensitivity to fluoroacetate is shown in the presence of glucose. The equivocal nature of the sensitivity data may suggest that the alcohol and acetate utilisation pathways of *A. niger* are not under the unique control of a '*cre*A' equivalent but instead indicate the existence and influence of more than one major CCR system and/or a multiplicity of enzymes differentially responsive to carbon source. We are currently searching for metabolic systems that are regulated solely by '*cre*A'.

ii) Cloning of the *A. niger cre*A gene.....(WAGENINGEN).

The *cre*A gene (encoding CREA) of *A. nidulans* has been shown to play a major role in the regulation of CCR in that organism. For this reason the identification and isolation of the equivalent gene in *A. niger* is of particular interest in order that the extent of its role in CCR may be investigated in this industrially important organism and compared to *A. nidulans*.

Southern blots of *A. niger* genomic digests probed at low stringency with an *A. nidulans cre*A gene probe supplied by Dr. Joan Kelly (Adelaide) showed discrete hybridisation signals. An *A. niger* phage library screened under the same stringency conditions yielded hybridising plaques which were re-screened and isolated to single clone purity. BamHI, BglII, and HindIII were used to generate pEMBL subclones from the phage recombinants. Mapping of the subclones showed insert sizes ranging from 6kb to 11kb. The equivalence of the *cre*A gene so isolated from *A. niger* with that of *A. nidulans* is strongly indicated by the observation that the *A. niger* gene itself is able to restore the CCR response to the *cre*A^{d}30 mutant of *A. nidulans* similarly to the *creA* gene of *A. nidulans* e.g. resistance to allyl and crotyl alcohols in the presence of glucose.

With reference to the program detailed in the original workplan, the cloning of the *A. niger cre*A gene has been accomplished (12-30 months, item 5).

Experiments in progress are also focussing on the nature of the CCR effect on the *pel*A gene of *A. niger*. It has been observed (Kusters-van Someren *et al* (1991) Curr. Genet. 20 p293) that this gene escapes repression in *cre*A$^+$ *A. nidulans* transformants. The isolation of the *A. niger cre*A gene will enable us to investigate whether or not the product of this gene is directly responsible for the repression of *pel*A i.e. do co-transformants of the *cre*A^{d}30 *A. nidulans* mutant containing copies of the *A. niger cre*A and *pel*A genes show repression of *pel*A in the presence of glucose? The outcome of this will, at a later stage, become relevant for 'Lyven' which is a pectinase producing company.

Recent work involving a seven week visit to the EC-BRIDGE participating labs of Dr. B. Felenbok and Prof. C. Scazzocchio (Orsay) by Dr. A. P. MacCabe in order to acquire technical expertise in nucleic acid/protein binding has provided interesting data regarding the interaction of a fusion protein consisting of the 'zinc-finger' domain of the *A. nidulans cre*A product and glutathione S-transferase (CREA::GST) and the upstream sequences of the *A. niger pel*A gene:

A ~ 1kb DNA fragment containing the fully sequenced upstream regions of the *A. niger pel*A gene was isolated and restriction digested to yield a number of fragments varying in size between 50bp-300bp. Methylation interference, methylation protection, and DNAaseI footprinting analyses revealed the presence of a specific interaction between the fusion protein and a DNA sequence located some 800bp upstream of the start of the *pel*A coding region. This sequence bears similarity to the concensus binding sequence deduced by Dr. B. Felenbok for the *A.nidulans* CREA protein. Interestingly, the complex formed between the fusion protein and the *pel*A upstream target is relatively easily disrupted in comparison to 'homologous' complexes i.e. those between the *A. nidulans cre*A 'zinc-finger' fusion protein and *A. nidulans* DNA target sequences. This may account for the observed absence of a CCR effect on the expression of the *A. niger pel*A gene in *cre*A$^+$ *A. nidulans* transformants.

iii) CCR effects on iso-penicillin N synthetase (IPNS) transcription.....(MADRID).

CCR of IPNS transcription in *A. nidulans* (as well as of penicillin production) has been shown. Levels of IPNS transcript are derepressed in penicillin production broths with 3% lactose, 0.1% fructose, 0.1% glucose or 1% L-arabinose (i.e. derepressing C-sources or derepressing levels of a repressing C-source) and repressed in broths with repressing C-sources (3% sucrose or 1% D-glucose). CCR can account for the temporal pattern IPNS gene expression during the growth cycle (previously observed by Peñalva *et al*) as derepressed transcript levels are detected at all tested moments of the growth cycle in

derepressing C-sources or in a repressing C-source when its concentration drops under the level necessary for repression. The behaviour of IPNS transcription across a range of C-sources tested suggests that this gene is under the control of the transcriptional repressor CREA. Several *creA*^{d} (derepressed) alleles (provided by the London Laboratory) were tested for IPNS derepression. Only the more hypofunctional (*creA*^{d}2 and *creA*^{d}30) derepress slightly (3 fold) during some periods of the growth cycle. Homogeneous CREA::GST fused protein, containing the CREA DNA-binding region, has been obtained in large quantities (constructs provided by the Orsay laboratory) and studies of CREA binding to IPNS^{P} (IPNS promotor fused to a reporter) are in progress. Other objectives scheduled for later reports have been accomplished partially. Two mutations simultaneously affecting expression of an *ipn*::*lacZ* fusion and penicillin production have been isolated. Formal genetic characterisation of these mutations (involving the London and Madrid laboratories) has been carried out; other screening strategies will be tested (see 'MAJOR PROBLEMS (A1)'). Functional analysis of IPNS^{P} is nearly finished and is especialy useful for the identification of *cis*-acting sites for CCR.

iv) Interaction of CCR with pathway-specific induction mechanisms of the *alc* and *prn* regulons of *A. nidulans*.....(ORSAY).

(a) The ethanol regulon (*alc*).....(Dr. B. Felenbok).

ALCR

The transactivator ALCR is the pathway specific protein mediating specific induction of the two structural genes *alcA*, encoding alcohol dehydrogenase I (ADHI), and *aldA*, encoding aldehyde dehydrogenase (AldDH). The activation of transcription of *alcA* and *aldA* is very strong and this property has been widely exploited for the expression of foreign proteins in biologically active forms. ALCR has also been shown to control its own expression. The ALCR protein (821 amino acids) possesses a zinc binding domain containing a sequence of six Cys residues. This motif is related to the highly conserved zinc DNA-binding domains of the transcription factors of the 'C6' class of the ascomycetes, as exemplified by GAL4. At variance with other structures of this class, an asymmetric sequence of sixteen amino acids - where six are usually present - was found between the third and fourth cysteines; moreover, splicing occurs within this loop.

A structural model has been constructed for the ALCR binuclear zinc domain showing that the motif could adopt a folded structure in which two zinc atoms are tetracoordinated by the six cysteines in a folded clover-leaf structure. In addition, the structural model of the ALCR DNA-binding region predicted that an helix-turn-helix structure appears but with the ß turn replaced by the two zinc atoms. Finally, C-terminal to this DNA-binding motif, a putative leucine zipper was found. The *in vivo* function of these two latter sequences remains to be demonstrated. ALCR could combine three types of DNA-binding structures, namely zinc cluster, helix-turn-helix and leucine zipper.

cDNA clones encoding the zinc binding domain of ALCR were used in conjunction with the prokaryoyic expression vector pGEX-2T (Smith, D. B. and Johnson, K. S. (1988) Gene 67 p31-40) to construct a recombinant in which the zinc binding region of ALCR was fused with the gene encoding glutathione S transferase (GST) - resident on pGEX-2T - thus giving rise to the production of a fusion protein ALCR::GST which could be purified by affinity chromatography from bacterial cell extracts. Targets for the ALCR protein common to *alc*R and *alc*A were identified on their cognate promotors by gel retardation assays. Zinc was shown to be necessary for the binding activity of ALCR since removal led to complete loss of binding; activity could be restored by addition of exogenous zinc. Footprinting experiments using DNAaseI protection and methylation interference revealed clear protection of a common sequence - CCGCA - which occurs in two arrangements, either as a palindrome or a direct repeat. Two targets were identified well upstream of the *alc*R gene transcriptional start point (-460nt) and three were located upstream of *alc*A at positions -120nt and -280nt. The data are consistent with a model in which the ALCR protein makes contacts only with the G bases of the targets in the major groove of the DNA.

CREA

CREA has been shown to contain two 'zinc fingers' of the Cys-His class which are closely related to the 'zinc fingers' of MIG1 (involved in glucose repression in yeast) and other mammalian 'zinc fingers' such as the Krox family, Sp1, etc. (Dowzer and Kelly (1991) Molec. Cell. Biol. 11 p5701-5709)). Dr. J. Kelly provided a *cre*A cDNA clone which was used in conjunction with the prokaryotic expression vector pGEX-2T to construct a recombinant in which the zinc binding region of CREA was fused with the gene encoding glutathione S transferase in order to produce a CREA::GST fusion protein.

Gel retardation assays and DNAaseI footprinting of complexes between CREA::GST and both *alc*R and *alc*A promotors have shown in each case that the protected sequence comprises a core rich in Gs, very similar to those found for MIG1 and the Krox family. Three CREA binding sites were identified upstream of the *alc*R gene, and two upstream of *alc*A. The existence of specific targets for CREA upstream of the *alc*R and *alc*A genes provides a rationale for the repression of these two genes under CCR conditions.

(b) The proline cluster (*prn*).....(Prof. C. Scazzocchio).

The proline utilisation cluster of *A. nidulans* is under the control of the pathway-specific activator PRNA (also belonging to the C6 class of zinc DNA-binding proteins) and the two wide-domain regulatory proteins AREA (responsible for nitrogen metabolite repression) and CREA (responsible for CCR). The *prn* cluster is efficiently repressed under both nitrogen metabolite and carbon catabolite repression conditions.

DNAaseI footprint and methylation protection experiments have shown that the sequence containing prn^d mutations - manifest as derepression of the *prn*B, C and D genes in the presence of both ammonium and glucose - defines the binding site for CREA. Two CREA binding sites were found in the *cis*-acting region, occurring as inverted repeats, with cores rich in Gs and very similar to those found in the *alc*R and *alc*A promotors. The prn^d22 mutation shows an impaired affinity for CREA and a smaller protected region in the footprint patterns, thus providing a rationale for the derepression of the *prn*B, C and D genes under glucose and ammonium repression conditions.

An *A. nidulans* mutant has been constructed by transformation, lacking that part of the *cis*-acting region of the *prn* cluster defined by the prn^d mutations which contains the CREA binding site. This transformant was found to be derepressed in the presence of glucose, both at the level of *prn*B transcription and by genetic analysis,but nevertheless repressible by ammonium. Thus, whereas the utilisation of proline is normally under the control of AREA, in the absence of the particular part of the cis-acting region, AREA is necessary even in the absence of CREA. Hence, evidence is provided for the presence of an enhancer-like element whose removal results in the absolute requirement for the product of the areA gene. The existence of another transcription factor necessary for the transcription of prnB is proposed, the binding or action of which is prevented by CREA. The AREA protein would bypass the need for this factor by CREA or in absence of the element to which the transcription factor binds.

B) pH REGULATION.

i) Sequencing of the *A. nidulans pac*C gene.....(LONDON).

The *Aspergillus nidulans* pH regulatory gene *pac*C complementing activity was subcloned to a 2.6kb HindIII-BamHI genomic fragment. A 3319bp genomic region including all of the HindIII-BamHI fragment sufficient to complement $pacC^-$ mutations when integrated ectopically in a single copy has been sequenced. In addition two long but not full length cDNA clones together covering 2179bp up to the beginning of the poly(A) tail have been sequenced. There are two introns, 53 and 85 nucleotides in length. The 5' end of the transcript remains to be mapped. However, a pyrimidine-rich region covering more than 80bp and likely to be a feature of the *pac*C promotor lies within the HindIII-BamHI fragment and ends about 230bp upstream of the most 5' nucleotide present in the cDNA clones. Examination of the sequence between the pyrimidine-rich region and the cDNA clone suggests that the initiator methionine codon lies just under 60 nucleotides upstream of the 5' end of the cDNA clone with no open reading frames of any significant length upstream to the pyrimidine-rich region and beyond to the HindIII site. Coupled with an mRNA size estimated from northern blots at ~2.4kb, this predicts that *pac*C encodes a protein of 573 amino acids. The derived amino acid sequence contains three 'zinc fingers' of the Cys_2His_2 class near the N-terminus. It is possible that the most N-terminal of these is actually a more novel Cys_3His 'zinc finger'. There are 13 copies of the S/TPXX motif which occurs frequently in DNA-binding proteins. The sequence also contains two very acidic regions which might be involved in gene activation and a very basic region in addition to the 'zinc finger' containing basic regions. Just over 60bp downstream from the termination codon and well upstream of the 3' end of the mRNA lie four tandem copies of an 18bp direct repeat whose significance is at present unclear.

Studies of the regulation of *pac*C mRNA and expression of the *pac*C protein in *E. coli* are beginning, involving both the London and Madrid laboratories.

ii) Cloning of *A.niger pac*C.....(WAGENINGEN).

In tandem with the cloning of the *cre*A gene of *A. niger* (A(ii) above), we have also cloned a *pac*C related sequence from *A. niger* using an *A. nidulans pac*C probe (supplied by Prof. Arst). *A. niger pac*C equivalent phages have been isolated to single clone purity and analysed by Southern blotting. Two fragments, one BamHI and one EcoRI each of about 5kb in length, have been subcloned into pEMBL

vectors. These constructs are currently being fine-structure mapped and will then be used in transformation experiments of defined *A. nidulans pac*C mutants in order to determine their equivalence to the *A. nidulans* gene.

The cloning of the *A. niger pac*C gene has been accomplished earlier than anticipated (12-30 months, item 5).

2. METABOLIC ANALYSIS AND PHYSIOLOGICAL pH REGULATION STUDIES

A) METABOLIC ANALYSIS OF *ASPERGILLUS* SPECIES.

The fermentation conditions for *A. nidulans* have been improved in order that the fungus grows in homogeneous suspension thus permitting monitoring of the time-dependent uptake of substrates and the formation of carbon metabolites during *in vivo* ^{13}C-NMR measurements under carefully defined conditions. Moreover, different perchloric acid extraction methods have been tested to ensure that important metabolites, such as polyols, glycolytic intermediates and TCA cycle products can be simultaneously isolated at high yield.

B) PHYSIOLOGICAL APPROACH TO STUDY PH REGULATION AND PH HOMEOSTASIS.

In order to understand the various ion transport systems which are involved in pH homeostasis and pH regulation, two approaches to measure the intracellular pH in *Aspergillus* species have been utilised.....(WAGENINEN):

i) *in vivo* ^{31}P-NMR measurements.

The *in vivo* ^{31}P-NMR experiments were performed at the Max Planck Institut für Systemphysiologie, Dortmund, Germany, where a capillary system for aeration of samples was used, and at the Agricultural University of Wageningen, where aeration was performed via gassing stones which led to very high oxygen values in the cell suspension. Both systems yielded different results concerning the steady state pH value in *A. nidulans*. High aeration of the sample tube led to very alkaline pH values in the range between 7.8 and 8.0; additionally, phosphate and glycerophosphorylcholine (GPC) levels decreased very rapidly so that no real steady-state conditions were reached. In contrast, lower aeration (between 7% and 50%) led to more acidic intracellular pH values (approximately 7.3) and more stable GPC and phosphate levels for at least 60 minutes, indicating more steady-state like conditions.

ii) Intracellular pH measurements using pH sensitive fluorescent indicator dyes.

In the last few years several fluorescent pH-indicator probes have been developed and employed in techniques such as digital imaging microscopy and spectrofluorimetry. So far, application of this method to fungal cells has been limited to the marine fungus *Dendryphiella salina*. (J. Cell Sc. (1990) 96 p731-736). Along with the *in vivo* ^{31}P-NMR technique, the parallel approach to measure intracellular pH spectroscopically with pH sensitive fluorescent probes was chosen because of a number of specific advantages: it allows *in vivo* measurements, is very accurate, non-invasive, and even faster than *in vivo* ^{31}P-NMR, monitoring rapid pH changes down to milliseconds.

Several fluorescent dyes such as HPTS-acetate (8-hydroxy 1-3-6-pyrenetrisulfonate), SNAFL, and BCECF-AM (2',7'-bis-(2-carboxyethyl)-5(and-6)-carboxyfluorescein) were tested. The membrane-impermeable pH-indicators HPTS-acetate and SNAFL were loaded into the fungi by application of the acid-shock method developed for *Lactococcus lactis* (Molenaar *et al* BBA (1991) 1115 p75-83). Possibly due to the acid shock, the viability of the fungal cells was reduced in comparison to cells which were incubated with the BCECF ester which is membrane permeable. The fluorescence microscopic investigations showed that the probes tested preferentially accumulated in the vacuole, which was itself also microscopically identified. The loss of bright fluorescence (indicating pH changes) in these cell compartments by addition of the proton ATPase inhibitor CCCP (carbonyl cyanide m-chlorophenylhydrazone), hints that these organelles are involved in pH regulation processes at the subcellular level. Thorough comparative transmission electron microscopic (TEM) investigations in *A. nidulans* and *A.niger* wild type and *A. nidulans pal* A, C, F and *pac*C pH mutants (provided by Prof. H. Arst) revealed that a) the size of this vacuolar compartment varies during the germination and developmental process of the hyphae, b) vacuolar size is dependent on the location within the fungal cell i.e. the size reduces from the spore head to the hyphal tip, and c) the number and size of these organelles is somewhat species dependent.

Determination of the intracellular pH by fluorescence measurements were performed on a Perkin-Elmer LS 50 spectrofluorimeter with computer controlled data acquisition and storage. This quick fluorescence method requires the measurement of the fluorescence intensity at two fixed wavelengths and computing the ratio of these intensities. For correlation of the measured fluorescence signal to cytoplasmic pH, the probe was calibrated intracellularly using nigericin and valinomycin in calibration-buffers of

different pH values containing high amounts of potassium in order to dissipate intra- and extracellular proton gradients. Calibration curves (fluorescence ratio as a function of pH) were constructed from these treatments for comparison with experimental pH measurements. Preliminary pH measurements revealed that the addition of 10 mM NH_4Cl to the external medium led to an alkalinisation of the vacuolar compartment of 0.24 pH-units. The uptake of easily utilised carbon sources, such as glucose, fructose and sucrose resulted in a temporary acidification of the vacuoles. These results show that this cell compartment plays an important function in pH regulation because externally applied bases induce pH transitions within the vacuole and it appears that this compartment helps to diminish cytoplasmic pH changes.

HIGHLIGHTS/MILESTONES:

1) Identification of the ALCR and CREA binding sites is the first to be performed with *A. nidulans* transcriptional regulators. The transactivator ALCR seems to share properties of the three main groups of DNA-binding proteins. The molecular mechanism of induction and repression of the ethanol regulon is now well understood, as is the mechanism of double repression of the *prn* cluster.....(ORSAY).

2) IPNS expression has been shown to be under CCR. As *cre*A^d alleles do not fully derepress IPNS transcription, three possibilities arise: (i) a gene different from *cre*A controls CCR of secondary metabolism; (ii) very little *cre*A function is required for IPNS repression (conclusion based on incomplete derepression by *cre*A^d30); (iii) the combination of a repressing C-source and a *cre*A^d30 allele might result in a derepressed but non-induced situation. Experiments are in progress to distinguish these posibilities. Each is of intrinsic biotechnological interest.....(MADRID).

3) Through the use of *A. nidulans* probes isolated from genes of defined wide-domain function, namely *cre*A and *pac*C, it has been possible to isolate the equivalent sequences from the industrially valuable organism *A. niger* thus providing a means by which metabolic regulation in this organism can be analysed at the molecular level.....(WAGENINGEN).

4) *In vivo* ^{31}P-NMR spectroscopy is a powerful method to measure the cytoplasmic pH in young *Aspergillus* hyphae in which the vacuoles contain very low amounts of inorganic phosphate and thus are not monitored by this method. Additionally, pH sensitive fluorescent dyes (as BCECF) can be used to monitor selectively the intracellular pH of the vacuolar compartment of the fungal cells which plays an important role in cellular pH regulation.....(WAGENINGEN).

5) The presence of 'zinc fingers' and a high frequency of S/TPXX motifs in the derived amino acid sequence of *pac*C suppport our hypothesis that it is a transcriptional regulator. It is the first transcriptional regulator mediating pH regulation to be identified in any organism.....(LONDON).

WIDER CONSIDERATIONS:

1) The identification of the ALCR targets on the *alc*R and *alc*A promotors will facilitate the construction of superinducible promotors able to express higher amounts of heterologous protein. Disruption of the CREA binding sites should result in complete glucose derepressed *alc*R-*alc*A expression, allowing growth of these *A. nidulans* strains on a glucose medium. This feature is of valuable interest for industrial purposes.

2) Penicillin production is under CCR; IPNS is under CCR. Non-lethal, pleitropic *cre*A^d mutations do not fully release IPNS transcription and are useless for penicillin over-production. Mutations affecting *cis*-acting regions should avoid inconveniences caused by pleiotropic mutations.

3) The ability to control gene expression so that it is appropriate to ambient pH is an important feature which enables microorganisms to adapt to their environment. Microorganisms alter the repertoire of both enzymes and small molecules they secrete in response to ambient pH. We are now on the way to understanding how they do so and to being able to alter it.

4) Present investigations concerning intracellular pH measurements indicate that the fungal vacuole plays an important role in cellular pH control and deserves increased attention.

COOPERATIVE ACTIVITIES:

1) Prof. H. Arst visited the Orsay laboratory and examined a doctoral thesis in July 1991. E. Espeso (Madrid lab) spent six weeks in the London lab in July-August 1991 learning classical genetic techniques, analysing transformants and preparing IPNS mRNA from pH regulatory mutants. A large number of *A. nidulans* strains has been supplied by the London laboratory to each of the laboratories in Madrid, Orsay and Wageningen. The *A. nidulans pac*C clone was supplied to the Wageningen laboratory for cloning the equivalent gene in *A. niger*. He also provided advice to the Madrid group on aspects of CCR.

2) Dr. J. Visser visited Madrid for scientific discussion and supplied a number of mutations impairing glycerol utilisation. Dr. B. Felenbok provided the construct expressing CREA::GST polypeptide in *E. coli* and Prof. C. Scazzocchio provided a number of strains and continuous flow of information on *cis*-acting regions of CCR in the proline cluster. Strains carrying IPNS::*lacZ* fusion have been supplied to Orsay.
3) Dr. B. Felenbok and Prof. C. Scazzocchio (Orsay) have hosted two post-doctoral fellows (Dr. A. Ravagnani and Dr. A. P. MacCabe) who have spent twelve week and seven week visits, respectively, learning the techniques of analysis of nucleic acid/protein complexes.
4) The pH measurements with fluorescent pH sensitive probes and the ultrastructural studies were done in collaboration with Prof. Dr. W. Konings and D. Molenaar, Dept. of Microbiology, State University of Groningen, NL. Dr. I. von Recklinghausen spent several weeks in Groningen for that purpose.

The NMR measurements have been performed on the NMR spectrometers of the Molecular Physics Department, Agricultural University Wageningen and at the Max-Planck Institut für Systemphysiologie, NMR-Group (Prof. A.W.H. Jans), Dortmund, Germany.

Dr. I. von Recklinghausen participated in the International Postgraduate Course 'NMR (Nuclear Magnetic Resonance) in Agriculture: Plants, Animals, Products' held from 15 - 26 October 1991 at the AMEV Opleidingscentrum in Wageningen.

PUBLICATIONS:

E. Espeso and M.A. Peñalva (1992) 'Carbon catabolite repression can account for the temporal pattern of expression of a penicillin structural gene in *A. nidulans*'. Molecular Microbiology - in press.

Kulmburg, P., Prangé, T., Mathieu, M., Sequeval, D., Scazzocchio, C. and Felenbok, B. (1991) Correct Intron Splicing Generates a New Type of a Putative Zn-binding Domain in a Transcriptional Activator of *Aspergillus nidulans*. FEBS Letters 280 (1) p11-16.

Kulmberg, P., Sequeval, D., Lenouvel, F., Mathieu, M. and Felenbok, B. (1992) Identification of the Promotor Region Involved in the Autoregulation of the Transcriptional Activator ALCR in *Aspergillus nidulans*. Mol. Cell. Biol. 12 (5) - in press.

Sophianopoulou, V., Suàrez, T. Diallinas, G. and Scazzocchio, C. (1992) Operator Derepressed Mutations in the Proline Utilisation Gene Cluster of *Aspergillus nidulans*. Mol. Cell. Biol. - in press.

Stability of Genetic Information in *Bacillus*

BIOT - CT910268 Official Starting date : 01-04-91

Coordinator : S.D. EHRLICH, Génétique Microbienne, INRA, Jouy en Josas, FR
Participants :
K.M. DEVINE, Dept. Genetics, Trinity College, Dublin, IE
S. BRON, Dept. Genetics, University of Groningen, Haren, NL
M. SALAS, Centro de Biologia Molecular, CSIC, Madrid, ES
L. BOE, Dept. Microbiology, Technical University of Denmark, Lyngby, DK
B. DIDERICHSEN, NOVO Nordisk, Bagsvaerd, DK

OBJECTIVES

Isolation of mutant plasmid replication proteins and origins
Cloning of plasmid segregational stability functions
Cloning of the phage PBSX lysis gene
Cloning of basic replicons of large plasmids
Isolation of topoisomerase I mutants
Isolation of phage Phi29 DNA polymerase mutants
Construction of systems to study stability of genes inserted into the chromosome

MAJOR PROBLEMS ENCOUNTERED

In most of the participating laboratories the onset of the studies was delayed by the unexpected length of the contract processing. Less progress than anticipated was therefore made towards several of the objectives. This problem being solved it is foreseen that the research will now proceed with the optimal speed. A technical problem arose concerning isolation of large plasmid DNA from Gram positive bacteria, envisaged within the strategy of replicon cloning. This was solved by cloning replicons from total DNA preparations.

RESULTS

Mutant (+) replication origins and proteins (INRA)

Active site of the replication protein of the plasmid pC194 was analysed by site-directed mutagenesis. Alignment of 12 proteins from the related plasmids has shown six conserved tyrosines. One of these was contained within a stretch of amino acids resembling the active site of the *Escherichia coli* phage ΦX174 Rep protein, in which the Tyr remains bound to

DNA upon cleavage initiating rolling circle replication (RCR). Each of the conserved Tyr in the pC194 protein was changed to Phe and the activity of the protein was measured in an *in vivo* assay. Five of the changes had little effect, since >50% of the parental protein activity was still present. In contrast, change of the Tyr which corresponds to ΦX174 protein active site had a strong effect, since only ≈5% of the activity remained. To test whether the residual activity is due to another hydroxyl-amino acid in the vicinity, another Tyr was changed to Phe and two Ser to Ala. None of the single mutations affected the protein activity and the multiple mutations in conjunction with a critical Tyr -> Phe change did not abolish the residual activity. This indicates that either a hydroxyl group is not required for the activity of the protein or that a hydroxyl group from a distant amino acid, or even from a water molecule, can catalyse the cleavage. Both alternatives are unexpected and novel for a Rep protein of a RCR plasmid.

Segregational stability functions (INRA, Trinity College, Technical University)

(-) origins affect stability of RCR plasmids. The features essential for functioning of the (-) origin of the plasmid pBAA1 and its role in the plasmid segregation stability were investigated. This analysis has revealed that at least two stem-loop structures are important for activity. Deletions which affect the integrity of stem-loop I result in total inactivation of the origin. Deletion of stem-loop III results in partial inactivation of the origin. The function of the (-) origin was restored by replacing stem-loop III by a different stem-loop structure placed at a correct distance from stem-loop I. This suggests that functioning of the minus origin requires at least two stem-loop structures: stem-loop I, both the structure and the sequence of which may be important, and stem-loop III, only the structure of which is required. The analysis has also shown that RNA polymerase is involved in conversion of the single-stranded plasmid to the double-stranded form. The minus origin of pBAA1 was shown to function not only in *B. subtilis* but also in *S. aureus*. In addition, another (-) ori was isolated from plasmid pTX14-3, and shown not to require RNA polymerase, which is different from previously described RCR (-) origins.

Insertion of foreign segments in RCR plasmids often leads to the appearance of high molecular weight plasmid forms (HMW), which, in turn, cause plasmid instability, possibly because of (i) lowering number of separate plasmid units in the cell and (ii) slowing the growth rate of the plasmid-carrying cells. Using *Escherichia coli* as a host it was shown that such forms arise if a CHI site (an 8 bp sequence) is introduced on a plasmid. This site protects HMW molecules, which are probably formed by accidental lack of termination of the RCR, from degradation by the RecBCD exonuclease. Plasmid stability should therefore be increased if the CHI sites were eliminated from the cloned inserts. A locus which suppresses HMW formation, by a presently unknown mechanism, was detected on plasmid pTX14-3.

Mutation rate on plasmid multimers was increased relative to monomeric plasmids in *E. coli*, raising a possibility that plasmids which form large amounts of HMW are hyper-mutable. It is possible that the high mutability is due to the formation of single-stranded regions, by homologous base pairing between plasmid copies out of register. Furthermore, the mutation rate was affected by the rate of translation errors in *E. coli*, possibly by generating error-prone DNA polymerase. However, such relation was not established yet in *B. subtilis*.

A system contributing to the stability of the large theta-replicating plasmid pAMβ1 and comprising a resolvase and the cognate resolution site was characterised.

Lysis gene of the phage PBSX (NOVO, Trinity College)

In an attempt to introduce the phage lysis gene under repressor control the IPTG-inducible SPAC promoter was integrated at various sites of the phage late operon. However, only a low level of phage-specific mRNA and proteins was detected upon induction and the cell lysis was not observed, which indicates that the promoter activity was relatively weak. In contrast, phage protein production was observed in cells carrying the phage thermosensitive repressor. Deletion of most of the phage sequences which encode head and tail proteins will be attempted, which should fuse the lysis gene to the late phage promoter. The lysis gene appears to map on the phage segment 39, as deduced by sequence homology of the segment with the *B. subtilis cwlA* gene, which encodes N-acetylmuramoyl-L-alanine amidase.

Replicons of large plasmids (INRA, Trinity College)

Basic replicons of several large plasmids were cloned, using a method which involved (i) cleavage of total DNA isolated from plasmid-containing strains by different restriction enzymes, (ii) ligation of the resulting segments to a replicon-cloning vector and (iii) transformation of *B. subtilis* competent cells. Structural and segregational stability of the derived plasmids is currently being investigated.

Replication region of pAMβ1 was shown to consist of two domains. One, named replication domain, comprises two essential elements, the Rep protein and the origin, which suffice to confer broad host range upon the derived plasmids. It also contains several non-essential elements, which appear to (i) help initiation of the lagging DNA strand and (ii) interfere with the formation of HMW. The other domain, named expression domain, consists of elements which ensure expression of the Rep protein. It appears to include two negative regulators, a protein and a counter-transcript driven transcription terminator. Two related plasmids, pIP501 and pSM19035, have a similarly organised replication region.

B. subtilis topoisomerase (Groningen University, INRA)

Two approaches are being followed for the isolation of the topoisomerase I gene. One is to amplify a segment of the gene by PCR, using primers corresponding to the conserved regions of different topoisomerases. Several segments were amplified with these primers and are presently being sequenced. If one of them derives from the topoisomerase gene it will be used as a probe for cloning of the entire gene. Another approach is to purify topoisomerase I and determine its N-terminal sequence, which should allow to design a probe for cloning of the corresponding gene. An assay was set to measure the topoisomerase activity in the *B. subtilis* cell extracts and the isolation of the protein has started.

Φ29 DNA polymerase mutants (CSCI)

Φ29 DNA polymerase mutants have been isolated in two regions at the carboxyl part of the protein, conserved among α-like DNA polymerases, characterised by the motifs "D-NSLYP" and "K—NS(L/V)YG" (regions 1 and 2A, respectively). The mutant proteins Tyr^{254} -> Phe (Y254F) and Tyr^{390} -> Phe(Y390F) or Tyr^{390} -> Ser(Y390S), in conserved regions 1 and 2A, respectively, were purified and characterised. The results obtained indicate that residues

Tyr^{254} and Tyr^{390} are involved, directly or indirectly, in Me^{2+}-dNTP binding.

A complete alignment of 33 DNA polymerases has allowed to identify, in addition to the most conserved regions, *ExoI, ExoII* and *ExoIII*, other segments of amino acid similarity. These contain residues involved in single-stranded DNA binding at the three 3'->5' exonuclease domains of *E. coli* polI. Φ29 DNA polymerase mutants in residues Tyr^{165} (Y165F and Y165C) and Asp^{169} (D169A) were isolated, purified and characterised. The results indicate that the two residues form part of the Φ29 DNA polymerase exonuclease domain. Interestingly, none of the three mutant polymerase were able to produce strand displacement, which is a property of the wild type Φ29 DNA polymerase. This suggests that the strand displacement and 3'->5' exonucleases active sites overlap and probably share the single-stranded DNA binding cleft.

The interaction of protein p6 with the Φ29 DNA ends was also studied by constructing head-to-tail concatemers of a 24 bp p6 binding sequence (p6 dimer binding unit). The protein binds to these sequences at the same position as in the replication origin but with higher affinity. Series of pUC19 derived plasmids contained in different number of p6 dimer binding units were used to measure changes in linking number induced by protein p6. The observed change was .25. Formation of p6 DNA complex was also confirmed by electron microscopy. Site directed mutagenesis of the amino end of protein p6, previously shown to be required for DNA interaction, has shown that the basic amino acid Lys^2 an Arg^6 are involved in DNA binding.

Stability of genes inserted in the chromosome (INRA)

Multiple tandemly repeated copies of foreign genes inserted in the chromosome of *B. subtilis* allow to obtain increased production of the proteins they encode. Recombination between directly repeated sequences affects stability of these genes. To study factors which affect such recombination, a system consisting of directly repeated sequences of 900 bp, which generate a functional tetracycline resistance gene by recombination was developed. The system allows to directly select the recombinants and therefore to measure relatively low recombination frequencies.

HIGHLIGHTS/MILESTONES

(a) Unexpected activity of the Rep protein of a RCR plasmid was revealed.

(b) CHI sequence was shown to be a critical factor in the formation of high molecular weight (HMW) plasmid multimers, which affect the stability of RCR plasmids.

(c) Phage PBSX lysis gene was identified.

(d) Replication region of a representative large plasmid was characterised.

(e) Minus origin of a RCR plasmid was characterised.

WIDER CONSIDERATIONS

Stable maintenance of foreign genetic information is essential for amelioration of industrially important microorganisms. Small plasmids which replicate as rolling circles are widely used for DNA cloning in Gram positive bacteria, which is an important class of industrial microorganisms, and their biology is relatively well known. In contrast, large theta-replicating plasmids have been used only recently for cloning in these bacteria and are less well characterised. However, the well-known small plasmids seem to be intrinsically less stable than the large less-known plasmids. Much of the research during the past year was orientated on the one hand towards understanding the instability and improving the stability of the small plasmids and on the other towards isolating new and better characterising the known large plasmids. *Bacillus subtilis* was used as a model host, and processes known to affect stability of genetic information, such as DNA replication of plasmids and phage were studied. Better understanding of the genetic stability is expected to have impact on a number of biotechnology applications.

COOPERATIVE ACTIVITIES

Two meetings between people from Novo Nordisk and Trinity College Dublin have taken place: (1) 10th-11th of January 1991, (2) 27th-28th of June 1991, both at Trinity College. One scientist from Nov Nordisk was working in the Genetics Department at Trinity College from January 3rd to July 1st, 1991. Exchange of materials has taken place during the whole period.

Also, two meetings took place between Génétique Microbienne (INRA) and the Department of Genetics (Groningen), one in Groningen in December 1991, and one in Jouy en Josas in January 1992. Exchange of material between these two groups took place at the second meeting. There was also a continuous exchange of material between the Department of Genetics (Trinity College) and Department of Genetics (Groningen).

PUBLICATIONS

Individual publications :

L. Boe (1992) Translational errors as the cause of mutations in *Escherichia coli*. Mol. Gene. Genet, in press.

The following manuscripts were submitted for publication since the beginning of 1992.

P. Dabert, S.D. Ehrlich, A. Gruss (1992) The chi sequence protects afainst recBCD degradation of DNA *in vivo*.

D. Vilette, M. Uzest, S.D. Ehrlich, B. Michel (1992) DNA transcription and repressor binding affect deletion formation in *Escherichia coli* plasmids.

I. Biswas, V. Vagner, S.D. Ehrlich (1992) Efficiency of homologous intermolecular recombination at different locations of the *Bacillus subtilis* chromosome.

E. Le Chatelier, S.D. Ehrlich, L. Jannière (1992) A family of theta replicating plasmids in Gram-positive bacteria.

E. Le Chatelier, S.D. Ehrlich, L. Jannière (1992) The replication region of the *Enterococcus faecalis* plasmid pAMβ1 contains and expression and a replication domain.

L. Seery and K.M. Devine (1992) Analysis of the features contributing to the activity of the origin of complementary-strand synthesis of the *Bacillus* plasmid pBAA1.

M.A. Blasco, J.M. Lazaro, A. Bernad, L. Blanco and M. Salas (1992) Φ29 DNA polymerase active site : mutants in conserved residues Tyr^{254} and Tyr^{390} are affected in dNTP binding.

TITLE: *Physiology and molecular genetics of amino acid production and secretion by corynebacteria: Flux of intermediates and feedback control mechanisms*

CONTRACT NUMBER: *BIOT-CT91-0264 (RZJE)*

OFFICIAL STARTING DATE: *01/07/1991*

COORDINATOR: *Juan F. Martín, Universidad de León, 24071 León, Spain*

PARTICIPANTS: *Hermann Sahm, Forschungszentrum Jülich GmbH, Germany*
Kieran Dunican, University College, Galway, Ireland
Alfred Pühler, Universität Bielefeld, Germany
Carlos Blanco, Université de Rennes I, France

OBJECTIVES SET FOR THE REPORTING PERIOD:
-Characterization of plasmid sequences required for stability
-Cloning genes involved in TCA cycle
-Characterization of phage int sequences
-Study of different corynephages
-Conjugal gene transfer
-Development of methods for construction of an encyclopedia of corynebacteria
-Gene expression of the tryptophan operon
-Characterization of promoters of corynebacteria: threonine biosynthetic genes

MAJOR PROBLEMS ENCOUNTERED: *No major problems have been encountered.*

RESULTS

1. Characterization of DNA sequences required for plasmid replication and stability (Martín)

The characterization of DNA sequences involved in stability and replication of plasmid pBL1 (an endogenous plasmid of B. lactofermentum from which most of the available vectors of corynebacteria are derived) is important to develop vectors with high stability in submerged cultures. Deletion studies and subcloning of essential regions for plasmid replication, stability and control of the copy number revealed that the HincII-SphI fragment (1.8 kb) of pBL1 is essential for its replication and it contains two ORFs, ORF1 which encodes a Rep protein and ORF5 of unknown function. The Rep protein encoded by ORF1 shows amino acid similarity with several known Rep proteins. The 0.8 kb HindIII-SphI of pBL1 is necessary for the stability of pBL1 derivatives in B. lactofermentum. We have obtained very high copy number (runaway) derivatives of pBL1. The copy number of pBL1 derivatives increased when the terminator of the tryptophan operon from B. lactofermentum (Ttrp) was cloned adjacent to the HindIII site which might prevent readthrough from a putative ORF

that negatively controls the copy number. There is a good homology between the nucleotide sequence of a DNA region present in the HindIII-SphI fragment of pBL1, and the RNA I of E. coli (which control the copy number in ColE1-derived plasmids) what suggests that an antisense RNA mechanism may be involved in the control of the copy number of plasmids in corynebacteria.

2. Characterization of enzymes and cloning genes involved in the TCA cycle (Sahm)

The specific activities of pyruvate kinase (PK), citrate synthase (CS), isocitrate dehydrogenase (ICDH), isocitrate lyase (ICL), malate synthase (MS), and glutamate dehydrogenase (GDH) have been determined in cell extracts from C. glutamicum after growth on different media. The results show that PK, CS, ICDH, and GDH were present constitutively with activities between 0.7 and 1.9 µmol/min mg protein. ICL and MS are 100-fold inducible by acetate and showed specific activities of 2.4 and 0.9 µmol/min mg protein, respectively. On activity level no significant regulation was observed for CS, for MS, and for GDH. PK is stimulated by AMP; ICDH was severely inhibited by oxaloacetate and glyoxylate and ICL by phosphoenolpyruvate.

The C. glutamicum CS gene (glt) and the C. glutamicum GDH gene (gdh) could be isolated by heterologous complementation of appropriate E. coli mutants. Subcloning and subsequent sequencing of respective DNA fragments identified open reading frames coding for polypeptides which show significant identity to CSs and GDHs from other organisms. Both cloned genes could be expressed in C. glutamicum. Transcriptional analysis of the gdh gene revealed that gdh is a monocistronic gene. The promoter region of gdh was identified by RNase protection assay.

3. Study of different corynephages. Characterization of corynephages isolated from soil (Blanco)

Two strategies were developped to isolate bacteriophages active on corynebacteria. First by direct isolation of phages in soil samples, and secondly by induction of integrated prophages in collection strains. All these studies were perfomed on 40 coryneform strains covering the genera Corynebacterium, Brevibacterium and Arthrobacter.

Various corynephages were isolated from soil samples. Restriction analysis of phage DNA indicated that 24 phages were unique. One of them was identical to the previously characterized phage CG33. There is only a minor difference on the restriction map resulting of the loss of a HindIII site. Twenty three of them are virulent phages, their hosts are essentially composed of Arthrobacter species.

The unique temperate phage, AAU2, made turbid plaques on Arthrobacter aureus. It is active only on Arthrobacter species. One virulent mutant was isolated that is unable to make plaques on the lysogenised strain, and thus seems affected on the repressor of lytic functions. AAU2 was characterized morphologically. It presents a polyhedral head

of 50 nm wide and a non contractile tail of 150 nm, and it was classified in Ackerman group B1.

Its genome is composed of a 45 kb double stranded DNA with cohesive ends. Its restriction map was achieved for 4 restriction enzymes. The chromosomal integration site was mapped for 10 independent lysogenic strains, and revealed to be unique. We have mapped some structural capsid proteins, and the phage attachment site. The cloning of integrative functions and chromosomal attachment site is in progress.

4. Induction of integrated prophages (Blanco)

Since no temperate phages infecting Corynebacterium were isolated from soil samples, integrated prophages on Corynebacterium and Brevibacterium strains were released by induction.

After UV induction 7 strains revealed to be lysogenic. Induced phages were able to infect other strains and in all cases caused the formation of turbid plaques. Their host spectrum is actually limited by restriction of DNA in the recipient strain. It could be enhanced by improving the titer of the phage stocks. The study of these phages is in progress; they constitute good candidates to construct broad-host range integrative vectors.

5. Conjugal gene transfer (Pühler)

The conjugal gene transfer from E. coli to C. glutamicum (Schäfer et al., 1990) was investigated in more detail with respect to factors which influence the efficiency of transfer of mobilizable plasmids. Besides a heat treatment step, we found other methods to render corynebacterial cells more susceptible to conjugal transfer. These include treatments with ethanol, detergents, acids and bases. The method using sublethal concentrations of ethanol prior to mating produces frequencies as high as the original heat treatment, whereas the other methods displayed lower frequencies.

Since all the treatments that led to high frequency transfer are thought to introduce changes in the cytoplasmic membrane, it is proposed that the restriction enzyme(s) influencing the transfer efficiency are membrane associated.

An attempt to clone the gene(s) responsible for the restriction phenomenon by complementing a restriction-deficient C. glutamicum strain was sucessfull. For this purpose, a mating method on agar plates was developed that allows the simultaneous testing of large quantities of clones for restriction.

The complementing fragment of 6.7 kb will be sequenced and a restriction deficient mutant will be constructed by gene disruption (Schwarzer and Pühler, 1991) using an internal fragment of the gene responsible for restriction.

6. Development of methods for construction of an encyclopedia of corynebacteria

A. (Pühler)

An important tool for the construction of an

encyclopedia is the cosmid vector. To expand the use of a cosmid gene bank, the vector should be equipped with a corynebacterial replication region and the mobilization region. To achieve this goal, the corynebacterial replicon pBL1 was choosen, which displays stable replication and a relatively low copy number. The plasmid was introduced into the mobilizable E. coli vector pK18::mob and the resulting vector was supplied with the cos-fragment from pSUP205 to give pEBMC1, a mobilizable shuttle cosmid vector with several single restriction sites for cloning and a high transfer frequency from E. coli to Corynebacteria. At the moment, the feasibility of this vector for cosmid cloning and conjugal cosmid transfer is investigated.

The construction of ordered cosmid clone banks will be greatly facilitated, if the location of all repetitive sequences in the corynebacterial genome that might cause confusion during linking of adjacent cosmid clones is known. For these purposes, we investigated known repetitive sequences as transposable elements and ribosomal RNA clusters in C. glutamicum.

An AEC-resistant mutant of C. glutamicum displaying a defective lysI gene coding for lysine and AEC uptake (Seep-Feldhaus et al., 1991) was investigated by Southern hybridization. Within the lysI gene an insertion of around 1.5 kb was detected. The region containing the insertion was amplified by using the PCR method and cloned. DNA sequencing of the inserted DNA region revealed a typical insertion element of ca. 1450 bp showing 24 bp inverted repeats. ISCg1 has 4 to 7 copies in the chromosome of C. glutamicum and is also present in B. flavum and C. herculis.

Bacterial ribosomal RNA clusters contain the coding regions for the three ribosomal RNAs and are present in multiple copies in the chromosome due to the requirement for huge amounts of these RNA species. To investigate the number and the location of rRNA clusters in C. glutamicum, PCR was performed with primers synthesized according to the known sequence (Park et al., 1987) in the case of 5S rRNA and according to conserved sequences in the case of 16S rRNA. Both rRNA genes were sucessfully amplified and the 5S rDNA directly sequenced confirming the identity of our C. glutamicum ATCC 13032 and ATCC 13058 strains with the C. glutamicum CNF 016 (ATCC 13032) type strain (Park et al., 1987).

The PCR products will be cloned and sequenced and the locations and copy numbers of the corresponding operons will be determined in the near future.

B. (Martin)

Using pulse field gel electrophoresis we have established that the genome of B. lactofermentum has a size of 2350 kb, and that of C. glutamicum 2400 kb. Restriction endonucleases recognizing AT rich sequences like PacI (target sequence TTAATTAA) and DraI (target sequence TTTAAA) cleave the genomes of B. lactofermentum, C. glutamicum or B. linens in fragments that can be resolved by pulse-field gel electrophoresis (PFGE). Eighteen PacI

fragments were found in the genomes of B. lactofermentum and C. glutamicum with a size range of 5-450 kbp. The genome of B. linens was resolved into 8 DraI fragments, ranging from 90 to 850 kbp. Other restriction enzymes recognizing GC-rich octanucleotides like NotI and SfiI also produced discrete bands in the genome of B. lactofermentum and C. glutamicum. Other restriction enzymes like XbaI, SspI and AseI have also been useful for mapping large fragments of B. linens.

Cloned genes for amino acid biosynthesis available in the laboratory have been located in the different fragments by Southern hybridization. Strategies for obtaining linking clones are being used to construct ordered encyclopedias of the genome of corynebacteria.

7. Gene expression of the tryptophan biosynthetic genes

A. Cloning and sequence analysis of the DAHP synthetase gene of C. glutamicum (Dunican)

The DAHP synthetase gene in coryneform bacteria catalyses the initial step in the general aromatic amino acid pathway. This major pathway controls the flow of intermediate metabolites for the synthesis of the amino acids tryptophan, phenylalanine and tyrosine. There are three isomers of the gene in E. coli each individually feed-back inhibited by one of the three aromatic amino acids. Biochemical evidence suggests that there is only one copy of this gene in the coryneform bacteria.

The DAHP gene was cloned using a synthetic oligonucleotide designed from a region conserved in the aroF, aroG and aroH genes of E. coli by probing an EMBL111 bank constructed from C. glutamicum 21850. A fragment was sub-cloned which was capable of complementing E. coli AB3257 which has mutations in all three DAHP genes. The gene has been sequenced and shows a remarkable similarity to regions of the three E. coli aro genes. Circa 45% of the amino acids were identical. The DAHP gene of C. glutamicum contained 375 amino acids which corresponds closely to the molecular weight of the protein isolated biochemically from C. glutamicum. Analysis of the cloned region showed that:

a) Upstream of the gene there is a region which could serve as a promoter for this gene. Sequence information of regions upstream and downstream suggests that this C. glutamicum aro gene is not part of a larger operon.
b) The gene is a little longer than the E. coli genes with short additional stretches at the 5' and 3' ends.
c) A conserved sequence at amino acid 148 shows very high homology with the allosteric site in the aroF gene of E. coli, i.e. the amino acid binding pocket for tyrosine (Weaver and Herrmann, 1990). For this reason it has been decided to designate this C. glutamicum gene aroF to maintain the E. coli nomenclature.

B. Regulation of the tryptophan operon (Dunican)

This operon and particularly the trpE (initial gene in the operon) has been studied intensely. Work in Galway is focused on this gene which was isolated from a derepressed

strain of C. glutamicum which was resistant to 5-methyl tryptophan. Results show that a region upstream of this trpE gene confers resistance to 5-methyl tryptophan when cloned into E. coli. The regulatory role of this gene is being studied by integrating a portion of this region into wild-type C. glutamicum.

C. Gene expression of the tryptophan operon (Martín)

We are approaching the characterization of the expression of genes involved in the tryptophan biosynthesis in corynebacteria. Our goal in León is to increase tryptophan production by gene dosage amplification and by removal of the feedback regulatory mechanism that limits tryptophan biosynthesis. Initially, four DNA fragments of B. lactofermentum that complement mutations in the trpB, trpC(F) and trpD genes of E. coli were cloned (Del Real et al., 1985). Later the whole operon was cloned into E. coli in a 9.6 kb BamHI DNA fragment, using pUC13 as cloning vector. This construction, called pULW7, has served as the starting point to subclone the operon regulatory signals (promoter, attenuator and terminator). These regulatory sequences have been characterized and altered by in vitro mutagenesis.

The main promoter of the operon was located in a 0.55 EcoRI-HindIII fragment that was subcloned in pUC118. The transcription initiation site was determined by S1 mapping. A protected fragment of 266-267 nt indicated that transcription starts in an adenine located 16 bp downstream from the putative -10 region. A ribosome binding sequence was found 9 bases downstream of the first transcribed nucleotide. An inverted repeat overlaps with the -10 region of the promoter.

Northern analysis of total RNA of B. lactofermentum using an internal fragment of the trpA-trpB region showed that a transcript of about 7 kb is formed, although the exact size of this large polycistronic transcript corresponding to the entire operon is difficult to estimate precisely. By subcloning experiments in which 2.2 kb NruI-BglII carrying the end of trpC and the trpB and trpA genes was subcloned in the vectors pULMJ51 and pULMJ55, it was shown that an internal promoter located of the end of the trpC gene appears to exist. No significant band of hybridization corresponding to this short transcript was observed in Northern hybridization of total RNA of B. lactofermentum what suggests that this internal promoter is not used significantly when the entire operon is being transcribed.

The promoter and attenuator of the operon have been changed by directed in vitro mutagenesis. These mutations have allowed us to construct mutants with altered expression of the tryptophan operon.

8. Caracterization of promoters of amino acid-producing corynebacteria (Martín)

A characterization of several promoters of corynebacteria has been made, including promoters of the

pBL1 plasmids, the threonine genes, and the tryptophan operon of B. lactofermentum. Two different types of DNA fragments with promoter activity were found in the cryptic plasmid pBL1. Four of the eight promoters cloned from pBL1 showed promoter activity in E. coli and B. lactofermentum while the other four are expressed only in corynebacteria. The first group has been named corynebacterial-E. coli-like promoters (CEP) whereas the second group has been designated corynebacteria-specific-promoters (CSP). The strength of such promoters has been determined by subcloning them in promoter-probe vectors (Cadenas et al., 1991). Several promoters have been sequenced and characterized by S1 mapping. CEP promoters are very similar to those of consensus E. coli with a standard spacing of 17 nt. The CSP promoters are probably similar to the promoters of Streptomyces genes involved in the biosynthesis of antibiotics and other secondary metabolites.

There are two promoters (Phom, PthrB) in the threonine cluster hom-thrB of B. lactofermentum that was cloned previously. Phom is the main promoter of the cluster in corynebacteria, but it is not functional in E. coli, i.e. it is a CSP type promoter. PthrB is located between hom and thrB, and is expressed both in E. coli and corynebacteria, i.e. it is a CEP-type promoter.

HIGHLIGHTS/MILESTONES:- *DNA sequences required for plasmid replication and stability in corynebacteria have been characterized.*

- *Citrate synthase and glutamate dehydrogenase genes of corynebacteria have been cloned.*
- *Several corynephages have been isolated from soil, and also by induction of integrated prophages.*
- *An efficient conjugal gene transfer from E. coli to C. glutamicum has been developped.*
- *A DNA fragment that complements a restriction deficient C. glutamicum strain has been cloned.*
- *Methods for construction of cosmid gene banks of corynebacteria have been established. A transposable element has been identified.*
- *Using restriction endonucleases that recognize very infrequent restriction sites and pulse field gel electrophoresis, the genome size of several corynebacteria has been determined. The genome of C. glutamicum, B. lactofermentum and B. linens has been resolved in discrete large fragments and the cloned amino acid biosynthetic genes have been mapped, by hybridization, in those fragments.*
- *The DAHP synthetase gene (aroF) was cloned and sequenced.*
- *Regulation of the tryptophan operon has been studied in the wild type and in a 5-methyl tryptophan resistant clone.*
- *The promoter region of the tryptophan operon of B. lactofermentum has been characterized by S1 mapping and their strenght studied in promoter-probe vectors.*
- *A 7 kb polycistronic mRNA for the entire tryptophan operon was found in B. lactofermentum, whereas no significative transcription occurred from an internal promoter.*
- *Two types of promoters were found in corynebacteria. The CSP type are only expressed in corynebacteria, whereas the CEP type promoters are expressed in E. coli and corynebacteria*

WIDER CONSIDERATIONS: *An array of basic techniques for genetic manipulation of amino acid producing corynebacteria and related Gram-positive organisms have been developed by the five groups involved in this project. New improvements for gene transfer technology has lead to cloning several genes involved in intermediary metabolism of corynebacteria, i.e. genes that provide precursors (building blocks) for amino acid biosynthesis, and also genes directly involved in the production of several amino acids (tryptopahn, threonine, lysine, etc.). New phage-derived tools are also being implemented for genetic modification of corynebacteria.*

Techniques of pulse field electrophoresis have been sucessfully used for isolating large frgments of the genome of several corynebacteria. In combination with cosmid gene libraries, this technique is being applied for construction of ordered encyclopedias of the genome of B. lactofermentum and C. glutamicum.

Characterization of corynebacterial promoters and regulatory sequences, and directed in vitro mutagenesis of those sequences, has led to construction of strong expression systems in which production of amino acids is deregulated.

COOPERATIVE ACTIVITY: *A close conection has been already established between the five participating laboratories. Dunican (Galway) and Martín (León) have exchanged strains, plasmids and gene fragments. Blanco (Rennes) has provided phages to Sahm. A scientist from Martín laboratory (Carmen Guerrero) was working for one month in Galway, and one scientist of Galway (Angela Fegan) worked for two months in León. Dr. Luis M. Mateos and Ms. Cristina Fernández from the group of Martín worked in Pühler's lab for three (August-November, 1991) and two months (September- November, 1991) respectively.*

All the groups involved have also scientific conections with the major amino acid-producing companies in Europe, Degussa, S.A., Orsan, and some smaller companies (Bioproces, S.A.). A first coordination meeting of the scientist involved in the project was held in Orsan at Les Ulis (Paris) on February 1991. The First Annual Meeting of the project is scheduled to take place in late May in Paris, after the initial 11 months of the project.

LIST OF JOINT PUBLICATIONS/PATENTS WITH TRANS-NATIONAL AUTHORSHIP: *Work is not yet published.*

OTHER PUBLICATIONS/ PATENTS:

- *Börmann, E.R., Eikmanns, B.J. and Sahm, H. (1992) Mol. Microbiol. 6:317-326.*
- *Cadenas, R.F., Martín, J.F. and Gil, J.A. (1991) Gene 98:117-121.*
- *Del Real, G., Aguilar, A. and Martín, J.F. (1985) Biophys. Res. Commun. 133:1013-1019.*
- *Guerrero, C., Mateos, L.M., Malumbres, M. and Martín, J.F. (1992) Appl. Microbiol. Biotechnol. 36:759-762.*
- *Martín, J.F., Cadenas, R.F., Malumbres, M., Mateos, L.M., Guerrero, C. and Gil, J.A. (1990) In: Genetics of Industrial Microorganisms, Strasbourg, pp. 285.*
- *Park, Y.-H., Hori, H., Suzuki, K.-I., Osawa, S. and Komagata, D. (1987) J. Bacteriol. 169:1801-1806.*
- *Schäfer, A., Kalinowski, J., Simon, R., Seep-Feldhaus, A.-H. and Pühler, A. (1990) J. Bacteriol. 172:1663-1666.*
- *Schwarzer, A. and Pühler, A. (1991) Bio/Technology 9:84-87.*
- *Weaver, L.M. and Herrmann, K.M. (1990) J. Bacteriol. 172:6581-65-84.*

TITLE : Valorization of non-conventional yeasts of industrial interest : exploration and molecular engineering of their genetic constituents.

CONTRACT NUMBER BIOT - CT91-0267 (DSCN)

OFFICIAL STARTING DATE: 1 April 1991

COORDINATOR: FUKUHARA, Institut Curie-Section de Biologie, Orsay, FR

PARTICIPANTS :

1) FUKUHARA, Institut Curie - Biologie, Orsay, FR
2) FRONTALI, University of Rome, IT
3) HOLLENBERG, University of Düsseldorf, DR
4) DOMINGUEZ, University of Salamanca, ES
5) MEACOCK, University of Leicester Biocentre, UK
6) FERRERO, Genetics Institute, University of Parma, IT
7) GAILLARDIN, INRA, Thiverval-Grignon, FR
8) FLEER, Rhône-Poulenc Rorer, Vitry-sur-Seine, FR
9) VERBAKEL, Unilever Laboratorium, Vlaardingen, ND

OBJECTIVES SET FOR THE REPORTING PERIOD :

* *PARTICIPANT 1* : Genetic and physical mapping of *K. lactis* genes ; assessment of strain variability ; isolation of maximal number of mutations ; construction of yeast-yeast shuttle banks ; characterization of killer plasmid promoters.

* *PARTICIPANT 2* : Identification of *ADH* genes from *K. lactis* genomic library ; identi- fication and sequencing of chromosomal *ARS* sequences from *K. lactis* ; identification of *ARS* consensus sequences.

* *PARTICIPANT 3* : Analysis of regulation of methanol oxidase promoter in *H. polymorpha* ; establishement of a *K. lactis* promoter based expression system.

* *PARTICIPANT 4* : Cloning of the acid phosphatase gene of *K. lactis* and of metalloproteins of *K. lactis* and *Y. lipolytica* ; genetic and physical mapping of *K. lactis* genes; isolation of *K. lactis* secretory mutations.

* *PARTICIPANT 5* : Analysis of the patterns of protein synthesis vs. environmental stresses ; search of stress-response genes ; analysis of specific mRNA abundancies vs. stress and growth phase ; attempts of cloning stress response genes or construction of a cDNA library from differentially expressed genes.

* *PARTICIPANT 6* : Isolation and genetic characterization of new Rag$^-$ mutations involved in sugar metabolism ; physiological characterization of Rag$^-$ mutations ; definition of parameters of catabolite repression in *K. lactis* and selection of catabolite repression mutants.

* *PARTICIPANT 7* : Improving conditions for chromosome separation in *Y. lipolytica* ; identification and characterization of *SEC* genes ; construction of genomic library of *Y. lipolytica* on replicative vectors ; assessing maintenance of *K. lactis* killer plasmids in *Y. lipolytica*.

* *PARTICIPANT 8* : Choice of a reporter gene, construction of expression cassettes ; construction of vectors allowing targetted integration of expression cassettes ; transfer of the same cassettes to stable plasmid vectors ; transformation of selected yeast strains.

* *PARTICIPANT 9* : Develop and evaluate general fermentation characteristics of commercial *Kluyveromyces* strains ; cloning of regulator sequences ; develop cloning system for selected commercial *Kluyveromyces* strains ; evaluation of engineered yeasts (*Kluyveromyces* and *Hansenula*) for possible application.

MAJOR PROBLEMS ENCOUNTERED : 8 months delay of the EC funding

RESULTS :

The research is progressing on the line defined above. Exchanges of researchers and materials, as well as interaction between participants are active. The situation of specific topics are summarized below.

* *PARTICIPANT 1* :

Mapping of genes in *K. lactis* has progressed by construction and use of multiply labelled strains and centromere linked markers. Data on physical mapping on chromosomes of cloned genes are accumulating in parallel, confirming genetic data. To support mapping work, many new mutations were isolated and several genes were cloned. Strain diversity is being assessed by electrophoretic karyotyping of most of the *K. lactis* isolates from the CBS collection. *K. lactis* - *S. cerevisiae* shuttle banks were constructed. A promoter from the killer plasmid k1 has been characterized and is being examined for a possible use in heterologous gene expression.

* *PARTICIPANT 2* :

Four genes encoding alcohol dehydrogenase isozymes have been identified by hybridization to *ADH2* probes of *S. cerevisiae*. The genes have been isolated and sequenced. Sequence analysis revealed high homology (near 80 %) between the *K. lactis* and *S. cerevisiae* genes. Two *ADH* genes of *K. lactis* have amino terminal extensions suggesting a mitochondrial localization of the encoded activities.

Identification of the gene products is in progress based on the ADH electrophoretic pattern of *adh* mutants obtained by gene disruption.

Chromosomal DNA fragments from *K. lactis* have been tested for the ability of efficiently sustaining autonomous replication of plasmids in *K. lactis* or in *S. cerevisiae.*
Some of these fragments have ARS function only in *K. lactis* while others support autonomous replication in both yeasts.
Comparison of the sequences responsible for ARS function is in progress and the role of a dodecanucleotide similar to the core consensus of *S. cerevisiae* is being assessed by site directed mutagenesis.

* *PARTICIPANT 3 :*

Studies on the strong promoter of the methanol oxidase gene in *Hansenula polymorpha* have revealed a complex mode of regulation. Cis-acting regulatory sequences have been identified. Binding of sequence specific factors to three of these elements could be shown both in vitro and in vivo. Mutants have been isolated that affect the down regulation of this gene in the absence of methanol whereas catabolite repression is retained. These mutants are currently being investigated. In *Kluyveromyces lactis*, the detailed knowledge on the structure and mode of regulation of the β-galactosidase (*LAC4*) promoter is being exploited to establish expression systems for heterologous genes. The system is based on integrative and centromere (collaboration with Dr. B. Zonneveld and Y. Steensma, Technical University of Delft) containing vectors allowing to construct stable strains expressing heterologous genes. As a first indicator gene the bacterial β-glucosidase gene (*GUS*) was chosen in agreement with other participants. To evaluate the effect of over production of the *LAC4* controlling transcriptional activator *LAC9*, host strains expressing different levels of *LAC9* have been constructed.

* *PARTICIPANT 4* :

The resistance of different strains of *Yarrowia lipolytica* was analyzed and copper-sensitive mutants have been obtained. Using an integrative bank of *Yarrowia lipolytica* supplied by participant 7, it has been possible to complement one of the mutations. The corresponding gene has been cloned and sequenced, proving to be a regulatory gene. In different strains of *K. lactis* provided by participants 1 and 3, we have examined the existence of repressible acid phosphatase. The enzyme has been purified and its aminoterminal end is now being sequenced. Mutants lacking in this activity have been obtained. Different plasmids have been constructed with genes donated by participants 2 and 9 to obtain mutants in the secretory pathway.

* *PARTICIPANT 5* :

Four clones of stress-inducible genes isolated from *Saccharomyces cerevisiae* (HSP12, HSP26, ESP30 and ESP35) were used as hybridization probes for a Southern blot analysis of *K. lactis* genomic DNA. Clones hybridizing with these probes (except HSP26) could be isolated. It has not been necessary to construct cDNA libraries for their isolation. Regions of the *K. lactis* DNA with homology to the *S. cerevisiae* probes have been used to examine the

expression of their cognate RNAs. The *K. lactis* sequences are transcribed and they have very similar expression to their *S. cerevisiae* counterparts. The transcription of each of the K. lactis genes is specifically regulated as a function of growth media (YPD and molasses were tested) and growth phase. Similar approach could be extended to search for homologous sequences to other *S. cerevisiae* genes that have a potentially interesting and useful pattern of expression (e.g. UB14, SSA3). The cloned *K. lactis* genes will be characterized in detail by DNA sequencing, RNA transcript mapping, etc, so that their promoter regions can be evaluated for heterologous gene expression. The *S. cerevisiae* probes have been sent to participant 4 who will search for homologues in *Yarrowia lipolytica*.

* *PARTICIPANT 6* :

Many new mutants of *Kluyveromyces lactis* affected in carbohydrate metabolism were isolated. Some of them belong to the "rag" group (cf. participant 1), some others do not. The genetic analysis demonstrated that the isolated mutants define several complementation groups. A gene involved in the energetic metabolism was isolated and sequenced. Parameters of catabolite repression have been defined. Several selection strategies were used to obtain mutants resistant to catabolite repression (non-repressible mutants) and mutants that do not derepress sensitive enzymes (non-derepressible mutants).

* *PARTICIPANT 7* :

Karyotyping of wild type *Y. lipolytica* isolates revealed a pronounced diversity of patterns and individual gene probes showed that gross chromosomal rearrangements are associated with chromosomal polymorphism. A minimal chromosome number estimate of 5 is proposed. A replicative genomic library of *Y. lipolytica* has been constructed to isolated new genes and successfully tested.

* *PARTICIPANT 8* :

We have started to work on the construction of recombinant *Kluyveromyces lactis* strains harboring expression cassettes either integrated at a defined chromosomal locus (*RAG2*) or contained on multi-copy vectors derived from the nature *Kluyveromyces* plasmid pKD1. The expression cassettes were identical in structure except for the expression signals used to initiate DNA transcription. The promoters used were S.c. *PGK*, K.l. *LAC4*, K.l. *ADH3* and K.l. *ADH4*. First results have been obtained in shake flask experiments with respect to product yield and transcriptional activity (Northern analysis). Fed-batch studies with a strain harboring the reporter gene under control of the *ADH4* promoter are scheduled for the month of April.

* *PARTICIPANT 9* :

The fermentation characteristics of *Kluyveromyces marxianus* have been studied. The fermentation process is aiming at a very high cell density and high production/secretion of the homologous enzyme inulinase. The following factors affecting the fermentation process were studied : temperature, mixing, sugar concentration, medium composition and carbon dioxide levels. Very important was to grow the yeast using high air/oxygen flows in order to avoid the generation of toxic metabolites such as acetaldehyde and acetylacetate. In the optimal fermentation process 100 gram dry weight per liter was achieved and up to 4 gram per liter inulinase was excreted. In this process 8 % of the total cellular protein is inulinase which shows that the production and secretion of an enzyme using this fermentation process is very deficient.

HIGHLIGHTS/MILESTONES : Nothing to report.

WIDER CONSIDERATIONS :

The annual ELWW Workshop, Biology of *Kluyveromyces* V, will be organized as a satellite meeting of the 16th International Conference on Yeast Genetics and Molecular Biology, August 1992 at Vienna. An International Conference on non-conventional yeasts, held at the Biozentrum, Basel, also in August 1992, will be an previledged place of information exchange. As non-conventional yeasts, the target organisms of the present project are among the most significant species in biotechnology.

COOPERATIVE ACTIVITIES :

1) The ELWW Workshop, Biology of *Kluyveromyces* IV, was held at Düsseldorf, 21-22 September 1991. A first meeting of all the project leaders was also held at this occasion.

2) A ELWW booklet on the activity of this consortium is being prepared by all the participants.

3) A predoctoral fellow's long term stay from laboratory 4 to laboratory 1 ; a post-doctoral fellow's long term stay from laboratory 4 to laboratory 7.

4) Working visits of researchers : two from laboratory 2 to laboratory 1, two visits from 1 to 2; one visit from 7 to 4, one visit from 5 to 7 ; one visit from 2 to 7.

5) Collaboration on the *K. lactis* expression system between participants 3 and 8.

6) Many exchanges of materials between the participant laboratories (yeast strains, vectors, DNA banks, clones genes, communication of methods).

JOINT PUBLICATIONS :

Participants 1 and 6 : Glucose transport in the yeast *Kluyveromyces lactis*. I. Properties of an inducible low-affinity glucose transporter gene. M. Wésolowski-Louvel, P. Goffrini, I. Ferrero and H. Fukuhara. Molec. Gen. Genet., in the press.

OTHER PUBLICATIONS/PATENTS

Participant 3 : Co-regulation of the *K. lactis* lactose permease and β-galactosidase genes is achieved by interaction of multiple LAC9 binding sites in a 2.6 kbp divergent promoter. A. Gödecke, W. Zachariae, A. Arvanitidis and K.D. Breunig. Nucl. Acids Res. 19, 5351-5358 (1991).

- 3 articles submitted for publication (participant 1)
- 1 article submitted for publication (participant 2)
- 2 articles submitted for publication (participant 4)
- 2 articles submitted for publication (participant 6)
- 2 articles submitted for publication (participant 7).

MOLECULAR AND GENETIC ANALYSIS OF GENES CONTROLLING FLOWER DEVELOPMENT

Contract : BIOT 900171

Official start : 1991-01-01

Coordinator : Dr E. Coen, John Innes Institute, Norwich, UK.

Participants : Dr Z. Schwarz-Sommer, Max-Planck-Institut, Koeln, Germany.
Dr J. Beltran Porter, Consejo Super. de Invest. Cientificas, Valencia, Spain.
Dr J. Almeida, Inst. Superior de Agronomia, Lisboa, Portugal.
Dr J. Newbury, Birmingham University, Birmingham, UK.

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.

MAJOR PROBLEMS ENCOUNTERED: Nothing to report.

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, 14 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 the 14 mutants were caused by insertions of various types of transposons at different positions in the *inc* gene. These transposons are now being characterised and any novel types will be cloned and used 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 (Norwich), three at *squamosa* (Koeln), three at *globosa* (Koeln) and two at *deficiens* (Koeln). The overall objective will be to establish a "library" of transposons available for general use.

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 *pallida* (*pal*) locus to the closely linked *divaricata* (*div*) locus. 2500 progeny from a cross between *div* and 100 different *Pal*$^+$ revertants are being grown to screen 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. In addition, they constructed a double mutant for a stable *pal* allele and *div*, to be crossed to plants carrying Tam3 at *pal* grown at 15°C. This should enable thousands of independent transpositions to be selected for at the seedling stage (*pal* mutants affect seedling pimentation). Furthermore, in collaboration with the Koeln and Norwich groups, the *flo* gene has been mapped relative to *div* and *pal*, to give the order *pal-div-flo* (*pal-div* = 5cM; *div-flo* = 10-15 cM). Because Tam3 insertions at *flo* are available, this may provide another source of linked Tam3 copies for mutagenesis of *div*. In parallel with these studies, the Lisbon group has also carried out a detailed phenotypic analysis of *div* and wild-type flowers at various stages of development using scanning electron microscopy.

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 is constructing 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 about 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 80 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, could be 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.

Current work of the Koeln Group is focussing on three areas. Firstly, the linkage map has to be refined by mapping further molecular markers which should allow the number of RFLP linkage groups to be reduced to eight. Secondly, the mapping of known morphological mutants should lead to the alignment of the classical genetic map with the RFLP map. For this reason, they will carry out an RFLP analysis of F2 populations segregating for morphological markers known to be located at two distant postions within each of the chromosomes of the classical linkage map. Thirdly, to facilitate use of the map by other groups, they are preparing recombinant inbreds by repeated selfing of all F2 individuals of the *A.majus x A.molle* population used to establish the current map. As this work progresses, a more extensive and accurate map will be produced which will be invaluable to all *Antirrhinum* scientists.

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;squa*. The phenotypes obtained have had important implications for models of how flower development is controlled. Many other mutant combinations are currently being made in the

collaborating laboratories.

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. An efficient procedure for regeneration from young stem explants is being developed by the Birmingham group.

They have determined suitable conditions for the production of hypocotyl-derived callus and the efficient regeneration of shoots and roots, using a range of different varieties of *A. majus*. Transformation experiments were carried out using hypocotyls and a range of wild-type *Agrobacterium tumefaciens* strains. Transformation, as assessed by the formation of opine-producing tumours, was achieved in all varieties tested. The strain C58 was identified as being the most virulent and agrobacteria containing C58 virulence have been chosen for later work with disarmed vectors.

T-DNA transfer rates are known to be influenced by a range of factors, including the concentration of certain phenolic compounds. The Birmingham group therefore performed transformations using combinations of different phenolics, wild-type *Agrobacterium* strains and varieties of *Antirrhinum* using media of varying pH. In general, transformation was favoured by low pH and the inclusion of acetosyringone in the co-cultivation medium. Maximal regeneration of the least susceptible variety, however, was obtained with the combination of high pH and the addition of syringaldehyde. This indicates that transformation conditions for each *Antirrhinum* variety will have to be optimised. A study to see if correlations exist between the frequency of transformation and the endogenous production of phenolic compounds by wounded *Antirrhinum* tissue is in progress. Initial results indicate large variations in both the type and quantity of compounds formed during 24 h after wounding.

Transformations have now been attempted using a range of different disarmed vectors. This work has included (1) the use of vectors containing selectable markers for either antibiotic or herbicide resistance and (2) the use of different transformation protocols using either hypocotyls, shoot bases or whole plants. To date, no transformed plants have been regenerated. However, the use of a herbicide resistance marker allowed fewer non-transformed "escapes" and its use should eventually lead to a more efficient transformation protocol.

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.

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 are using several strategies to eliminate common proteins from the extracts before they are injected into mice. Early results with subtracted carpel extracts are encouraging and a similar approach is now being extended to other organ types. However, in some organs there is the further complication of abundant proteins that may be immuno-dominant. For example, anthers contain an abundant 27kD protein, probably located in the pollen sacs, that is present at very early stages of development. To eliminate this protein from anther extracts by immunoprecipitation, they are raising a monoclonal antibody that will recognise it. This antibody will also be useful as a marker for anthers. 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. Initiation of targetted tagging

5. Characterisation of monoclonal antibodies recognising floral organs

6. Optimisation of a regeneration system for *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 one major meeting which was held in Koeln from 17/4/91-21/4/91 and involved presentations from all of the participants. A report on this meeting was published in Flowering Newsletter (see below). The next meeting will be held in Valencia from 14/5/92-17/5/92.

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 exchaged, 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.

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.

In addition, we have also submitted a report on the programme to be published as a brochure by the Commission.

OTHER PUBLICATIONS

Most of the work carried out in this programme has not yet been published. Two reports on transformation experiments are:

Holford, P., Newbury, H.J. (1992) The effects of antibiotics and their breakdown products on the *in vitro* growth of *Antirrhinum majus*. **Plant Cell Reports** (in press).

Holford, P., Hernandez, N, Newbury, H.J. (1992) Factors influencing the efficiency of T-DNA transfer during the co-cultivation of *Antirrhinum majus* with *Agrobacterium tumefaciens*. **Plant Cell Reports** (in press).

THE MOLECULAR BASIS OF CELL-CELL INTERACTIONS IN SELF-INCOMPATIBILITY

Contract :BIOT 900172
Signed start :1990-12-01
Duration :36.0 Months

Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V.
Institut für Züchtungsforschung

Coordinator:
Dr. R.D. Thompson, MPI für Züchtungsforschung, D-5000 Köln

Participants:
Dr. A. Tiezzi, Univ. of Siena, Italy
Dr. E. Jacobsen, Univ. of. Wageningen, Netherlands
Dr. G. Wricke, Univ. of Hannover, Germany
Dr. G. Wullems, Univ. of Nijmegen, Netherlands
Dr. M.v. Grinsven, Zaadunie B.V., Enkhuizen, Netherlands

Objectives set for the reporting period
The objectives set for the first year of this project as defined by the original workplan focussed on characterization of the potato S-RNAse alleles S1 and S2, specifically the genomic sequence determination and expression of recombinant protein in *E. coli*/Baculovirus. Further, it was planned to test for S-RNAse sequences in potato anther RNA using PCR, to set up PCR tests for S-RNAse-related sequences in Rye, and to construct a cDNA bank from anther RNA. All of these experimental approaches have been pursued and completed. In the case of Rye S-RNAse-related sequences, no products were identified, and in view of the apparent similarity of this system to *Brassica* SI (see results), efforts to identify SRK-related sequences by PCR will be attempted instead. A cDNA bank has been constructed to fertilised styles rather than anthers, in order to include transcripts produced only in germinated pollen (see results).

Major problems encountered

1. Propagation of potato lines of defined "S" genotype
2. Identification of pollen transcript of the S-locus

1. Previous molecular characterization of the potato "S"-locus was carried out on dihaploid lines identified by Prof. Hermsen which are derived from the cross G-254 x B16. As these lines are derived from tetraploid material, they display in many cases defects at loci previously masked in the tetraploid. Thus, many of the lines have yellow leaf margins, and in some cases growth and pollen fertility is reduced. At the start of the programme, research was impeded as only this material was available. The plant material supplied to BRIDGE labs. needed to be redeveloped to select against and thereby minimize these deleterious effects on plant performance. This work has now

been carried out by Professor Jacobsens laboratory (see results), and much improved lines are available for study.

2. A central objective of this project is the identification of the pollen component of the S-locus. Since the discovery and cloning of SLG cDNAs in 1986, a number of labs have tried to demonstrate the presence of a homologous pollen *S*-locus product, without success. The alternative approach, of looking for a different, *S*-linked pollen protein has also not met with success. Therefore not unexpectedly, this remains on a problem for our project. Efforts to use PCR to identify SLG-homologous pollen-transcripts have been so far negative, and alternative approaches are being pursued. On the assumption that the pollen *S*-linked gene is distinct in sequence from the SLG, but tightly linked to it, RNA filters carrying pollen RNA are being hybridized with a series of DNA fragments covering the entire S1 and S2 SLG-genomic clones.

Results:
Identification of S-locus-defined potato plants (Wageningen)

The potato clones previously used for molecular analysis of S-locus gene products (Kirch et al., 1989) suffered from genetic defects and therefore the development of new S-locus-defined clones was of high priority in our investigations. For this purpose, the Wageningen group have identified clones of all six possible SI genotypes from the same seed stocks studied previously. These genotypes were selected on the following criteria:

1) S-allele constitution as assessed by isoelectric focussing gel protein pattern
2) Plant vigour
3) Tuberization

30 clones of the newly developed material have been distributed to other BRIDGE partners.

New methods for identifying S1 genotypes (Wageningen)

In order to screen large populations for their S-genotypes, an IEF method was established which permits the analysis of up to 52 samples on one gel. A technique for producing homozygous plants more efficiently has been developed, in which during an incompatible cross-pollination, pollen from *Solanum phureja* is also supplied; this enhances seed-set and hence yield of homozygotes. The diploid clone of *S. phureja* used carries a dominant embryo seed spot marker which allows hybrid seed to be distinguished from the desired selfed product. Finally, a bilateral compatible system has been established between diploid potato (*S. tuberosum*) and *S. verrucosum*. *S. verrucosum* is self-compatible, but cannot normally be used as male partner in the interspecific cross. However, SI *S. tuberosum* acceptor lines have been identified which overcome this problem. This research paves the way for understanding the basis of unilateral interspecific incompatibility, as it will allow the genetic factors involved to be mapped.

Ultrastructural analysis of fertilisation in SI protato lines (Siena)

Observations were carried out using transmission and scanning electron microscopy on pollen and stigma from S1S3 and S2S3 clones, with the following conclusions. Firstly, a discrepancy was noted between the maturation time of pollen and stigma on the same plant. When anthers release mature pollen grains, the stigma is still immature and first produces stigmatic exudate a few days later. A scanning E.M. comparison of S1S3 and S2S3 pollen grains revealed differing exine structures. Cross sections analysed by transmission E.m. showed no internal differences between pollen grains of the two genotypes. Potato pollen is "bicellular" as it contains a vegetative mucleus and a generative cell. The generative cell is spindle-shaped and located close to the plasma membrane. The generative cell is located at the centre of the mature pollen grain. In some cases a close association between the generative cell and vegetative nucleus is observed.

Stigma from both S1S3 and S2S3 plants have been compared by scanning E.M. In both cases the stigmatic surface is composed of papillae which at maturity are completely covered with stigmatic exudate. S1S3 stigma are single hemisperical lobes whereas S2S3 stigma exhibit a deep central furrow. It will be of interest to establish whether the genetic basis for morphological differences observed can be related to the S-locus.

Biochemical basis for gametophytic self-incompatibility (Hannover)

The physiology of the SI response varies with different species. In Hannover, the two loci S and Z system of Rye is being investigated. In this case, the SI reaction occurs within a few minutes after pollination. Incompatible pollen tubes usually do not penetrate the stigmatic tissue but are arrested at the stigma surface. In view of the involvement of protein kinases in mammalian fast transduction pathways, and the finding of an S-related receptor-like kinase at the *Brassica S*-locus, experiments were initated to check the involvement of kinases in Rye SI.

An involvement of Ca^{2+} in the SI response has been established by incubating stigma with two Ca^{2+} blockers (Verapamil and La^{3+}), and monitoring the effect of an attempted self-pollination. The vast majority of pollen tubes grew down to the ovary, indicating a strong delay in the SI response.

A panel of six inhibitors acting on Tyrosine-specific protein kinases was tested for effects on the Rye SI-reaction. In two cases, Lavendustin A and its chemical precursor 2-OH-5-(2,5 di OH Benzyl) aminobenzoic acid, "self" pollen tubes behaved as in a compatible pollination, suggesting similar kinases are involved in the SI response. Isoelectric fosussing of ^{32}P-phosphorylated pollen membrane preparations demonstrates the

presence of a number of acidic phosphorylated polypeptides. Part of the physphorylating activity can be inhibited by Lavendustin A.

Characterization of the potato stylar S-linked RNAse (S-RNAse) gene (Köln)

The S-RNAse S1 and S2 genes have been isolated (Kaufmann, Salamini and Thompson, 1991) and sequenced, thus permitting us to study the sequences necessary for style-specific expression. The maps of both S-clones with several restriction enzymes show no common features, indicating the highly polymorphic character of the S-locus. Both genes possess one small (113, 117 bp) intron, inserted in the same region of the open reading frame. The coding sequences have been expressed *E. coli* as glutathione-S-transferase fusion proteins, and antibodies raised. Using these antibodies, the existence of an antigenically-related pollen protein has been investigated. No such protein was identifiable on Western blots. The ability of isolated S2 protein to associate with specific pollen proteins was also analysed using anti S2-AB. No specific association was detected.

S1 and S2-RNAse gene sequences were introduced into tobacco and potato plants via *Agrobacterium*-mediated transformation. Using translational fusions of the S2 promoter to β-glucuronidase, S2-promoter expression was found in pistils and pollen of transgenic tobacco plants. Similar results were obtained from transient expression experiments in which the DNA had been introduced via particle bombardment. In contrast, the S1 promoter was very weakly expressed, and when introduced as the entire gene, no S1 polypeptide or transcript could be detected. Possibly the S1 promoter fragment used lacks an enhancer sequence located further away from the coding sequence than in S2. The promoters possess sequence homology restricted to a few short (< 20 bp) motifs, which may have a regulatory role. cDNA and genomic clones for SK2 have been isolated. The sequence shows SK2 to be a pistil-specific endochitinase, and this activity has been demonstrated for the purified protein. Monoclonal antibodies to SK2 are being developed by the Siena group.

Pollen-specific gene expression in SI (Nijmegen)

During pollen tube development, pollen-specific mRNAs accumulate, which may have a function in pollen-tube growth and in the pollen-stylar tissue recognition process leading to SI. A pollen-specific gene, NTP 303 has been isolated. NTP 303 is transcribed in developing pollen, after the mid-binucleate stage, and in growing pollen tubes (Weterings et al., 1992). Its mRNA remains present in growing pollen tubes up to 72 h after pollination (Reijnen et al., 1991). Now that *de novo* transcription in germinating pollen has been demonstrated, efforts are underway to identify S-locus-determined transcripts in a library constructed using mRNA from styles 24 h after pollination (S1S3 x S1S4). The library will be

subtracted with mRNA from mature unpollinated styles (S1S3) and screened differentially against pollen (S2S4).

Transformation methodology for tomato (Enkhuizen)

Although the majority of participants in this BRIDGE programme investigate the gametophytic system of SI in potato, Zaadunie's interest is directed towards exploiting this knowledge in tomato, a crop in which F1-hybrid seed is produced commercially by hand-emasculation. Work in this phase of the contract period has focussed on the establishment of a transformation protocol for elite lines of tomato. Preliminary experiments suggested that many variables can dictate the success rate of a protocol. Several protocols were tested in order to identify a usable and if possible, genotype-independent transformation method. Variables under investigation include: source of the explant and pre-treatment of the explant donor, ploidy level of starting material and regenerated plants, effect of varying media composition, and effectiveness of different *Agrobacterium* strains. To date, although a generally applicable protocol for elite lines of tomato is not yet available, sufficient progress has been made that efficient transformation of S-protein genes in tomato elite lines is now realistic.

Highlights/Milestones:
The discovery of *de novo* mRNA synthesis in germinating pollen is particularly significant, as it had previously been assumed that gene expression at this stage is directed predominantly by stored mRNA. Our strategies for characterizing the SI pollen-pistil interaction have been modified accordingly.

Wider considerations:
The molecular analysis of gametophytic SI progressed significantly following the isolation of the gene encoding the female component of the system, and the genetical, cytological and biochemical studies summarized here. We are nearer to understanding how to exploit this natural system of fertilization control, both for the production of hybrids and for overcoming certain species barriers.

Cooperative activities:
The BRIDGE project members have met together twice in the past twelve months to communicate data and review collaborative activities. One such meeting was held together with the second Brassica SI BRIDGE group in Enkhuizen, Holland. In addition, a number of visits have taken place between the laboratories for the purpose of acquiring materials, data or techniques, for example, between Wageningen and Köln, Hannover and Köln, Nijmegen/Wageningen, and Siena/Köln. Materials exchanged include S-locus-defined potato clones, DNA clones, antibodies, and oligonucleotide probes.

Joint publications:

Thompson RD, Uhrig H, Hermsen JGTh, Salamini F, Kaufmann H (1991) Investigation of a self-compatible mutation in *Solanum*

tuberosum clones inhibiting S-allele activity in pollen differentially. Mol Gen Genet 226: 283-288.

Other publications:

Cresti M, Tiezzi A (1992) Pollen tube emission, organization and tip growth. In: Research in sexual plant reproduction (Cresti M, Tiezzi A, eds), Springer-Verlag, in press.

Kaufmann H, Salamini F, Thompson RD (1991) Sequence variability and gene structure at the self-incompatibility locus of *Solanum tuberosum*. Mol Gen Genet 226: 457-466.

Palevitz BA, Tiezzi A (1992) The organization, composition and function of the generative cell and sperm cytoskeleton. In: International Review of Cytology, Vol. Sexual reproduction in flowering plants, in press.

Rijnen WH, van Herpen MMA, de Groot PFM, Olmedilla A, Schrauwen JAM, Weterings KAP, Wullems GJ (1991) Cellular localization of a pollen-specific mRNA by *in situ* hybridization and confocal lase scanning microscopy. Sex Plant Reprod 4: 254-257.

Tiezzi A, Moscatelli A, Cai G, Bartalesi A, Cresti M (1992) An immunoreactive homolog of mammalian kinesin in *Nicotiana tabacum* pollen tube. Cell Mobility and the Cytoskeleton 21: 132-137.

Weterings K, Reijen W, Van Aarssen R, Kortstee A, Spijkers J, van Herpen M, Schrauwen J, Wullems G (1992) Characterization of a pollen-specific cDNA clone from *Nicotiana tabacum* expressed during microsporogenesis and germination. Plant Mol Biol, in press.

TITLE: "GAMETE DIFFERENTIATION AND FERTILIZATION

CONTRACT NUMBER: BIOT-0180-NL

OFFICIAL STARTING DATE: 1 january 1991

COORDINATOR: J. van Went, Wag. Agr. Univ., Wageningen, The Netherlands

PARTICIPANTS: M. Cresti, Univ. Siena, Siena, Italy
J. Callow, Univ. Birmingham, Birmingham, United Kingdom
J. Dunwell, ICI Seeds, Bracknell, United Kingdom
F. Hoekstra, Wag. Agr. Univ., Wageningen, The Netherlands
M. Kreis, Univ. Paris-sud, Orsay, France
P. Olesen, Maribo Seed, Copenhagen, Denmark
M. Steer, Univ. College Dublin, Dublin, Ireland

OBJECTIVES REPORTING PERIOD: Extraction of mRNA from isolated embryo sacs and ovules. Construction of cDNA library. Cytological analysis of isolated embryo sacs, egg cells; development of an in vitro culture system of *Petunia* pollen tubes for the isolation of sperm cells; development a bio-assay for the analysis of directed pollen tube growth; selection of fertilization mutants.

MAJOR PROBLEMS: due to limited financial support, the available manpower became a limiting factor. This seriously hampered progress, and it was decided to focus on one model species: *Petunia*.

RESULTS: mRNAs were extracted from isolated *Petunia* embryo sacs and ovules (PARIS); cell biological aspects of isolated embryo sacs were studied (WAGENINGEN); an in vitro culture system for pollen tubes is being developed for the isolation of sperm cells (WAGENINGEN-SIENA-COPENHAGEN); the in vitro fusion system is installed and tested (WAGENINGEN); the testing of gamete specific monoclonal antibodies is organized (BIRMINGHAM-SIENA-WAGENINGEN); Petunia mutants are collected to be analyzed for fertilization defects (BRACKNELL); the bio-assay for directed pollen tube growth (gametophyte interaction) is being developed (DUBLIN).

HIGHLIGHTS: The actual isolation gametophytic mRNA from isolated embryo sacs for the construction of a cDNA library. This strongly enhances the prospects for successful characterization of gametophyte and gamete specific gene expression.

WIDER CONSIDERATIONS: Our achievements, as well as results obtained by others, indicate that in vitro fusion of isolated gametes may become a powerful biotechnological tool in plant breeding for the realization of interspecific hybridization.

COOPERATIVE ACTIVITIES: - on january 28, 1991 a plenary meeting was held at Wageningen to discuss the project in view of the EC comments and the limited financial support (see report); - Plant materials were transferred from WAGENINGEN to SIENA, PARIS, and DUBLIN; - Staff exchange: V. Ferrant (PARIS) stayed from 1 february to 1 april at WAGENINGEN to learn the embryo sac isolation technique, and to collect isolated embryo sacs; F. Doris (DUBLIN) stayed from 15 february to 15 march at WAGENINGEN to learn the embryo sac isolation and pollen tube culturing techniques; L. Bruun (COPENHAGEN) visited WAGENINGEN on april 17 to discuss the Copenhagen contribution to the project, and she stayed 1 month in SIENA; K. Theunis (WAGENINGEN) worked in SIENA on sperm cell isolation; Cresti (SIENA) visited WAGENINGEN from 19 - 22 april to discuss and organize the Siena participation in the project; C. Del Casino (SIENA) stayed in October at WAGENINGEN to learn the in-vitro culturing of *Petunia* pollen tubes, and techniques for micromanipulation and micro-injection of pollen tubes; V. Ferrant (PARIS) stayed from 1 September to 1 November at WAGENINGEN to develop a technique for the extraction of mRNAs from isolated embryo sacs; - The transfer of gamete specific monoclonal antibodies from BIRMINGHAM to WAGENINGEN was arranged, for testing on isolated egg cells; - With dr. Kranz

(HAMBURG) and dr. Verhoeven (WAGENINGEN) arrangements were made for collaboration and future participation in the project.

JOINT PUBLICATIONS: Murgia M, Detchepare S, Van Went J, Cresti M (1991) Sex Plant Reprod 4: 176-181
Cresti M, Ciampolini F, Van Went J (1991) Ann Bot 68:105-107
Theunis K, Pierson E, Cresti M (1991) Sex Plant Reprod 4: 145-154

OTHER PUBLICATIONS: Van Aelst A, Van Went J (1991) Acta Bot Neerl 40:319-328

TITLE: Molecular genetics and physiology of self-incompatibility in *Brassica* crops
CONTRACT NUMBER: BIOT CT90-0174(SSMA)
OFFICIAL START DATE: JAN 01 1991 (SEE GENERAL REMARKS)
COORDINATOR: H.G. DICKINSON, Oxford University, Oxford OX1 3RB, UK.

PARTICIPANTS:
INRA, Lyon, France. Professor C. Dumas.
University of Durham, Durham, UK. Dr R. Croy.
University of Nijmegen, Nijmegen, The Netherlands. Professor G. Wullems.
Nickerson-Zwaan B.V., Barendrecht, The Netherlands. Dr E. Van Breukelen.
Zaadunie B.V., Enkhuizen, The Netherlands. Dr Mart Van Grinsven.
Maribo Seeds, Copenhagen, Denmark. Dr P. Olesen.
JICPSR, Cambridge Lab., Norwich, UK. Dr M. Trick.
University of Birmingham, Birmingham, UK. Dr C. Franklin.
HRI, Wellesbourne, UK. Dr D. Ockendon.
Royal Sluis B.V., Enkhuizen, The Netherlands. Dr L. Davidse.

OBJECTIVES SET FOR THE REPORTING PERIOD (YEAR 1 ONLY)
(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 yeast artificial chromosome (YAC) libraries (Durham)
* Characterisation of putative male determinants (Oxford)
* Immunoscreening of expression libraries (Nijmegen)
* Development of bioassays (Oxford)

2. BIOCHEMISTRY AND PHYSIOLOGY OF THE SI RESPONSE
(Managing contractor: Oxford)
* Structural and physiological changes in pollen and stigma (Oxford)
* Comparisons with other species (Maribo Seeds)
* Modelling of S-locus linked glycoprotein (SLG) and other SI molecules (Oxford)
* Polypeptide changes in pollen following self and cross pollinations (Nijmegen and Oxford)
* Pollen coating bioassays (Oxford)

3. ORGANISATION AND EXPRESSION OF THE S-LOCUS, RELATIONSHIPS BETWEEN S-ALLELES
(Managing contractor: JICPSR; Norwich)
* Immunological and molecular studies of "mutants" (Lyon).
* Molecular genetics (Norwich, Birmingham)
* Construction of YAC libraries (Durham)
* Development of S-allele specific probes (HRI; Wellesbourne)

4. TRANSFER OF S-ALLELES TO NEW LINES
(Managing contractor: JICPSR; Norwich)
* Development of transformation strategies (Norwich, Birmingham)

MAJOR PROBLEMS ENCOUNTERED:
1.MALE COMPONENT
* For genomic libraries; availability, and efficiency of P1 packaging system; now working satisfactorily (Durham)

* Failure of pollen grown *in vitro* in pollination experiments; alternative strategy adopted (Nijmegen)
* Fractionation of low M_r pollen coat proteins; now achieved (Oxford)
* Availability of S-specific monoclonals; help received from Lyon (Oxford)

2. SI RESPONSE
* Measurement of Ca^{2+} in stigmatic papillae; now achieved (Oxford)

3. ORGANISATION OF S-LOCUS
* Problems with P1 packaging system; see above (Durham)
* Inefficient transformation strategies; see below (Birmingham and Norwich)
* Ignorance of SI genetics of sugar beet (Maribo Seeds)
* Making cDNA libraries from small amounts of material (Maribo Seeds)

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 (e.g. Nottingham) and the use of transient assay systems has helped, but whole-plant transformation remains a serious barrier to progress

GENERAL
* The quality and pace of research was significantly affected by extreme delays in receiving funds from the EC. Many institutes would not allow positions to be advertised before funds had arrived, and thus some participants could not commence work until late 1991/early 1992.

RESULTS

1. MALE COMPONENT
In **Lyon**, a preliminary study based on the use of PCR has demonstrated transcripts homologous to the SLG gene in anther tissues during early microsporogenesis (Guilluy *et al.* 1991). Interestingly, these transcripts are 4 kb instead of the 1.6 kb usually found for stigma SLG products. Since January 1992, a post-doctoral researcher (Dr M.J. Cock) has adopted a similar approach on two *P57* genotypes. The *P57* self-compatible line will also give information on the possible changes that may have occurred in S-locus expression in the pollen which have resulted in compatibility.
For the purpose of long range mapping of the S-locus, genomic libraries are being constructed in **Durham** using very high molecular DNA from *Brassica oleracea*. This DNA, estimated (by pulsed field electrophoresis) to be about 150 kB - 200 kB, has been digested to yield the required sizes of genomic fragments for cloning, and the digests size fractionated on sucrose density gradients. Desired lengths have been pooled and ligated into the precut, phosphatase-treated vector and packaged. Clones obtained are currently being analyzed to assess the quality of the library.This vector system, which is also being used in **Lyon**, is regarded as an alternative to YAC libraries.
New methods have been developed at **Oxford** for fractionating exine proteins and the characterisation of a peptide that interacts with the SLG class of molecules (Doughty *et al* 1992). The technique for isolating exine proteins has also been used by the **Nijmegen** group, and antibodies have been raised in rabbits against these proteins from mature pollen of plants with S_5S_5 alleles. A cDNA expression library is under construction at Nijmegen using mRNA from S_5S_5 anthers of different age, ranging from the uni- to the tri-nucleate stage of pollen development. The expression products of the cDNA library will be screened against the specific 'exine' antibody pool.

2. THE SI RESPONSE
The initial SLG modelling programme at **Oxford** has now been successfully completed (Dickinson *et al* 1992); Database searches involving consensus sequences from the SLG family of stigmatic glycoproteins have revealed strong homologies with domains in several classes of animal proteins,

principally the von Willebrand factor and type VI collagen. These polypeptides, which are also heavily glycosylated, are held to be involved in protein-protein interactions and extracellular matrix formation - processes which may also be involved in the initial stages of pollination. Detailed modelling of these sequences indicate that, despite small differences in amino acid composition, these domains are strikingly similar in their 3D molecular architecture.

The effect of the interaction of pollen coat fractions with stigmatic molecules on male and female cells has been studied at **Oxford**. Changes in polypeptide synthesis and phosphorylation following compatible and incompatible pollinations are being followed *in vivo*, both in the pollen and during a stigmatic response that results in expansion of the papillar wall. Changes in Ca^{2+} in the papillar cells have also been examined.

The **Maribo Seeds** group has been attempting to characterise the genetics and cell biology of SI in sugar beet, which may be related to *Brassica*. Extraction of mRNA from the very small beet styles has been optimized and mRNA has been converted to first strand cDNA and used in PCR using primers representing conserved gametophytic SI-sequences. Identified PCR products have been purified, cloned and sequenced, but the sequences obtained did not resemble those of gametophytic SI-systems. A cDNA library from mRNA of beet styles is under construction. SI-clones have been received from different laboratories within the BRIDGE programme and have been transformed into *E. coli*, followed by amplification and purification. The clones are ready for Southern blotting and hybridization tests.A positive response has been obtained with gametophytic SI-antibodies which have been used for immunolabelling of sectioned beet flowers. The allele-specific *Petunia* antibodies cross-react with glycoproteins in the transmission tissue.

3. ORGANISATION AND EXPRESSION OF THE S-LOCUS

The **JICPSR; Norwich** laboratory has addressed the question of the implication of the SRK gene in SI by the isolation and analysis of a genomic clone from S_{63} *Brassica oleracea* which appears to contain a receptor kinase sequence. Further DNA sequencing 3' to the previously characterised SLG-like open reading frame has revealed the presence of sequences similar to those reported for SRK-2 and SRK-6. A large intron, about 2.5 kb in length, separates the two domains. The complete (9 kb) sequence should soon be available for analysis and dissemination. Already, certain features at the first exon/intron junction suggest that this gene may be different to the other SRKs characterised. A kinase-specific subclone is currently being used to reprobe genomic blots of segregating F_2 families in order to establish genetic linkage to the S-locus. We have synthesised PCR primers which have been proven to amplify directly a defined fragment from homologous kinase domains from a variety of genomic DNAs. It should now be possible, using these advances, to isolate other SRK genes and to eventually co-transform *B. oleracea* with appropriate SLG/SRK gene-pairs to define the S-locus at the molecular level.

The major aim of the **Birmingham** group is to delineate the nucleotide sequences and trans-acting proteins that are involved in the control of expression of the members of the S-gene family. In the longer term we aim to determine how the expression levels of the different family members are modulated and if the allelic dominance that occurs between different S-alleles is controlled at the transcriptional level. A series of constructs has been produced in which sequences 5' to the translation initiation codon of the S_{63} SLR (S-locus related) gene have been fused to a GUS reporter gene. These have been used to transform tobacco plants and GUS activity within the transgenic plants determined. This has revealed that sequences located within a region of 1500 bp upstream of the SLR coding sequences are sufficient to specify correct spatial and temporal expression of the transgene. Further analysis has demonstrated that sequences controlling spatial expression are located within a 450 bp region directly upstream of the gene. Nucleotide sequencing studies of the S_{63} promoter have indicated regions of sequence homology with the comparable region from the S_{63} SRK gene and with elements from other plant genes.

For each *P57* line, the **Lyon** laboratories have identified specific SLG- and SLR1-glycoproteins according to N-terminal amino-acid sequence data (Gaude *et al.* 1991). The striking result of our analysis is that the 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. In order to establish the homology between pollen recessive S-alleles (i.e. S2, S5 and S15) and the *P57Sc*-allele, the **Lyon** group has started an investigation to identify the pollen recessive SLGs by using the molecular probes established from the Sc-SLG products (monoclonal antibodies and Sc-SLG cDNA). This work has been undertaken with the collaboration of Dr D. Ockendon (**HRI, Wellesbourne**) who supplied "pollen recessive" seeds.

The aim of the **HRI; Wellesbourne** group is to use molecular techniques to identify the 50 different self-incompatibility [S] alleles which have already been recognised by conventional methods. Genomic DNA from a range of genotypes with different S-alleles has been amplified using PCR. The primers used in the PCR were selected using data from a multiple sequence alignment of published SLG and SLR sequences, together with sequence information provided by other members of the BRIDGE group. Initially, primers were selected from conserved regions located towards the two ends of the sequence alignment. Using these primers, the amplified PCR product is approximately 1150 bp long.

In parallel with this work, a start has been made on the development of probes which will allow particular S-alleles to be distinguished. Initial studies using probes derived from the whole 1150 bp amplified fragment from various cloned SLG and SLR sequences provided by other members of the BRIDGE group showed a degree of cross-hybridisation between different members of the S-gene family. However, a much shorter probe of 380 bp derived from the cloned SLG-S5 sequence not only seems to be SLG specific, but also enabled the recessive alleles S2, S5 and S15 to be distinguished from a number of dominant alleles.

4. TRANSFER OF S-ALLELES

Brassica oleracea remains difficult to transform (see above). Work in **JCIPSR; Norwich** and **Birmingham** has generally involved transgenic *B.napus* or transient assays. Collaboration with the Nottingham group, who are using antisense technology to study SI, may prove worthwhile. In the meantime both BRIDGE participants are both engaged in active programmes to transform *B.oleracea.*

HIGHLIGHTS/MILESTONES

- ***SI transcripts from anthers (Lyon)***
- ***Working packaging system for large DNA quantities (Durham)***
- ***Coating factors that interact with SLG/SLRs (Oxford)***
- ***Identification of physiological response in pollen and stigma (Oxford).***
- ***PCR primers for investigating SRK genes (Norwich)***
- ***Characterisation of promoter regions (Birmingham)***
- ***"SLG" cDNA clones from self compatibles (Lyon)***
- ***Promising probes for SLG identification (HRI, Wellesbourne).***
- ***Potential SLG cDNAs from sugar beet (Copenhagen)***

WIDER CONSIDERATIONS

The system by which *Brassica*, an important European crop, prevents inbreeding is under study 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 gene controlling 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 with this width of expertise, can hope to challenge

the current supremacy of large American laboratories.

COOPERATIVE ACTIVITIES

1. Meetings

A two day joint meeting of all participants in the project, and those of the gametophytic SI group (coordinated by Dr R. Thompson), was held in Enkhuizen, April 18-19, 1991, hosted by Zaadunie Ltd. A similar meeting is planned for April 1992 in Cologne.

2. Newsletter

SINEWS; A collaborative newsletter is published approximately every 4 months containing up-to-date information, literature and addresses from all participants from both self-incompatibility groups. SINEWS also incorporates an expertise register, containing information on the technology available in each laboratory and the willingness/ability of the laboratory in question to host visitors.

3. Visits between laboratories

a) Andrew McCubbin from Oxford spent 4 days in Lyon working with Thierry Gaude (April 1991).

b) Rene Ruiter from Nijmegen visited Oxford (December 1991) to work for 4 days with Andrew McCubbin isolating protein coating from pollen grains.

c) Steen Petersen from Maribo Seeds, visited Richard Thompson at the Max Planck Institute in Cologne (February 1991) and again for a gametophytic SI meeting in December 1991.

d) Richard Thompson visited the Maribo laboratory in Copenhagen and Professor Dickinson (Oxford) for one day in December 1991.

4. Exchange of materials

a) Seeds of homozygous S2, S5 and S15 *Brassica* from HRI; Wellesbourne to Lyon.

b) cDNA sequence and clone of P57Sc-SL9 supplied by Lyon to Durham, HRI; Wellesbourne and Maribo Seeds.

c) DNA probes for S-loci supplied by Martin Trick (IPSR; Norwich) to Maribo Seeds and Lyon.

d) Richard Thompson (Cologne) exchanged an oligonucleotide and a cloning vector for other oligonucleotides and an antibody with Steen Petersen (Maribo Seeds).

JOINT PUBLICATIONS

JICPSR; Norwich with Lyon

Guilluy, C.M., Trick, M., Heizmann, P. & Dumas, C. 1991. PCR detection of transcripts homologous to the self-incompatibility gene in anthers of *Brassica*. Theor. Appl. Genet. 82, 466-472.

Trick, M. & Heizmann, P. 1992. Sporophytic self-incompatibility systems: *Brassica* S-gene family. Int. Rev. Cytol. (In press)

Oxford with Lyon

Dickinson, H.G., Crabbe, M.J.C. & Gaude, T. 1992. Sporophytic self-incompatibility systems: S-gene products. In "Sexual Reproduction in Flowering Plants". Eds. C. Dumas & S. Russell. Int. Rev. Cytol. Series 5.3. (In press)

Oxford with Birmingham

Elleman, C.J., Franklin-Tong, V. & Dickinson, H.G. 1992. Pollination in species with dry stigmas: the nature of the early stigmatic response and the pathway taken by the pollen tubes. New Phytol. (In press)

OTHER PUBLICATIONS

(Lyon) Gaude, T., Denoroy, L. & Dumas, C. 1991. Use of a fast protein electrophoretic procedure for N-terminal sequence analysis to identify S-locus related proteins in stigmas of *Brassica oleracea*. Electrophoresis 12, 646-653.

(Birmingham) Hackett, R.M., Lawrence, M.J. & Franklin, F.C.H. *Brassica* S-locus related gene promoter directs expression in both pollen and pistil of tobacco. The Plant Journal. (In press)

(Oxford) Doughty, J., McCubbin, A.G., Hedderson, F., Elleman, C.J. & Dickinson, H.G. The role of the pollen grain coating in pollination and self-incompatibility in *Brassica oleracea*. In "Angiosperm Pollen & Ovules". Eds. D. Mulcahy & E. Ottoviano. Springer Verlag. (In press)

Doughty, J., Hedderson, F., McCubbin, A. & Dickinson, H.G. Coating-borne peptides of the *Brassica* pollen grain interact with S(incompatibility)-locus linked stigmatic glycoproteins. Submitted to Proc. Natl. Acad. Sci.

TITLE:		MOLECULAR CONTROL OF GENETIC INSTABILITY IN REGENERATION OF CROP PLANTS
CONTRACT NUMBER:		BIOT-0154-UK
OFFICIAL STARTING DATE:		January 1991
COORDINATOR:	1.	Angela Karp (IACR, LARS, UK)
PARTICIPANTS:	2.	P. Brown (Univ. Hamburg, Germany)
	3.	M. Buiatti (Univ. Florence, Italy)
	4.	F. Sala (Univ. Pavia, Italy)
	5.	L.P. Pijnacker (Univ. Groningen, The Netherlands)
	6.	Y. Henry (Univ. Paris-Sud, France)
	7.	P.J. Kaltsikes (Agric. Univ., Athens)
	8.	G. Wersuhn (Humbodt Univ., Germany)
	9.	M. Humphreys (AFRC, IGER, UK)
	10.	A. Vazquez (Univ. Madrid, Spain)

OBJECTIVES:

To identify an integrated research strategy to determine the causes of genetic instability in plant tissue culture (somaclonal variation). Somaclonal variation is an important problem in genetic manipulation of crop plants and in the micropropagation of ornamental and horticultural plant species, where it results in unwanted variations in regenerated plants and losses in fertility. Some of the changes which arise can be useful for plant breeding, for mutant isolation (when used in conjunction with *in vitro* selection) or as a means of achieving introgession asexually in hybrids. However, these applications are currently limited by the unpredictable and uncontrolled way in which the variations arise. From both the viewpoint of removing unwanted variations and of generating useful variations, it is essential that somaclonal variation is controlled. The main objective of the group is to **determine the underlying mechanisms** and to identify a co-ordinated research effort directed towards this goal.

MAJOR PROBLEMS ENCOUNTERED:

Present efforts are limited by the funding available which can only be used to integrate and co-ordinate and which cannot be used for research purposes. Consequently, although a clear, co-ordinated research strategy has been identified, it cannot be placed into operation, as it requires the initiation of new work. Present research activities of the group's members are funded by completely separate programmes (see below) and any direct co-ordination is limited under the present contract. Additional funding is needed to strengthen and to integrate the research activities by supporting a new, focused activity which combines the expertise and efforts of the group's members.

RESULTS:

The following research activities were carried out by the participants during the period of the grant, under separate research funding:

Participant 1

Previous work in the group has studied external and internal factors involved in somaclonal variation. Even when external factors are constant, the degree of variation induced may differ depending on genotype, suggesting that specific components of plant genomes may be involved. Studies are now under way to determine which regions of the plant genome change in tissue culture and how these processes relate to evolutionary change. The stability of the three genomes of bread wheat in cell suspensions has been studied cytologically in relation to morphogenetic response. Instability was greatest in the AABBDD hexaploid *Triticum aestivum*, intermediate in the AABB tetraploids, *T. dicoccum* and *T. durum*, and least in the diploids *T. monococcum* (AA) and *T. tauschii* (DD). Differences in stability were also observed between species of the same ploidy and stability was greatest in the D genome. In rye (*Secale cereale*), more variation was observed in outbred lines compared with inbred lines. Interesting variants were identified in seed storage proteins some of which are consistent with the suspected presence of a transposable element. Variation in copy number of a 380bp repeated sequence in telomeric heterochromatin has been detected by *in situ* hybridisation (collaboration with participant 7).

Participant 2

One of the main limitations with the study of somaclonal variation is the difficulty of determining how to measure quickly and effectively the genetic variation. Studies in our group have previously used RFLP analysis to determine how tissue culture induced stress induces alterations in known characterised gene sequences. The development of the polymerase chain reaction (PCR) and the associated technique of random amplification of polymorphic DNA (RAPD), means that these processes can be easily used in the study of variation. Using known gene primers for transforming DNA sequences such as the NPT gene which encodes kanamycin resistance, we have been able to study how and when variability begins in tissue culture. Refining both techniques we are now able to examine variability in single isolated protoplasts. Our results demonstrate that little or no variation occurs in transformed DNA sequences until the protoplast begins to divide. Consequently, variation levels in micro-calli can be quite high. RAPD analysis of single protoplasts shows also that single cells isolated directly from leaf tissue are generally identical to controls. It is only when callus formation begins that variability appears. To attempt to resolve what levels of variability are induced by tissue culture and the effect on transformation, we have studied the stability of two transformed DNA sequences in tobacco plants. Over 300 regenerants are being examined for alterations in over forty different restriction sites. The plants are all genetically siblings, in that all are derived from one single transformed protoplast. The results confirm that tissue culture-induced variation considerably affects the efficiency of both transformation and subsequent gene expression.

Participant 3

The overall aim of the work carried out until now has been to study the effect of changing physiological conditions and of biotic and abiotic stresses on genome stability. The systems analysed have been (a) *Nicotiana tabacum* cell clones grown in the presence or absence of atrazine, (b) *Lycopersicon esculentum* cells in dual culture with a pathogen (*Fusarium oxysporium f.sp. lycopersici*) and tomato cotyledons from plantlets infected by the same fungus, and (c) tomato cells grown on media containing different auxin\cytokinin equilibria. In all cases, cultures were scored for sequence rearrangements, amplifications and methylation using homologous and heterologous unique and repeated sequences. In (b), the same screening was carried out also in the fungus in the presence or absence of plant cells. Results showed a low frequency of sequence rearrangements (changes in restriction patterns) in all cases. Atrazine increased the interclonal variability in the copy number of repetitive sequences but depressed the average multiplicity level: the presence of the pathogen induced amplification in repetitive tomato sequences, particularly in the case of incompatible combinations and in cotyledons and changed methylation patterns; multiplicity levels of repetitive sequences varied greatly in tomato according to phytohormone levels in culture being, however, generally lower than in the leaf amplification events, showing an interesting correlation with hypomethylation.

Participant 4

Our aim is to assess how much diversity is generated in plant DNA by biotic stresses and to propose molecular methods for its evaluation. One approach used was to clone highly repeated DNA sequences from two important crop species (tomato and rice) and to follow their degree of amplification in different stress conditions. This work was carried out in collaboration with M. Buiatti as described above. In rice, amplification of repeated DNA sequences in tissue culture is a massive phenomenon. In fact, all tested sequences were amplified when cells were grown in a de-differentiated state. In a long established cell line, we have also revealed the appearance of a circular, extrachromosomal DNA molecule and shown that this molecule includes a long stretch of chloroplast DNA. Using specific probes, we are now studying the molecular structure of this circular molecule, its function in the cells and its mode of replication. This system appears to be a good tool to study the plasticity of the plant chloroplast genome. The stability of foreign genes in transgenic tobacco plants has been studied and found to show little variation even after an extensive culture phase.

Participant 5

Microsporogenesis of a tetraploid *Solanum tuberosum* (+) *S. phureja* and a hypotetraploid *S. tuberosum* (+) desynaptic mutant has been compared with dihaploid fusion partners. The somatic hybrid had a first meiotic division like tetraploid potato. Abnormal spindle orientation occurred at second meiosis, leading to unreduced gametes at various frequencies. Pollen fertility could not be predicted from the fertility of the fusion partners.

Cell cultures of wheat genotypes were initiated and sister chromatid exchanges studied to detect factors influencing DNA duplication in cell cultures. Correlations will be made with meiotic aberrations in regenerants from the same calli.

Karyokinesis and cytokinesis of leaf protoplasts of tobacco and potato were compared with those of protoplasts embedded in alginate. Embedding did not influence the frequency of acytokinesis but the calli became more compact through more cells. Acytokinesis decreased through evacuolisation.

Participant 6

A large range of variation was found between aneuploid stocks of Chinese Spring tested for their *in vitro* response to tissue culture. Results indicate genes with major effects located on the long arms of homologous group 3 chromosomes and on 1AL. Experimental data provide evidence that (1) the induction of the somatic cell towards embryogenesis is limited to the scutellar cells, (2) the second step is concerned with the development of the induced cells into embryos or meristems, (3) plants are produced mostly from somatic embryo development and (4) particular steps are controlled by few genes. Most of the chromosome variation originated in the immature embryos produced from unbalanced gametes but accumulated *in vitro*, suggesting that somatic embryogenesis does not ensure normality. Changes in mitochondrial DNA organisation which occur during tissue culture appear to be under nuclear control and are correlated with the embryogenic capacity of the lines. The heritability of these changes was shown to be maternal in reciprocal crosses, but some progenies had changed mitochondrial DNA compared with the female parent.

Participant 7

Extensive field trials have been carried out on somaclones of rye and triticale. Somaclonal families were found to differ not only with respect to means but also with respect to the correlations between the traits. The somaclonal families also had more variance. This variance is genetic in origin and by affecting heritability values it also affects response to selection. In all lines, some plants were identified with traits superior to the controls.

Participant 8

Work in this group has been directed towards methodical elaborations. This comprised: (1) introduction of techniques to test the rate of sister chromatid exchange (SCE) and karyotype aberrations using *in vivo* cultivated material, short term *in vitro* cultivated explants and cultivated cells, (2) search for an experimental system for combined analysis of SCE rate and genetic recombination rate which allows a direct comparison between both criterions - (the first tests using this system) and (3) standardization of procedures to induce mitosis starting from differentiated somatic cells of *in vivo* grown plants and analysis of genetic diversity of first mitosis after transfer of tissues to *in vitro* conditions.

HIGHLIGHTS\MILESTONES:

Tissue culture-induced variation considerably affects the efficiency of transformation and subsequent expression of transformed genes. Changes can be detected in single isolated cells using PCR and RAPD technology. The stability of the wheat genome in cell suspension and protoplasts depends upon its composition. Nuclear genes control changes in the mitochondrial genome during somatic embryogenesis and major genes on chromosomes 1AL, 3AL, 3BL and 3DL control embryo induction in somatic cultures. Changes in copy number of repeated sequences occur in response to biotic and abiotic stress. In rice, amplification of the genome is a massive phenomenon and this includes sequences in the chloroplast genome. Sister chromatid exchanges can be used as a measure of genetic change *in vivo* and *in vitro*.

WIDER CONSIDERATIONS:

Research on genetic instability in tissue culture provides clear evidence of the extreme plasticity of the plant genome under stress and has implications for obtaining efficient transformation and for our understanding of the generation of genetic diversity. In effect, tissue culture provides an experimental system for studying evolutionary processes in action.

JOINT PUBLICATIONS:

Karp, A., Owen, P., Steele, S.H., Bebeli, P.J and Kaltsikes, P.J. (1992) Variation in telomeric heterochromatin in somaclones of rye. Genome (in press)

OTHER PUBLICATIONS\ PATENTS:

Nothing to report.

ADDENDUM

Participant 9

Although belonging to different genera of grasses, fescue species have strong evolutionary links with ryegrasses and gene exchange is possible albeit at low frequency. Compared to ryegrasses, fescues are more broadly adapted to less fertile conditions, greater climatic extremes and more extensive grassland use. Considerable potential exists to combine adaptive traits from fescues with the excellent growth and nutritive quality features of ryegrasses through reciprocal introgression of genetic material between species. Such introgression programmes rely on recombination through meiosis. Cell culture induces chromosome breakage and reunion in ryegrass/fescue hybrids and has led to improved frequency of interspecific chromosome association in regenerated plants. The tissue culture induced chromosome rearrangements and genetic exchange should enhance recovery of ryegrass-like plants with translocated genes from fescue species for characters such as drought resistance. Using unique drought resistant ryegrass lines derived from this programme at IGER, drought responsive genes will be isolated and identified which are functionally related to drought resistance. These will contribute significantly to our understanding of how plants respond to drought at the biochemical and physiological levels.

TITLE: The molecular biology of the cell-to-cell movement of plant viruses in relation to plasmodesmatal function.

CONTRACT NUMBER: BIOT 900156

OFFICIAL START DATE: 1/01/91

COORDINATOR: (01) Prof. R. Hull, John Innes Institute, Norwich, UK.

PARTICIPANTS: (02) Prof K. Roberts, John Innes Institute, Norwich, UK.
(03) Dr. T. Colburn, IBMP, Strasbourg, France.
(04) Dr. J. Kallerhoff*, BIOCEM, Aubiere, France.
(05) Dr. I. Garcia-Luque, CIB-CISC, Madrid, Spain.
*Dr. J. Kallerhoff was replaced by Dr. S. Ben Tahar during the year.

OBJECTIVES for 1991:

A) How many apparently different mechanisms are there of cell-to-cell movement of viruses? [It was decided at the group meeting to change this objective to - Are there different mechanisms in the cell-to-cell spread of the viruses being studied in the project?].

i) Cloning and sequencing of RNA 3 from CMV.
ii) Antiserum against CMV movement protein.
iii) *In vivo* localization of the movement proteins of various viruses.

B) What are the basic interactions between the virus, movement proteins and plasmodesmata?

i) Optimization of *in vitro* expression systems, use of purified protein in binding studies, mutagenesis of protein in infectious clones.
ii) *In vitro* expression of proteins of various viruses, raising antisera against these proteins and mutagenesis of genes.

C) What is the physical structure of plasmodesmata and how do receptor proteins fit in?

i) Setting up microinjection system, high resolution study of normal plasmodesmata.

D) What are the infection units of different viruses which move through plasmodesmata?

Nothing planned for year 1.

E) Methods for blocking viral movement through plasmodesmata.

i) Development of transformation and regeneration systems for beet and melon.

MAJOR PROBLEMS:

The problems fell under three main headings:-

- Participant 01 had difficulties with obtaining a suitable postdoctoral scientist who, soon after he started was offered a permanent position elsewhere. The post-doctoral position has been refilled, hopefully for the duration of the project.
- Technical problems, which were initially encountered with the systems for microinjection and high resolution microscopy of plasmodesmata, are being worked on and there appear to

be promising solutions. Several groups have had problems with yield and solubility of expressed movement proteins. This was discussed at the group meeting in Strasbourg and various ideas were suggeted for overcoming it.

- Two groups find that the initial choice of plant species for transformation was probably not the best. *Nicotiana benthamiana* has proved difficult to regenerate and gives only low yield of seeds. Participant 03 is changing to *N. tabacum* for transformation. The transformation and regeneration of melon and sugar beet have been found to require such long periods of time that the experimental throughput using these hosts would be very limiting. Participant 04 is changing to *N. tabacum* for the cucumber mosaic virus (CMV) studies and is seeking a suitable host for beet necrotic yellow vein virus (BNYVV).

RESULTS: (Principal investigator name and laboratory number in parentheses).

Most of the effort in this first year has been directed at developing systems, solving problems and obtaining experimental materials from which the main cooperative aspects of the project can be built.

Expression of movement proteins.

The gene 1 product of cauliflower mosaic virus has been expressed in yeast (Lebeurier, 03), *E. coli* and a baculovirus system (Maule, 01). The recombinant protein from all systems was shown not to be N-glycosylated and that from the baculovirus system was not phosphorylated. The expressed proteins had the same apparent mol. wt. (42 kDa in contrast to 38 kDa predicted from the sequence) as the protein synthesised in infected plants.

The movement protein of alfalfa mosaic virus (AlMV) expressed in yeast and *E. coli* has the same mobility as that from plants which suggests that none of the proteins is post-translationally modified. A series of 8 contiguous in-frame deletions covering the whole sequence of the AlMV movement protein gene have been made and are being expressed in *E. coli* (Godefroy-Colburn, 03).

The nucleotide sequence of RNA 3 of a Spanish strain of CMV (CMV-24) has been determined and the open reading frame, encoding the putative movement protein, shown to be 279 amino acids long. The nucleotide sequence of this region is very similar to that of Fny-CMV which was isolated from muskmelon in New York State, USA and the deduced protein sequence is identical. The CMV movement protein has been expressed using the T7 RNA polymerase system in *E. coli* by cloning into pT7-7. Expression levels were higher in *E. coli* strain K38 than in BL21 (DE3) at 2h post-induction but at 7h post-induction they were higher in BL21 than in K38 (Garcia-Luque, 05).

A comparison has been made of the production and properties of the 30 kDa movement protein of tobacco mosaic virus (TMV) in the baculovirus system, in plants, from *in vitro* translation of mRNA transcribed from a cDNA clone and in *E. coli*. The protein from translation and from plants was produced at low level and that from plants was difficult to purify. Baculovirus and *E. coli* expression gave high yields with the maximum level of P30 production in the baculovirus system between 48 and 72 h post infection of the SF21 insect cells. However, in both systems the major problem was insolubility (Hull, 01).

Genomic location of movement protein genes in different viruses.

BNYVV RNA 1 is infectious to protoplasts which shows that it can autoreplicate but RNA 2 is required for infection of a local lesion host such as *Chenopodium quinoa*. This indicates that RNA 2, which has 6 open reading frames, carries functions for cell-to-cell movement. Disabling mutations have been introduced into each of the open reading frames in RNA 2 and the ability of transcripts carrying each mutation to replicate when coinoculated

with RNA 1 was tested both in protoplasts and on leaves. Three adjacent genes encoding proteins of 42, 13 and 15 kDa (the 'triple block') were found to be non-essential for virus multiplication in protoplasts but required for local lesion formation in leaves. This suggests that the 'triple block' proteins are required for cell-to-cell movement. Mutations in the viral coat protein did not interfere with local lesion production whereas those in the 3' proximal gene, encoding a protein of 14 kDa (P14), were not lethal but dramatically down regulated production of viral RNAs and proteins. This suggests that P14 regulates expression of other RNA 2 gene products and thus indirectly influences cell-to-cell movement (Richards, 03).

Grapevine fanleaf virus (GFLV) RNA 2 encodes a 122 kDa polyprotein with the viral coat protein in its C-terminal portion. The viral proteinase encoded by GFLV RNA 1 processes the RNA 2 polyprotein into the 56 kDa coat protein and a 66 kDa product which is further processed to 37 and 29 kDa proteins. The 37 kDa protein shows amino acid sequence homology to TMV 30 kDa movement protein. A fusion product of the 37 kDa protein with the N-terminal portion of the C1 repressor from phage λ has been produced in *E. coli* and a specific antiserum reacting with a protein of apparent MW of 44 kDa in extracts from infected leaves was obtained (Pinck, 03).

Properties of movement proteins.

As noted above, problems have been encountered with insolubility of expressed movement proteins. A wide range of treatments have been tested in attempts to improve solubility. For instance, with the TMV P30 some of the protein could be solubilized by treating it first with Sarkosyl and then replacing the Sarkosyl with Triton X-100 (Hull, 01).

The AlMV movement protein (obtained from yeast or *E. coli*) has been shown to bind single-stranded nucleic acids cooperatively. The binding is biphasic: the first phase is very fast and non-cooperative whereas the second phase is slow, temperature dependent and cooperative (Godefroy-Colburn, 03).

Baculovirus-expressed CaMV gene 1 product aggregates as regular fibrillar arrays. The protein can be solubilized in acid and under denaturing conditions (e.g. >6M urea, 6M guanidine HCl or 4M urea, 1M NaCl at 70°C). The CaMV gene product expressed from *E. coli* binds single-stranded RNA, confirming the report from Citovsky (*Proc. Natl. Acad. Sci. USA* 88:2476, 1991). Constructs of CaMV with various deletions in gene 1 have been introduced into leaf disks by agroinoculation. This leaf disk assay showed that the deleted genomes multiplied in single cells but did not spread into whole tissues. This is the first formal identification of CaMV gene 1 product as the movement protein (Maule, 01).

In attempts to recognise one or more host proteins to which TMV P30 bound, extracts of host proteins were electrophoresed into a polyacrylamide gel and blotted onto a nitrocellulose membrane. This membrane was then treated with Sarkosyl/Triton solubilized baculovirus-expressed P30, washed and then any binding of P30 to proteins transferred from the gel was detected with anti-P30 antiserum. No bands were reliably detected. To enhance the sensitivity of this sandwich hybridization method ^{35}S-labelled P30 was used to probe the nitrocellulose blot. Even this approach did not relaibly detect any host protein to which P30 bound (Hull, 01).

An antiserum (supplied by participant 03) was used to localize BNYVV P42 in sugar beet plants which had been infected for 5 weeks. The protein was found in subcellular fractions containing membranes and cell walls (Kallerhoff, 04).

Ultrastructure of plasmodesmata.

A reliable method has been developed for the preparation of clean cell wall fragments containing intact plasmodesmata. This method involved grinding plasmolysed tobacco leaves

or maize roots in liquid $N2_2$ followed by extrusion through a French Press and then repeated washing with extraction buffer. This produced cell wall fragments ranging in size from 1µ to several µ which, on embedding and sectioning, were shown by electron microscopy to contain seemingly intact plasmodesmata. After negative staining these preparations have proved suitable for high-resolution conventional electron microscopy with a tilting stage to give 3-D imaging. In initial experiments using uranyl acetate or methylamine tungstate as negative stains, substructure was revealed in the plasmodesmatal neck (Roberts, 02).

Transformation of plants.

Using several previously produced lines of *N. tabacum* expressing the complete and N-terminally deleted AlMV movement protein lacking respectively 12 and 77 amino acids (designated Δ[1-12] and Δ[1-77]), it was shown that Δ[1-12], as well as the unmodified protein, fractionated with cell walls whereas Δ[1-77] occurred only in cytoplasmic fractions. This suggested that the domain covered by amino acids 13-77 is involved in cell wall targetting. Various other transformations of *N. benthamiana* and *N. tabacum* have been made with the complete protein and with the deletions described above. Those in *N. benthamiana* expressed at a relatively low level. Examination of the transgenic *N. tabacum* by immuno-electron microscopy revealed the unmodified and the Δ[1-12] protein in plasmodesmata and the Δ[1-77] in the cytoplasm and vacuole, thus confirming the cell fractionation results (Stussi-Garaud, 03).

Arabidopsis thaliana plants which have been transformed with CaMV gene 1 and the TMV 30K gene are currently being tested for expression and complementation (Maule, 01).

For transformation of plants with the BNYVV gene encoding P42 (obtained from participant 03) the gene was placed downstream of the TMV 'Ω' sequence and cloned between the cauliflower mosaic virus 35S promoter and the nos terminator. This was then cloned into the transformation vector pGA 492, which has successfully been used to transform sugar beet with the BNYVV coat protein gene, to give pGA MP42A. pGA MP42A was introduced into *Agrobacterium* to transform sugar beet and tobacco but, in the first round of experiments, no transformants were obtained which expressed P42 (Kallerhoff, 04).

HIGHLIGHTS/MILESTONES:

- Localization of cell wall targetting region of AlMV movement protein to an N-terminal domain of the gene.
- Single-stranded nucleic acid binding properties of AlMV movement protein.
- Cloning of movement protein gene of CMV.
- Recognition that each of the 'triple block' genes of BNYVV are involved in cell-to-cell movement.
- Development of a method for the preparation of cell wall fragments containing plasmodesmata

WIDER CONSIDERATIONS:

During this year we have made good progress at developing a strong research base from which we will build up the main thrust of the project. While there have been no real 'major' new findings we have added significantly to the knowledge of these movement proteins.

COOPERATIVE ACTIVITIES:

- March 18-19th 1991, R. Hull visited CIBC, Madrid to discuss programme with I. Garcia-Luque.
- 13-20th September 1991, G. Demangeat visited R. Hull to discuss progress of project.
- January 8-10th 1992, group meeting at IBMP Strasbourg attended by all participants and coworkers in Strasbourg. During this meeting there was detailed analysis of progress and problems and planning of future experiments and collaborations.
- Six issues of the group newsletter, View through the Plasmodesma, bringing news and information to members of the group.

JOINT PUBLICATIONS:

Atkins, D., Hull, R., Wells, B., Roberts, K., Moore, P. and Beachy, R.N. (1991) The TMV 30kD movement protein in transgenic tobacco plants is localized to plasmodesmata. J. Gen. Virol. **72**:209-211.

Atkins, D., Roberts, K., Hull, R., Prehoud, C. and Bishop, D.H.L. (1991) Expression of the tobacco mosaic virus movement protein using a baculovirus expression system. J. Gen. Virol. **72**:2831-2835.

OTHER PUBLICATIONS:

Gilmer, D., Bouzoubaa, S., Hehn, A., Guilley, H., Richards, K. and Jonard, G. (1992) Efficient cell-to-cell movement of beet necrotic yellow vein virus requires 3' proximal genes located on RNA 2. Virology (in press).

Kirchherr, D., Wurch, T., Mesnard, J.M. and Lebeurier, G. (1991) Expression of cauliflower mosaic virus gene 1 in *Saccharomyces cerevisiae*. Res. Virol. **142**:297-302.

Maule, A.J., Usmany, M., Wilson, I.G., Boudazin, G. and Vlak, J.M. (1992) Biophysical and biochemical properties of baculovirus-expressed CaMV P1 protein. Virus Genes **6**:5.

Schoumacher, F., Erny, C., Berna, A., Godefroy-Colburn, T. and Stussi-Garaud, C. (1992) Nucleic acid-binding properties of the alfalfa mosaic virus movement protein produced in yeast. Virology **188**: (in press).

TITLE:	Genes required for pathogenicity of bacteria to plants and application of knowledge in biological control of diseases of crops.
CONTRACT NUMBER:	BIOT-CT90-0168 (UK)
OFFICIAL STARTING DATE:	1st February, 1991
COORDINATOR:	DR. M.J. DANIELS, The Sainsbury Laboratory (SL) Norwich, UK.
PARTICIPANTS:	DR. U. BONAS, Inst. für Genbiologische Forschung (IGF), Berlin, GE. DR. A. VIVIAN, Bristol Polytechnic (BP), Bristol, UK. DR. C. BOUCHER, Lab. de Biol. Molec. des Relations Plantes-Microorganisms (LBMRPM), Toulouse, FR. MR. M. GUILLON, Calliope, Beziers, FR. DR. M. KOKKINIDIS, DR. N. PANOPOULOUS, Inst. for Molecular Biology & Biotechnology (IMBB), Heraklion, GR.

OBJECTIVES SET FOR THE REPORTING PERIOD:

hrp genes of *Pseudomonas* and *Xanthomonas* (required for pathogenicity and resistance induction): sequencing, characterisation of products, control of expression, role in pathogenicity, and regulation of other genes.
avr genes of *Pseudomonas* and *Xanthomonas* (determine inability to colonise certain hosts): sequencing, role in non-host interactions, structure-function relationships.
rpf genes of *Xanthomonas* (regulate pathogenicity functions): characterisation of Rpf C protein, sequencing of *rpfN* gene, interaction of RpfN protein with promoters of pathogenicity genes.
hrp mutants of *Pseudomonas solanacearum*: use as biological control agents against bacterial wilt.

MAJOR PROBLEMS ENCOUNTERED: NONE.

RESULTS:

hrp genes:

IGF: Cross-complementation of *Xanthomonas campestris* pv. *vesicatoria (Xcv) hrp* mutants by *hrp* sequences from *Xcc* that were cloned by SL indicate functional conservation of *hrp* genes between the two bacteria. The DNA sequence of a 13 kb portion of the *hrp* region of *Xcv* containing 4 operons was determined. Several putative *hrp* proteins show homology to putative *hrp* proteins from *Pseudomonas solanacearum* the sequences of which

were determined by LBMRPM. In addition, striking similarities to *Yersinia* proteins were found. The homologous *Yersinia* proteins are involved in protein export and necessary for pathogenicity indicating that pathogenicity determinants might be conserved between plant and animal pathogenic bacteria. Expression studies in *Xcv* showed that *hrp* gene activity is suppressed in complex media but induced in the plant. Furthermore, for one of the *hrp* loci, *hrpF*, a minimal medium was defined that allows gene induction without a plant-derived molecule. A more detailed analysis of the *hrp* promoters and of the *hrpB* operon is in progress. So far, four HrpB proteins can be expressed in *E.coli* so that we will be able to study these proteins using specific antibodies in the near future.

LBMRPM has concentrated its efforts on sequencing the *P. solanacearum hrp* gene cluster. 18 kb out of 23 kb has been sequenced, allowing the identification of 18 open reading frames (ORFs) with high coding probabilities. Seven of them are predicted to have transmembrane domains and are therefore probably located in the bacterial cell envelope. Search for homologous proteins in data banks has revealed that at least five of the putative Hrp proteins have homology with the products of pathogenicity genes of the animal pathogen *Yersinia*. The homologous *Yersinia* proteins are all essential and specific for the secretion of protein extracellular pathogenicity determinants called Yop(s). Therefore we propose that in *P. solanacearum hrp* genes might be involved in the secretion of macromolecular compound(s) which might directly or indirectly act as mediator(s) in the interaction of the bacteria with the plant. DNA sequence analysis has also revealed the presence of two possible regulatory genes within the *hrp* gene cluster. The role of the protein encoded by one of these genes in activating the transcription of 4 out of the 6 transcriptional units present in the *hrp* gene cluster is now established.

Finally, preliminary work shows that at least certain *hrp* genes are conserved among *P. solanacearum*, *X. campestris*, *P. syringae* and *Erwinia amylovora* (LBMRPM, SL, IGF).

In *X.c. campestris*, *hrp* genes are expressed most strongly under starvation conditions (SL). Functional *hrp* genes are not required for expression of the avirulence gene *avrXca* (which interacts with *Arabidopsis thaliana*), or for production of pathogenicity determinants such as extracellular enzymes and polysaccharide. On the other hand, mutants defective in these factors can still induce the hypersensitive resistance response (HR) in appropriate plants, whereas *hrp* mutants cannot. Thus, at least two independent groups of genes are required for plant-bacterial interactions (SL).

In the bean halo blight pathogen, *Pseudomonas syringae* pv. *phaseolicola*, a 22 kb cluster contains several *hrp* genes/operons (L, A, B, C, D, E, F and RS) that share common regulation. Three other *hrp* genes (M, Q, and T) are found in distinct locations in the genome. The expression of the genes in the *hrp* regulon is under complex genetic and physiological regulation by 1) a plant factor(s), 2) pH, 3) osmotic strength, 4) nutritional status, 5) the S, R, L, T, and Q genes, and 6) the sigma-54 subunit of RNA polymerase.

At IMBB an open reading frame of 302 codons has been identified as the region encoding a 34.5 kD polypeptide with homology to the highly conserved domain of several regulatory proteins of bacteria that promote transcription from sigma-54-dependent promoters. This gene, designated *hrpS*, was shown to be a regulator of several *hrp* operons in *P. s. phaseolicola*.

One objective is to characterize the molecular structure of the HrpS protein and its interaction with *hrp* gene promoters. 2.5 mg HrpS protein with a purity better than 90% was prepared with the aim of growing crystals of sufficient quality for high resolution diffraction studies. Various forms of crystals were obtained using the hanging-drop technique, and

experiments are presently under way to improve their quality. Freshly purified HrpS protein tends to form dimers and to some lesser extent higher oligomers.

The emphasis on overproduction and purification of the HrpS protein is linked to efforts to investigate two other questions regarding the mechanism of *hrp* gene expression control. 1. Does *hrpS* regulate the other *hrp* operons directly.?. Purified HrpS protein is being used in DNA footprint and gel retardation analysis of *hrp* gene promoter regions. Several *hrp* gene promoters may be controlled by sigma54-dependent activator proteins (such as HrpS). 2. Does the HrpS protein act in transcription as is or does it require either post-translational modification or inducer binding. Recent findings suggest that *hrpS* may be controlled by the Q and T genes and by a plant factor(s). Unpublished DNA sequence data for *P. s. phaseolicola* and *P. solanacearum hrp* genes was exchanged with LBMRPM, showing Hrp protein homologies between the two pathogens.

Calliope and LBMRPM have been developing biological control against *P. solanacearum* based on the introduction of bacterial antagonists (avirulent *hrp* mutants) into the vascular system of the host plant. The mutants are unable to produce disease in young susceptible tomato plants, even though most of them retain the ability to colonise and to multiply to some extent in the plant. The mutants prevent subsequent colonization of the plant by a virulent strain. The group is extending these promising results, obtained under P3 containment laboratory conditions in Toulouse, to field experiments in naturally infested soils in Guadeloupe. Further protective mutants have been obtained by insertion into the *hrp* genes of an Omega-Km cassette, which is no longer transposable. These mutants are derived from native virulent strains isolated in Guadeloupe. Experiments in a contained environment, over a 12 month period in Guadeloupe showed that effective protection against bacterial wilt with such mutants affects neither plant development nor fruit production. Isolations from root, collar and stems of protected tomato plants indicate that the mutants are restricted to root and collar level. Bacteriocins are produced *in planta* by some strains. These strains possess a competitive advantage which will be used in selecting the appropriate protective mutants.

avr genes:

The avirulence gene *avrBs3* from *X. c. vesicatoria* contains repeat motifs in the central region. The importance of the repeats in *avrBs3* was studied at IGF by generating deletion derivatives containing a smaller number of repeats. The avirulence protein function and the specificity of the interaction was changed with several of the new derivatives, some of which induce now a hypersensitive response on formerly susceptible pepper or tomato cultivars. We thus "unmasked" hitherto unknown resistance genes in the plant. Furthermore, genetic analysis of one of the "new" resistance genes revealed linkage or identity to the "recessive" allele of the known resistance locus *Bs3*. The sequence analysis of a naturally occurring *avrBs3*-allele which induces resistance in tomato but not in pepper confirmed the importance of the repeats for the specificity of the avirulence protein.

At BP, the DNA sequence of an *avr* gene (A2) determining race 2 specificity in *Pseudomonas syringae* pv. *pisi (Psp)* has been obtained. Database searches have failed to reveal significant homology to any DNA or protein sequences. The derived protein sequence indicates a cytosolic location for the gene product. Attempts are being made to express this gene in *E. coli*. The cloning of this gene led to the cloning by homology of an *avr* gene from *P. s.* pv. *maculicola (Psm)*, which confers race 2 cultivar-specificity in *Psp* toward pea *(Pisum sativum)*. Collaboration with J. Dangl at MPI, Koln has shown that this homologue acts as an *avr* gene in ecotype-specificity of *Psm* in *Arabidopsis thaliana*. A second *avr* gene (A3),

determining race 3 specificity in *Psp*, is currently being sequenced. This gene has been shown to be plasmid-borne and is the only gene responsible for race 3 incompatibility towards pea cultivar Belinda.

Non-host resistance: We have shown (with J.W. Mansfield (Wye) and J.D. Taylor (HRI) that the A2 gene from *Psp* behaves as an *avr* gene in *P. s.* pv. *phaseolicola* toward certain cultivars of its host bean. This has enabled the investigation of non-host resistance genes in bean and shown that the A2 gene interacts with two resistance genes.

Introducing *avr* genes into different races of *Psp* sometimes resulted in anomalous reactions towards the host. The results imply that the *avr* gene responsible for race 4 specificity is suppressed in race 6 strains, and attempts are in progress to identify and clone this putative suppressor.

At SL an avirulence gene from *X.c. campestris* has been characterised and sequenced. The gene interacts with gene(s) in *Arabidopsis thaliana* to confer avirulence on bacteria carrying it. However virulence to *Brassica* hosts is unaffected. The avirulence gene is not dependent on functional *hrp* genes for its expression.

rpf genes:

rpf genes in *X.c. campestris* are pleiotropic regulatory genes which control synthesis of pathogenicity factors such as extracellular enzymes and polysaccharide. One group of clustered genes, *rpfA-G*, act positively. Sequencing has shown that *rpfC* and *rpfG* products belong to the class of sensor-regulator proteins. The *rpfC* product is unusual in combining both sensor and regulator domains in the same protein. The protein is being purified to allow biochemical and genetic studies of its regulatory activity, probably involving phosphorylation, and its interaction with other *rpf* genes, and with the target genes. *rpfN* by contrast acts negatively on the same targets. The gene has been sequenced, but comparison of the sequence of the presumptive protein product with databases has yielded no information about relatedness to known proteins. A conserved sequence upstream of the coding regions of two of the regulated genes appears to be the site of RpfN action, demonstrated by DNA binding studies and "footprint" analysis. The relationship of the *rpfA-G*, *rpfN*, and other regulatory genes (e.g. *clp*), is under investigation, with a view to understanding the modulation of pathogen activity during disease development.

HIGHLIGHTS:

(i) *hrp* gene products of *P. solanacearum*, *P. syringae* and *X. campestris* are related to pathogenicity proteins of the human pathogen *Yersinia*, suggesting that certain aspects of plant and animal disease involve analogous mechanisms.
(ii) Repeat structures in some *avr* genes are important in determining specificity. Some avirulence genes are able to give both race-cultivar specificity and pathovar-non-host specificity, suggesting that a basic fundamental mechanism underlies host-pathogen specificity.
(iii) A suppressor of *avr* gene action may have been discovered.
(iv) Production of pathogenicity factors is under control by a series of overlapping systems.
(v) Pretreatment of plants with avirulent *hrp* mutants of *P. solanacearum* protects against subsequent infection by virulent strains.

WIDER IMPLICATIONS:

Our work has shown that a diverse group of plant pathogens use very similar mechanisms for

causing disease. Thus, if we can determine how to interfere with the pathogenicity process, this is likely to have general applicability. The use of avirulent mutants for biological control is a promising strategy for limiting disease, and will in addition give information about plant disease resistance mechanisms.

COOPERATIVE ACTIVITIES:

The group had a joint meeting at IMBB, kindly arranged by M. Kokkinidis, at which the background and progress of the project was fully discussed and collaborative activities were planned. Numerous exchanges of biomaterials and unpublished information have taken place between the groups.

PUBLICATIONS:

Tang JL, Liu Y, Barber CE, Dow JM, Daniels MJ (1991) Genetic and molecular analysis of a cluster of *rpf* genes involved in positive regulation of synthesis of extracellular enzymes and polysaccharide in *Xanthomonas campestris* pathovar *campestris*. Mol. Gen. Genet. 226: 409-417.

Arlat M, Gough CL, Barber CE, Boucher C, Daniels MJ (1991) *Xanthomonas campestris* contains a cluster of *hrp* genes related to the larger *hrp* cluster of *Pseudomonas solanacearum*. Mol. Plant-Microbe Interactions 4: 593-601.

Daniels MJ (1992) Interaction of *Xanthomonas* with crucifers: Neth. J. Plant Pathol. (in press).

Daniels MJ (1992) Bacteria. pp. 35-43. In: Molecular Plant Pathology, a Practical Approach ed. SJ Gurr, MJ McPherson & DJ Bowles. IRL/OUP, Oxford.

Bonas U, Schulte R, Fenselau S, Minsavage GV, Staskawicz BJ, Stall RE (1991) Isolation of a gene cluster from *Xanthomonas campestris* pv. *vesicatoria* that determines pathogenicity and the hypersensitive response on pepper and tomato. Mol. Plant-Microbe Interactions 4: 81-88.

Canteros B, Minsavage G, Bonas U, Pring D, Stall R (1991) A gene from *Xanthomonas campestris* pv. *vesicatoria* that determines avirulence in tomato is related to *avrBs3*. Mol. Plant-Microbe Interactions 4: 628-632.

Knoop V, Staskawicz BJ, Bonas U (1991) The expression of the avirulence gene *avrBs3* from *Xanthomonas campestris* pv. *vesicatoria* is not under the control of *hrp* genes and is independent of plant factors. J. Bacteriol. 173: 7142-7150.

Schulte R, Bonas U (1992) A *Xanthomonas* pathogenicity locus is induced by sucrose and sulfur-containing amino acids. Plant Cell 4: 79-86.

Schulte R, Bonas U (1992) The expression of the *hrp* gene cluster from *Xanthomonas campestris* pv. *vesicatoria* that determines pathogenicity and hypersensitivity on pepper and tomato is plant-inducible. J. Bacteriol. 174: 815-823.

Herbers K, Conrads-Strauch J Bonas U (1992) Race-specificity of plant resistance to bacterial spot disease determined by repetitive motifs in a bacterial avirulence protein. Nature 356: 172-174.

Arlat M, Gough C, Zischek C, Barberis P, Trigalet A, Boucher C (1992) Transcriptional organization and expression of the large *hrp* gene cluster of *Pseudomonas solanacearum*. Mol. Plant-Microbe Interactions. In press.

Gough C, Genin S, Arlat M, Zischek C, Boucher C (1992) Conservation of pathogenicity determinants among animal and plant pathogenic bacteria. Submitted.

Bavage AD, Vivian A, Atherton GT, Taylor JD, Malik AN (1991) Molecular genetics of *Pseudomonas syringae* pathovar *pisi*: plasmid involvement in cultivar-specific incompatibility. J. Gen. Microbiol. 137: 2231-2239.

Fillingham AJ, Wood J, Bevan JR, Crute IR, Mansfield JW, Taylor JD, Vivian A (1992) Avirulence genes from *Pseudomonas syringae* pathovars *phaseolicola* and *pisi* confer specificity towards both host and non-host species. Physiol. Molec. Plant Pathol. 39, in press.

Moulton PJ, Vivian A, Bavage AD, Muir LAJ, Hunter PJ, Orvis JD, Taylor JD (1992) Transfer of plasmid RP4 and other incompatibility group P replicons to *Pseudomonas syringae* pv. *pisi* can cause changes in cultivar-specificity toward pea. Manuscript in preparation for Submitted.

TITLE : Molecular basis of signalling in *Rhizobium meliloti-Medicago* interactions and genetic improvement of nodulation ability

CONTRACT NUMBER : BIOT 900159-C
OFFICIAL STARTING DATE : 0.1.12.1990
COORDINATOR :
01 : Dr. A. Kondorosi, Inst.des Sciences Végétales CNRS, 91198 Gif/Yvette FR
PARTICIPANTS :
02 : Dr. M. John, Max-Planck-Institut für Züchtungsforschung, 5000 Köln 30, DE
03 : Dr. J. Olivares, Estacion Experimental del Zaidin CSIC, 18006 Granada, ES
04 :Dr. J. Hache, Bertin and Cie, 78373 Plaisir, FR

OBJECTIVES SET FOR THE REPORTING PERIOD

Rhizobium bacteria, in symbiosis with leguminous host plants, are able to fix atmospheric N_2 in the absence of combined nitrogen. The formation of nitrogen-fixing root nodules requires specific interactions of certain combinations of plants and *Rhizobium* strains. The host-specific recognition is established at multiple stages during the development of symbiosis. Signals are emitted by both partners and are recognized by the other. The aim of this project is to elucidate how the signal molecules determined by the *Rhizobium* nodulation (*nod*, *nol*) genes evoke responses in the plant host leading to root nodule development. Furthermore, the molecular basis for increased competitiveness conferred by the *nod* genes and by another class of bacterial genes, the *nfe* genes are being investigated. Based on these studies, methods for improving the efficiency and competitiveness of *Rhizobium* strains to be used as field inoculants will be developed. Finally, modern analytical technology will be developed and applied for plant molecular genetic studies of the plant partner.
The following tasks were planned to be started:
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.

MAJOR PROBLEMS ENCOUNTERED: Nothing to report.

RESULTS:

Topics i.01, ii.01 and 02 has progressed faster and completed earlier, while iii.03 and iv.03 starts only in 1992.
i) 01 : At the ISV positive as well as negative transacting factors and their genes in *Rhizobium meliloti* have been characterized: the transcriptional activators NodD3 and SyrM and the repressor NolR (Kondorosi et al., 1991b ; 1991c). Studies on NolR were done together with MPI. We showed that these factors, in addition to NodD1, NodD2 and

specific plant signals, flavonoids, regulate the expression of the bacterial nodulation (*nod*) genes which are involved in the production of signal molecules, the so-called Nod factors. It has been demonstrated that the different regulatory proteins form an interdependent network. This involves amplifying circuits by positive autoregulation as well as the induction of different activator proteins by each other. Fine tuning is achieved by the inhibitory action of the NolR. Other, yet uncharacterized proteins were shown to participate in the interactions between the major regulatory elements. This complex regulation enables the bacterial cell to respond efficiently to a broad spectrum of environmental stimuli like different flavonoids excreted by a variety of host plants, as well as the concentration of combined nitrogen, and allows the production of optimal amounts of Nod signals. Understanding the determinants of this regulatory network has already allowed to extend the host range of *R. meliloti* (Kondorosi et al., 1991c) and will offer the possibility to improve the nodulation efficiency.

02: Various rhizobial genes (such as *nodABC* and *hsnD*) had been shown to be involved in catalyzing steps in the synthesis and specific modification of the Nod factors. Recently it was demonstrated at the MPI that the NodA and NodB proteins are sufficient to produce compounds that stimulate the mitosis of plant protoplasts. In order to test whether this new class of plant growth factors could also play a role in non-legumes at the MPI, the *nodA* and *nodB* genes were introduced and expressed in tobacco. Transgenic tobacco plants expressing *nodA* and *nodB* under the control of the relatively weak dual TR promoter showed an effect on cell differentiation leading to the formation of bifurcated leaves and to the formation of two or more stems emerging independently of the leaf axle.

Transgenic tobacco plants expressing only the *nodA* gene under the control of the strong 35S CaMV promoter exhibited slightly reduced growth, a reduction in the internode distance and an altered leaf morphology. Expression of the single *nodB* gene (35S promoter) was shown to be responsible for strongly reduced growth and a compact inflorescence. Many flowers had only four petals and four anthers instead of five. In all cases heterostyly with increased stigma size was found, so that the plants are unable to self-pollinate.

To express *nodA* and *nodB* in tobacco both under control of a strong promoter, the *nodA* transgenic plants have been crossed with *nodB*-transgenics. These plants combined the properties of the parental plants, resulting in an extremely reduced growth, small internode distances, heterostyly, reduced apical dominance and strongly curled leaves.

The generation of *nodABC*, *nodC*, and *hsnD* (*nodH*) transgenic tobacco plants is in progress. In collaboration with the ISV the same *nod* genes and their combinations are also being transformed into alfalfa (*M. sativa*). The comparison of the effects in tobacco versus alfalfa will permit to discriminate between the unspecific and symbiosis-specific functions of the Nod signals in plant development.

To explain the effects on growth and differentiation caused by the *nod* genes, the biochemical functions of the corresponding *nod* gene products have to be elucidated. Since the *nodB* transgenic tobacco showed the most pronounced phenotype, first the function of NodB was investigated at the MPI. Therefore, the protein has been overproduced in *E. coli* and was purified by immunoaffinity chromatography or by renaturation of active NodB from inclusion bodies. A function of the NodB protein has been demonstrated which is now being confirmed. Several other *nod* gene functions are under study, in collaboration between the MPI (providing purified Nod proteins and antibodies) and the ISV (providing precursor molecules and purified standards from over-producing *R. meliloti* strains).

An interesting observation was made at the MPI by showing that the *nod* inducers stimulate also the production of the phytohormone IAA in *R. meliloti*. (Prinsen *et al.*, 1991).

03: The success of the inoculation practice of legume seeds requires that the inoculant strains be highly effective in nitrogen fixation and highly competitive against soil strains in nodule occupancy. At the EEZ, the *nfe* genes were found to control competitiveness in *R. meliloti*. 9 Kb of the *nfe* DNA region of *R. meliloti* GR4 have been sequenced and preliminary results indicate the existence of 8 ORFs. Data bank search revealed that ORF3 and ORF4 have homology with the *ocd* gene product of *Agrobacterium tumefaciens* and with the insertion sequence ISRm3, respectively. The *ocd* gene, that has been reported only

in few bacterial species, encodes for ornithine cyclodeaminase that converts ornithine to proline. In *Agrobacterium* it was shown to be involved in opine catabolism. Although this gene locus seems to have its own functional promotor in *R. meliloti* GR4, it can also be transcribed, from the *nif*-like promoter upstream of ORF2. DNA sequence data predict a protein of 320 residues wich was supported also by SDS-PAGE. The functionality of the *ocd* gene product has been proved using labelled ornithine. Implication of this enzyme activity in the expression of the competitiveness of *R. meliloti* is under study.

The ORF4, located downstream of the *ocd*-like gene, corresponds to IS element ISRm3 which is present only in one copy in the GR4 genome. The significance of this IS in the genetic organization of the *nfe* region is not known.

When a clone (pRmNT40) containing the *nfe* genes was transferred to different *R. meliloti* strains, most of them showed an increased competitiveness (strains 2011, 41, etc.). Strain L5.30 however, did not change, and surprisingly, AK631 a derivative of 41 (obtained from the ISV) hast lost its competitive ability. No explanation has been found so far for this behaviour since the nodulation kinetics for AK631 and AK631 (pRmNT40) in single infections are similar. The genetic determinant influencing competitiveness in strain AK631 will be investigated in collaboration with the ISV.

ii) 01 and 02: Using the new knowledge about the regulatory factors (see i 01) has allowed the manipulation of *R. meliloti* strains lacking the negatively acting factors but containing higher amounts of positive transacting factors. These strains produced more than thousand fold higher quantitites of Nod signals in comparison to the wild type strains (Schultze et al., 1992). This engineering step was a prerequisite to purify and characterize the Nod metabolites. From one of the overproducing strains high amounts of a family of sulfated lipo-oligosaccharides were extracted and the chemical structures of the molecules were determined (Schultze et al., 1992). The ability of the Nod factors to induce morphological and developmental changes on plant roots, like root hair deformation and induction of nodule structures were investigated. The differential activity on host versus non-host plants of related lipo-oligosaccharides allowed to identify some of the structural features (e. g. oligosaccharide chain length) influencing the host plant-specific recognition.

The purified lipo-oligosaccharides are now being used at the ISV to elucidate the specific signal perception and transduction by the host plant. For this aim the Nod signal molecules were radiolabeled and used to identify receptor molecules within host plant tissues. To study the signal transduction the molecular effects, like induction of gene expression, influences on the cell cycle control and physiological changes like pH and Ca^{2+} concentration, either in plant roots, root hairs or cells in suspension culture are being studied. Understanding the signal perception and transduction as well as the structural requirements may allow to engineer *Rhizobium* strains producing optimal concentrations of Nod factors, and possibly in long term, to generate plant varieties responding more efficiently to the bacterial signals.

iii) 01 : In a search for plant genes activated by the Nod factors at the ISV a *Medicago* genomic clone homologous to the already characterized pea early nodulin Enod12 has been isolated. This clone was sequenced and fusions of the putative promoter to the GUS reporter gene were constructed. These constructions have been introduced into *Medicago sativa* . These plants will be useful tools to analyze specific expression, and inducibility of this early nodulation gene by the Nod factors. Other genes activated by the Nod factors and cloned by differential screening of a cDNA bank prepared from *Rhizobium* -infected root hair RNA are being characterized.

04: A complete procedure of DNA extraction is a combination of unit operations including pre-treatment, lysis, removal of debris, extraction and eventually resuspension. BERTIN has developed a process consisting of an unique combination of novel techniques including, in a same cartridge, isolation of nuclei, nuclear lysis and dialysis. This process was compared for *Medicago sativa* to classical techniques. In a first time, DNA was perfectly digestible by restriction enzymes, but was of poor quality (DO 260/280 = 1.25). Improvements comprising grinding in the presence of liquid nitrogen followed by a rapid lysis and a solvent extraction before the lysis/dialysis process in the cartridge, allowed to

obtain a better quality (DO 260/280 = 1.85). At present semi-automatic prototypes allowing to extract DNA from various plant materials are available at BERTIN.

A multisouthern/northern electrophoresis and blotter system has been developed and used to obtain first blots starting from *M. sativa* . The technology is perfectly defined and during a simple run one can obtain automatically 4 blots with 24 lanes per blot, plus 3 lanes for markers. This system is now available to establish RFLP maps. The major advantage of RLFPs in variety identification being lied in the availability of an almost infinite number of combinations of the restriction enzymes and probes. The establishment of the RFLP map of *M. truncatula* has not begun as foreseen, since it was preferred to fully automatize the system in order to make more efficient the preparation of blots before starting. The map will now be established by using markers selected in collaboration with all the partners.

iv) 01: The phenylpropanoid pathway seems to have special importance in the development of *Rhizobium*-legume symbiosis. Certain flavonoid products are *nod* gene activators. In addition, rhizobia might control the expression of genes of this pathway, thus avoiding the activation of plant defense involving the synthesis of certain isoflavonoids. To study the possible control of the pathway by *Rhizobium*, the control of expression of these genes and the effect of their altered expression by the antisense DNA approach are under investigation at the ISV.Genomic or cDNA clones for proteins involved in the phenylpropanoid pathway (chalcone synthase, flavanone-3-hydroxylase, dihydroflavanole-4-reductase, isoflavone reductase), in lignin synthesis (peroxidase) as well as for pathogenesis related proteins were isolated from *M. sativa*.The genes are being used to determine in what extent *Rhizobium* is controlling the plant defense mechanisms during symbiosis and the synthesis of *nod* gene activator or inhibitor flavonoids. The clones are also available for projects iii.03 and iv.03. The flavonoid production by the plant in response to different growth conditions and during nodulation was studied. Plants grown in the presence of a nitrogen source where no symbiosis is needed, were shown to produce flavonoids which can repress *R. meliloti nod* gene expression, while plants grown in the absence of nitrogen source produced *nod* gene activators. Moreover, nodulated plants produced reduced amounts of activating compounds. All these compounds are now being purified and analyzed.

HIGHLIGHTS/MILESTONES:

The knowledge gained about the regulation of *nod* gene expression has enabled us to engineer *Rhizobium meliloti* strains having an extended host range as well as strains producing several thousand fold higher amounts of Nod signals. This allowed us to describe a one step purification scheme and to characterize Nod signal molecules which were previously undetectable.

The factors produced by the NodA and NodB protein alone or in combination are active in non-legumes and the expression of these genes affects the phytohormone balance in transgenic tobacco. Morphological abnormalities of the transgenic plants further indicate that tobacco must contain the necessary substrates allowing the *nodA* and *nodB* encoded proteins to synthesize growth controlling factors and also the necessary receptors to respond to the presence of these regulatory molecules.

A gene (*ocd*) encoding the enzyme ornithine cyclodeaminase that converts ornithine into proline has been found in *R. meliloti* GR4 as one of the *nfe* genes. This gene could provide bacteria with better abilities to nodulate *Medicago*.

A semi-automatic prototype for DNA extraction is available. It will be used to confirm the protocols on various plant materials and could allow to furnish DNA to various groups. An automatic system to realize blots has been developed and improved. It will allow to furnish blots to various groups and to establish a RFLP map of *Medicago truncatula* more efficiently.

WIDER CONSIDERATIONS :

The ability of this new class of oligosaccharide signals to control plant growth and development could increase our knowledge on cellular mechanisms of plant differentiation. This knowledge could also have a significant impact on plant breeding and agricultural yields.

COOPERATIVE ACTIVITIES :

Cooperation in the various research topics are specified in the RESULTS.

A. SAVOURE from BERTIN has been working full-time at ISV. Six meetings were held during the year either at BERTIN or at ISV. Another person has worked closely with ISV on plant DNA extraction during three months.

ISV, MPI and EEZ had regular exchange of plasmids, bacteria and information.

With other laboratories participating in the BRIDGE. Integrated Action, with Univ. Bielefeld (Prof. A. Pühler) : One week stay of two Spanish scientists and a three month stay of a fellow in Bielefeld and two German scientists in Granada.

20-22 January 1991 :	Meeting of all participants of the BRIDGE project at ISV in Gif.
26-29 August 1991 :	Visit of Dr. E. Kondorosi (ISV) at MPI for discussion of results, collaborations and exchange of strains.
7 October 1991 :	Visit of Prof. H. Van Onckelen (Univ. Antwerp, Participant of other BRIDGE project) at MPI for discussion of results, collaboration and collection of samples.
27 October 1991 :	BRIDGE ELWW meeting on "*Rhizobium*-legume symbiosis" in Capri.
5-8 November 1991 :	Visit of Dr. M. Schultze (ISV) at MPI for discussions.
24 February 1992 :	Visit of Prof. H. Van Onckelen at MPI for discussion and collection of samples.
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.

LIST OF JOINT PUBLICATIONS/PATENTS WITH TRANS-NATIONAL AUTHORSHIP :

Kondorosi A., Kondorosi E., John M. Schmidt J. and Schell J. (1991a) The role of nodulation genes in bacterium-plant communication. In : Genetic Engenering, vol. 13 (J.K. Setlow, ed.) Plenum Press, New York and London, pp. 115-136.

Prinsen E., Chauvaux N., Schmidt J., John M., Wieneke U., De Greef J., Schell J. and Van Onckelen H. (1991). Stimulation of indole-3-acetic acid production in *Rhizobium* by flavonoids. FEBS Lett. **282**, 53-55.

Schmidt J., John M., Kondorosi E., Kondorosi A. and Schell J. (1991). Recent progress in the elucidation of the function of *nodAB* and *C* genes of *Rhizobium meliloti*. Israel J. Bot. **40**, 165-169.

Kondorosi E., Pierre M., Cren M., Haumann U., Buiré M., Hoffmann B., Schell J. and Kondorosi A. (1991b) Identification of NolR, a negative transacting factor controlling the *nod* regulon in *Rhizobium meliloti*. J. Mol. Biol. **222**, 885-896.

OTHER PUBLICATIONS/PATENTS:

Kondorosi E. , Buiré M., Cren M., Iyer N., Hoffmann B., Kondorosi A. (1991c) Involvement of the *syrM* and nodD3 genes of *Rhizobium meliloti* in *nod* gene activation and in optimal nodulation of the plant host. Mol. Microbiol. **5**, 3035-3048.

Baev N., Endre G. , Petrovics G., Banfalvi Z., Kondorosi A. (1991) Six nodulation genes of *nod* box locus 4 in *Rhizobium meliloti* are involved in nodulation signal production : *nodM* codes for D-glucosamine synthetase. Mol. Gen. Genet., 228, 113-124.

Györgypal Z., Kondorosi E., Kondorosi A. (1991) Diverse signal sensitivity of NodD protein homologs from narrow and broad host range rhizobia. Mol. Plant-Microbe Interact. **4**, 356-364.

Schmidt J., John M., Wieneke U., Stacey G., Röhrig H. and Schell J. (1991) Studies on the function of *Rhizobium meliloti* nodulation genes. In : Molecular Genetics of Plant-Microbe Interactions (H. Hennecke and D.P.S. Verma, Eds.) Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 150-155.

Schultze M., Quiclet-Sire B., Kondorosi E., Virelizier H., Glushka J.N., Endre G., Géro S.D., Kondorosi A. (1992) *Rhizobium meliloti* produces a family of sulfated lipooligosaccharides exhibiting different degrees of plant host specificity. Proc. Natl. Acad. Sci. USA, **89**, 192-196.

Soto M.J., Zorzano A., Olivares J. and Toro N. Characterization of *Rhizobium meliloti* GR4 insertion sequence ISRm3 linked to the nodulation competitiveness locus *nfe*. (submitted).

Procédé de fabrication d'une cartouche pour l'extraction d'ADN et cartouches ainsi obtenues (EN91.08579, BERTIN -Patent)

Automate de séparation de macromolécules ou de fragments de celles-ci (EN88-11627, BERTIN-Patent).

THE MOLECULAR BASIS OF PATHOGENICITY, AVIRULENCE AND RESISTANCE IN THE INTERACTION BETWEEN THE FUNGAL PATHOGEN *CLADOSPORIUM FULVUM* (SYN. *FULVIA FULVA)* AND TOMATO.

CONTRACT NUMBER: BIOT-CT90-0163(TSTS)

OFFICIAL STARTING DATE: 31/03/91

COORDINATOR: P.J.G.M. DE WIT, AGRICULTURAL UNIVERSITY, WAGENINGEN, NL.

PARTICIPANTS: R.P. OLIVER, UNIVERSITY OF EAST ANGLIA, NORWICH, UK.
H.W.J. VAN DEN BROEK, AGRICULTURAL UNIVERSITY, WAGENINGEN, NL.
B.J.C. CORNELISSEN, MOGEN INTERNATIONAL NV, LEIDEN, NL.
S.C. FRY, UNIVERSITY OF EDINBURGH, EDINBURGH, UK.
F. CERVONE, UNIVERSITY OF ROME, ROME, IT.

OBJECTIVES SET FOR THE REPORTING PERIOD

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 P1 and P17), Cornelissen (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 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 different 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 P17 and the avirulence gene *avr9* of *C. fulvum*. Also gene targeting would be initiated. Cornelissen would optimize the transformation and regeneration procedure for tomato and tobacco; the plants would be transformed with different cDNAs, especially those encoding ß-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 isolate the bean cDNA encoding PGIP and purify PGIP from tomato.

MAJOR PROBLEMS ENCOUNTERED

Nothing to report.

RESULTS

Lab 01, de Wit's research group. Upon inoculation of tomato leaves with *C. fulvum* several pathogenesis-related (PR) proteins accumulate which could be involved in induced resistance. Some of the purified PR proteins were shown to be hydrolytic enzymes; they have ß-1,3-glucanase or chitinase activity and are potentially able to degrade fungal cell walls which consist of glucans and/or chitin. In *in vitro* bioassays growth of the fungus *Trichoderma viride* was inhibited by combined activities as low as 0.5 nkatal for the ß-1,3-glucanases and 1500 cpm for the chitinases. *C. fulvum* was shown to be insensitive to the hydrolytic enzymes; combined activities as high as 14 nkatal for the ß-1,3-glucanases and 50.000 cpm for the chitinases did not inhibit growth of the fungus.

Genomic and cDNA clones encoding the putative pathogenicity proteins P1 and P17 have been isolated and and sequenced, and in addition the first fungal avirulence gene *avr9* was cloned and sequenced. The final proof that a true fungal avirulence gene had been cloned came from transformation studies of races virulent on Cf9 genotypes with the *avr9* gene. The transformants became avirulent on Cf9 genotypes of tomato. The putative product of avirulence gene *avr4* has been purified and partially sequenced. To this end proteins present in apoplastic fluid isolated from the interaction between tomato cultivar Cf5 and race 5 of *C. fulvum* were fractionated by gel filtration on a Sephadex G-50 column and the fractions obtained were screened for the presence of the putative product of avirulence gene *avr4* by assaying for necrosis-inducing activity on tomato cultivar Cf4. Active fractions were further purified by reversed-phase FPLC. Several fractions were obtained that specifically induced necrosis in tomato cultivar Cf4. Some of the necrosis-inducing fractions contained only one protein which migrated as a 12 kD band on SDS-polyacrylamide gels. The amino acid sequence of the N-terminal part and of a peptide that was obtained after digestion of the 12 kD protein with trypsin was determined and degenerated oligonucleotide probes were synthesized. These probes should enable us to isolate the gene encoding the 12 kD protein by performing a PCR reaction on DNA isolated from race 5 of *C. fulvum* or cDNA synthesized from mRNA isolated from a Cf5/race 5 interaction.

Lab 02, Oliver's research group. Hundred transformants created with the vector pAN8-1 have been screened for change in pathogenicity. Two of them appeared to be significantly reduced in pathogenicity. The two mutants will be analysed in detail. Progress has been made with the purification of the enzyme mannitol dehydrogenase, an enzyme involved in the primary carbon sequestration of *C. fulvum*. The pale and white forms of *C. fulvum* which occur frequently during subculturing have been analyzed in some detail. The white form is non-pathogenic and sporulates at only 1% of the wild-type. The pale form is still pathogenic. The white form has only 5% of the mitochondria of the wild type and is rather sensitive to UV. The white form grows rapidly on saccharose or glucose. About a hundred progeny from a protoplast fusion cross between race 4 and race 5 have been collected and purified by single spore collection. They have been assayed for race-specific virulence, nitrate phenotype and hygromycin resistance. In the coming year they will be screened for other characteristics as well. In the region of avirulence gene *avr9* a genomic walk has been initiated in order to get more information on the flanking sequences. Several probes have been exchanged between lab 01 and lab 02 for karyotyping *C. fulvum* on CHEF gels. From *C. fulvum* two telomeric sequences have been obtained which will be used to construct a replicating vector in the future. Finally the role of the retrotransposon in creating races with new virulences has been addressed.

Lab 03, van den Broek's research group. The avirulence gene *avr9* and the putative pathogenicity gene P17 have been subjected to *in vivo* directed mutagenesis. Disruption of P17 has been realized following the transformation of a wild-type strain of race 5 with a plasmid construct containing the P17 encoding sequence interrupted by a hygromycin resistance gene. In two out of about a hundred transformants, the resident gene was replaced by the mutated one. However, these transformants could still infect tomato seedlings, while producing a P17 protein lacking 23% of its amino acids. A new construct has now been made in which the complete coding sequence of P17 has been removed. In addition transformants of race 5 which overpoduce the P17 protein upto a 10 times higher level than the wild-type have been selected. However, this overproduction did not seem to affect pathogenicity. Similarly, a plasmid construct has been made in which the complete coding sequence of the *avr9* gene has been removed. This plasmid has been used to disrupt the *avr9* gene in wild-type *avr9*-containing races, which will be assayed for virulence on Cf9 genotypes of tomato. To identify upstream regulatory sequences in the promoters of the different cloned pathogenicity and avirulence genes, promoter deletions have to be generated and reintroduced in the genome at a specific site. The establishment of a gene targeting system in *C. fulvum*, using a pyrimidine biosynthetic gene as the target genomic locus, is one of the priorities.

Lab 04, Cornelissen's research group. Various parameters have been changed in the existing protocol to optimize and improve the transformation and regeneration efficiency of tomato. Cotyledons of

seedlings harvested two days after germination have been used as explants. Different light and cold regimes did not significantly influence regeneration efficiencies. Regeneration could be improved by incubating the explants upside down. 2,4 D did not improve regeneration when compared with *Petunia* feeder layers. Rooting of shoots was found to occur earlier when kanamycin concentrations of 30 mg /l were used rather than 100 mg/l. The influence of various *Agrobacterium tumefaciens* helper plasmids on the transformation frequency was investigated. Of the three types of Ti helper plasmids which were compared the leucinopine helper plasmid gave the best results. Cervone was provided with four oligonucleotide primers to facilitate the cloning of bean PGIP. In addition, plasmids pMOG402 and pMOG464 were provided: pMOG402 is a binary vector containing the kanamycin resistance marker gene and pMOG464 is a pUC derivative containing a gene-expression cassette consisting of the 35S promoter and the nopaline synthase transcription termination signal. After cloning the PGIP gene in the expression cassette and subsequently in the binary vector, it will be introduced in tomato. Several tomato plants transgenic for chimeric β-1,3-glucanase and chitinases genes are available now for analysis by Fry.

Lab 05, Fry's research group. Various purified polysaccharides from plants and other sources have been subjected to enzyme- and acid-catalysed hydrolysis under various conditions and the released oligosaccharides have been purified and assayed for biological activity by observing their effects on L-(U-14C)leucine incorporation by suspension cultured tomato cells. A low molecular weight sub-class of rhamnogalacturonan II was most active in inhibiting the incorporation of L-(U-14C)leucine into suspension cultures up to 60%. The effect of precursors other than L-(U-14C)leucine was investigated. Rhamnogalacturonan II did also inhibit the incorporation of 14C from D-(6-14C)glucuronic acid into D-galacturonic acid residues of pectins and of 14C from D-(1-14C)glucosamine into glycoproteins.The effect of rhamnogalacturonan II seems to be on a number of rather narrowly-defined biosyntheses. Apoplastic fluids from both healthy and *C. fulvum*-infected tomato leaves containing different classes of oligosaccharides inhibited the L-(U-14C)leucine incorporation in tomato suspension cells. However, it appeared that the endogenous L-leucine possibly interferes with the uptake and incorporation of L-(U-14C)leucine. This means that components of apoplastic fluids need to be purified before assessing their ability to affect the incorporation of L-(U-14C)leucine by possible oligosaccharides. IAA did not influence the incorporation of L-(U-14C)leucine. Apoplastic fluids from *C. fulvum*-infected leaves contain enzymes which not only release fragments from (U-14C)-labelled plant cell walls but also from (U-14C)-labelled fungal walls. The free sugars which were released contained glucose, glucosamine and N-acetyl glucosamine but no mannose, while the released basic oligosaccharides contained glucose, glucosamine and sometimes mannose. The released plant and fungal oligosaccharides at the interface between host and pathogen may be of importance if they act synergistically to enhance (or even suppress) the host defence response.

Lab 06, Cervone's research group. The polygalacturonase-inhibiting protein (PGIP) from bean has been partially cloned by the polymerase chain reaction (PCR) on tomato DNA using primers which were designed based on the amino acid sequence of the N-terminus and a tryptic peptide of the bean PGIP. In this way a 758 bp fragment was isolated which was subsequently used as a probe to islate the PGIP gene from a genomic library of *Phaseolus vulgaris* cv. Saxa. A single open reading frame of 1026 nucleotides (342 amino acids) was present in the genomic clone. Northern analysis with the labelled PCR-cloned fragment on poly(A)$^+$ RNA isolated from different bean tissues showed a 1.2 kb transcript present in suspension-cultured cells and to a lesser extent in leaves, hypocotyls and flowers. A cDNA clone containing two third of the coding region of the PGIP gene was sequenced. The nucleotide and deduced amino acid sequences of the PGIP gene from two different cultivars of bean showed 96.4% and 97.4% identity, respectively. The entire coding region of the cloned genomic PGIP gene, which is not interrupted by introns, has been inserted in the plasmid pMOG402 for transgenic expression in tomato. Apoplastic fluids from tomato exhibited very low PGIP activity. Preliminary experiments using bean PGIP nucleotide sequences on Southern blots containing total tomato DNA revealed that a very high homology existed between the bean and the tomato gene. So, the original

plan of cloning the tomato PGIP starting from the purified tomato protein has been abandoned and the approach utilizing the heterologous bean probe is now being undertaken.

HIGHLIGHTS

1. **The first fungal avirulence gene, *avr9*, of *C. fulvum* that fits a gene-for-gene relationship has been cloned. Cloning of another avirulence gene *avr4* is within reach.**

2. **Gene disruption has been successfully performed in *C. fulvum*.**

3. **Transformation of *C. fulvum* with the vector pAN8-1 has resulted in a number of mutants with reduced pathogenicity.**

4. **Two telomere sequences of *C. fulvum* with the sequence TTAGGG have been isolated.**

5. **Tomato plants transgenic for chimeric β-1,3-glucanase and chitinase genes have been obtained.**

6. **Apoplastic fluids of *C. fulvum*-infected tomato leaves contain enzymes that release oligosaccharides from plant and fungal cell walls.**

7. **The cloning of a polygalacturonase inhibitor protein (PGIP) from *Phaseolus vulgaris* is completed.**

WIDER CONSIDERATIONS

Molecular characterization of gene-for-gene systems is in progress. Of the many bacterial genes and one fungal avirulence gene 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 two-day workshop at the Agricultural University in Wageningen on June 6-7, 1991. Fifteen lectures and five posters 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. Detailed and overall research plans were discussed and arrangements were made for the exchange of materials and joint experiments. The exchange of materials between the labs 01, 02, 03 and 04 was most frequent. In the coming year the exchange of material between labs 04, 05 and 06 will be intensified. Several visitors came to the different labs to discuss research, exchange of expertise and ways of cooperation in the near future. Two scientists applied for a scolarship at EC to work for one year within the framework of BRIDGE, but their reseach proposals were not granted by EC. Oliver has made a Bursary scheme application with Thomas Sandal, a Danish PhD student, linked to this grant. All participants will have their next two-day workshop in Rome on June 12-13, 1992.

LIST OF JOINT PUBLICATIONS WITH TRANS-NATIONAL AUTHORSHIP

Dore, I., Legrand, M., Cornelissen, B.J.C. and Bol, J.F. (1991). Subcellular localization of acidic and basic PR proteins in tobacco mosaic virus-infected tobacco. Archives of Virology **120**, 97-107.

Joosten, M.H.A.J., Bergmans, C.J.B., Meulenhoff, E.J.S., Cornelissen, B.J.C. and de Wit P.J.G.M. (1990). Purification and serological characterization of three basic 15-kilodalton pathogenesis-related proteins from tomato. Plant Physiology **94**, 585-591.

OTHER PUBLICATIONS

Aldington, S., McDougall, G.J. and Fry, S.C. (1991). Structure-activity relationships of biologically active oligosaccharides. Plant Cell and Environment **14**, 625-636.

Aldington, S. and Fry, S.C. (1992). Plant cell wall-lysing enzymes in the apoplast of *Fulvia fulva*-infected tomato leaves. Canadian Journal of Botany. (In press).

Aldington, S., Fry, S.C. (1992) Oligosaccharins. Advances in Botanical Research, **19**. (In press).

De Wit, P.J.G.M., van Kan, J.A.L., van den Ackerveken, A.F.J.M. and Joosten, M.H.A.J. (1991). Specificity of plant-fungus interactions: molecular aspects of avirulence genes. In: Advances in Molecular Genetics of Plant-Microbe Interactions, Vol 1. (H. Hennecke and D.P.S. Verma, eds). Kluwer Academic Publishers. pp 233-241.

De Wit, P.J.G.M. (1992). Molecular characterization of gene-for-gene systems in plant-fungus interactions and the application of avirulence genes in control of plant pathogens. Annual Review of Phytopathology **30**, 000-000.

Garner, N., Van den Elzen, P.J.M. and Cornelissen, B.J.C. (1992). The potential for the control of fungal disease in crop plants using gene transfer technology. In: Biotechnology International, issue 1992, Century Press Ltd., London, pp 111-116.

Goosen, T., Bos, C.J. and van den Broek, H.W.J. (1991). Transformation and gene manipulation in filamentous fungi: An overview. In: Handbook of Applied Mycology, Vol 4. Fungal Biotechnology (D.K. Arora, R.P. Elander, K.G. Mukerji, eds). Marcel Dekker, Inc, New York. pp 151-195.

Hoekema, A., Huisman, M.J., Posthumus-Lutke Willink, D., Jongedijk, E., Van den Elzen, P.J.M. and Cornelissen, B.J.C. (1991). Transgenic potato cultivars resistant to potato virus X. In: Plant Molecular Biology 2. (R.G. Hermann and B. Larkins, eds). Plenum Press, New York, pp 183-192.

Huisman, M.J., Van Vloten-Doting, L. and Cornelissen, B.J.C. (1992). The stability and utility of plant virus replicons. In: Genetic engineering with plant viruses. (J.W. Davies and T.M.A. Wilson, eds). CRC Press, London, pp 000-000.

Huisman, M.J., Jongedijk, E., Posthumus-Lutke Willink, D., Van der Wilk, F. and Cornelissen, B.J.C. (1992). Molecular breeding for virus resistant potato plants. Netherlands Journal of Plant Pathology, 000-000.

Jongedijk, E., De Schutter, A.J.M., Stolte, T., Van den Elzen, P.J.M. and Cornelissen, B.J.C. (1992). Increased resistance to potato virus X and preservation of cultivar properties in transgenic potato under field conditions. Bio/Technology **10**, 000-000.

McHale, M.T., Roberts, I.N., Noble, S.N., Beaumont, C., Whitehead, M.P., Seth, D. and Oliver R.P. (1992). *CfT-1*. An LTR-Retrotransposon in *Cladosporium fulvum*, a fungal pathogen of tomato. Molecular and General Genetics. (In press).

Oliver, R.P., Farman, R.L., Talbot, N.J. and McHale, M.T. (1991). Molecular biology of fungal plant pathogenicity. In: Applied Molecular Genetics of Fungi. (J.F. Peberdy, C.E. Caten, J.E. Ogden, J.W. Bennet, eds). Cambridge University Press. pp 170-183.

Oliver, R.P., Talbot, N.J., McHale, M.T. and Coddington, A. (1991). Molecular Genetics of the tomato pathogen *Cladosporium fulvum*. In: Plant Molecular Biology 2. (R.G. Hermann, B. Larkins, eds). Plenum Press, New York. pp 179-182.

Oliver, R.P. (1992). A model system for the study of plant-fungal interactions: Tomato leaf mould caused by *Cladosporium fulvum*. In: Molecular Signals in Plant-Microbe Communication. (D.P.S. Verma ed). CRC Press. pp 297-308.

Talbot, N., Coddington, A. and Oliver, R.P. (1991). Pulsed field gel electrophoresis reveals chromosome length differences between isolates of *Cladosporium fulvum* (syn. *Fulvia fulva*). Molecular and General Genetics. **229**, 267-272.

Toubart, P., Desiderio, A., Salvi, G., Cervone, F., Daroda, L., De Lorenzo, G., Bergmann, C., Darvill, A. and Albersheim, P. (1992). Cloning and characterization of the gene encoding the endopolygalacturonase-inhibiting protein (PGIP) of *Phaseolus vulgaris* L. The Plant Journal (in press).

Van Kan, J.A.L., Van den Ackerveken, G.F.J.M. and de Wit, P.J.G.M. (1991). Cloning and characterization of cDNA of avirulence gene *avr9* of the fungal pathogen *C. fulvum*, causal agent of tomato leaf mold. Molecular Plant-Microbe Interactions **4**, 52-59.

Van den Ackerveken, G.F.J.M., van Kan, J.A.L. and de Wit, P.J.G.M.(1992). Molecular analysis of the avirulence gene *avr9* of the fungal tomato pathogen *C. fulvum* fully supports the gene-for-gene hypothesis. The Plant Journal **2**, 000-000.

Wolosluk, C.P., Meulenhoff, E.J.S., Sela-Buurlage, M.B., Van den Elzen, P.J.M. and Cornelissen, B.J.C. (1991). Pathogen-induced proteins with inhibitory activity toward *Phytophthora infestans*. The Plant Cell **3**, 619-628.

TITLE : GENETIC AND MOLECULAR APPROACHES OF THE PHYSIOLOGY OF BACTEROIDS IN RELATION TO THE PLANT NODULE METABOLISM
CONTRACT NUBMER : BIOT-CT90-0166-C-(EDB)
STARTING FROM : 1.12.1990
COORDINATOR : P. Boistard, INRA-CNRS, Toulouse, France
PARTICIPANTS : M. Iaccarino, IIGB, CNR, Naples,Italy
F. O'Gara, University College, Cork, Ireland
R. Kron-Morelli, Agrifutur, Brescia, Italy
A. Puhler and U. Priefer, University of Bilefeld, Germany
M. O'Connel, Dublin City University, Ireland

OBJECTIVES

Symbiotic nitrogen fixation occurs in a specialized plant organ, the nodule, whose endodermis and parenchyma cells constitute a diffusion barrier resulting in a low oxygen pressure in the zone invaded by the bacteria. It has been shown recently that bacterial *nif* and *fix* genes can be induced in microaerobic cultures, which points to oxygen as a most likely key signal for triggering nitrogen fixation inside the plant.

The energy required for nitrogen reduction is provided to the bacteroids in the form of dicarboxylic acids (dCA) as evidenced by the fact that mutants deficient in sugar utilization are Fix^+ whereas *dctA* mutants affected in the transport of dCA are Fix^-. Recent data indicate that the regulatory genes which control *dctA* expression in bacterial cultures do not operate in bacteroids which implicates a specific signal and regulatory pathway for the expression of *dctA* inside the plant.

The relationship between nitrogen fixation and nitrogen metabolism in a symbiotic association, although crucial for the full understanding of the possible regulatory circuits, remains an open question. It is known that the bacterial regulatory pathway which connects nitrogen compounds catabolism and assimilation to the availability of utilisable nitrogen sources does not control the expression of *nif* and *fix* genes in symbiosis. The expression and/or activity of bacterial glutamine synthetases is turned down by a still unknown symbiotic signal. On the other hand recent data point to a possible role of plant glutamine synthetase(s) in modulating the efficiency of the bacterial nitrogen fixing apparatus.

Molecular mechanisms involved in gene regulation, signal perception and transduction will be studied. Because of the similarities of these mechanisms throughout the whole living world, knowledge gained from the sudy of prokaryotic systems especially suited for genetic analysis will be of general interest.

The integrated approach of the project will result in an overall picture of regulatory networks operative in the nodule.

The knowledge gained at these two levels will permit strategies to be developed to genetically manipulate the system in order to modify the regulatory circuits and metabolic fluxes and eventually increase the symbiotic nitrogen fixation potential.

The two symbiotic systems that our project will help improving are of agricultural importance all over Europe. A major environmental problem currently exists due to the extensive use of fertilizers in the European agricultural sector. Much of the synthetic nitrogen applied to crops is lost from the soil, leading to problems such as eutrophy in lakes and rivers and drinking water pollution. Therefore besides reduction in costs resulting from reduced use of fertilizers, symbiotic nitrogen fixation provides a buffered mineral nitrogen source which is less susceptible to being washed out into the ground water and thus less harmful to the environment.

The genetic components of the *nif fix R. meliloti* regulatory pathway which responds to oxygen have been identified. Molecular mechanisms responsible for signal perception and transduction by the FixL sensor and for target genes activation by FixJ will be studied (1). The genetic data available in *R. meliloti* will be used for the identification of the functionally homologous pathway which operates in *R. leguminosarum* and the possible variations between both pathways (2). Comparative studies of the homologous components of the two regulatory pathways will allow an identification of the conserved domains of the regulatory proteins and common regulatory sequences in the target genes (3). When differences will be observed, the resulting information will be used to construct chimaeric regulatory proteins or promoters (4). The signal and regulatory protein(s) responsible for symbiotic *dctA* expression will be identified (5). The mechanism by which the dct regulatory system which senses dCA concentration in bacterial cultures, although not essential for *dctA* symbiotic activation interferes with *nif* gene expression will be investigated (6). The various factors which regulate the expression or activity of bacterial glutamine

synthetases will be identified (7). The role of GSIII in bacteroid nitrogen metabolism will be examined (8). Genetic analysis of the export of fixed nitrogen towards the plant cell will be undertaken (9). The effect of various nitrogen and carbon sources on the microaerobic expression of *nif* genes will be investigated (10). The glutamine synthetase genes of the autogamous diploids *Medicago sativa* will be identified (11) and modulation of glutamine synthetase levels will be achieved by expression of antisens messenger RNA (12). Bacterial strains and plant lines with modified regulatory circuits will be assayed for their symbiotic nitrogen fixation efficiency (13).

Task	Year 1	Year 2	Year 3
1	----------	----------	----------
2	----------	----------	----------
3		----------	
4			---------
5			---------
6	----------	----------	----------
7	----------	----------	----------
8	----------	----------	----------
9	----------	----------	----------
10	----------	----------	
11	----------	----------	
12		----------	----------
13		----------	----------

MAJOR PROBLEMS ENCOUNTERED : Nothing to report

RESULTS

1. Oxygen regulation of nitrogen fixation gene expression in *R. meliloti* and *R. leguminosarum* (objectives 1, 2 and 3 ; Toulouse and Bielefeld)

In the course of the BAP programme we had provided evidence that FixL is an oxygen sensor which allows the transcriptional activator FixJ to activate the expression of two *R. meliloti* regulons needed for symbiotic nitrogen fixation, the *nif* regulon and the *fixN* regulon, in response to low oxygen concentration. FixJ mediated activation of the two regulons involves the FixJ controlled expression of two transcriptional activators, FixK active on the *fixN* regulon and NifA active on the *nif* regulon. The expression of *nifA*, in addition to being activated by FixJ is repressed by FixK which provides an additional link between the two regulons. Sequence examination allowed us to predict that FixL consists of three domains : a N-terminal hydrophobic domain which probably anchors the protein to the cytoplasmic membrane, a central cytoplasmic domain and a C-terminal domain which is common to all sensors and responsible for the activation of the cognate transcriptional activator. We constructed FixL derivatives in which either the hydrophobic domain or the central cytoplasmic domain were deleted. Whereas the derivative lacking the hydrophobic domain was still able to activate FixJ at low oxygen concentration, the FixL protein lacking the central cytoplasmic domain was able to activate FixJ independently of oxygen concentration (de Philip *et al.*, submitted).

The *fixK* promoter has been analysed by deletion and oligonucleotide directed mutagenesis. A putative FixK binding sequence has been identified -487 bp from the transcription start and its mutation partly alleviates negative regulation. Two regions involved in activation by FixJ have been identified. One of them from -33 to -54 shows strong conservation with other FixJ responsive regulatory sequences such as *R. meliloti pnifA* or *Azorhizobium caulinodans pfixK*. A second region at -60 is not present in *pnifA* which could explain the differential behaviour of the two promoters (Waelkens *et al.*, in press). We are presently devising an *in vitro* transcription system to study the role of FixJ in the activation of the *nifA* and *fixK* promoters.

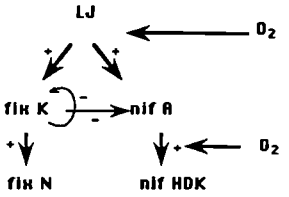
In *R. leguminosarum* circumstantial evidence indicates that regulatory genes homologous to FixLJ could be absent. Hybridization experiments using *R. meliloti* probes have failed to show any homologous *R. leguminosarum* sequence. Heterologous complementation studies did not allow us to show the presence in *R. leguminosarum* of genes functionally equivalent to *fixLJ* whereas they permitted the identification of *fnrN*, a *fixK* homologue. However by contrast with the situation in *R. meliloti fnrN* is subject to positive

autoregulation and furthermore this autoregulation is oxygen dependent. Expression of a *fnrN-lacZ* fusion in *R. meliloti* depends on the presence of a functional *fixK* gene. This proves that *fixK* can replace *fnrN* as a transcriptional activator and provides additional evidence that *fnrN* is not activated by *fixLJ*. Whereas FixK activity does not seem to be controlled by oxygen cell the available data indicate that FnrN responds to oxygen. This may be attributed to the fact that contrary to FixK FnrN contains a N-terminal cystein cluster.

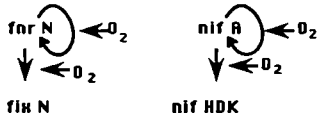
fnrN was first identified by its ability to complement a *R. meliloti fixJ* mutant for *fixN* expression. Therefore the question arose whether genes equivalent to *fixN* were present in *R. leguminosarum*. Hybridization studies have revealed the existence of two *fixN* copies in *R. leguminosarum*, like in *R. meliloti*. Work is in progress to study the regulation of these genes and particularly determine whether they are under the control of fnrN.

The likely absence of *fixLJ* homologues in *R. leguminosarum* raises the question of the control of *nifA* expression. We are currently testing the hypothesis that *nifA* could be subject to autoregulation in *R. leguminosarum*.

Comparison of oxygen control in *R. meliloti* and *R. leguminosarum* suggests that *fix* and *nif* genes could belong to completely separate regulons in *R. leguminosarum* whereas they are connected through *fixLJ* central control in *R. meliloti*.



O_2 regulatory cascade in *R. meliloti*



Hypothetical O_2 regulatory cascade in *R. leguminosarum*

2. Carbon metabolism and efficiency of nitrogen fixation (objectives 5, 6 and 10 ; Cork)

In order to study the relationship between symbiotic nitrogen fixation and carbon metabolism we paid special attention to the regulation of dicarboxylic acid transport since dCA appears to be a major carbon source for nitrogen fixing bacteroids.

Because the *dctA* gene which is needed for dcA transport can be expressed in symbiotic conditions independently of the *dctBD* regulatory genes which operate in free living conditions, we isolated spontaneous secondary mutants of an *R. meliloti dctD* strain that are capable of growth on dcA as role carbon source. Such mutants should provide a way for identifying an alternative symbiotic activator of *dctA* expression. Alternatively they could allow the identification of a repressor of *dctA* expression in free living conditions.

A link between *dct* regulatory system and *nif* gene expression was suspected because of a repressing effect of some *dctB* mutations on *R. meliloti nifHDK* expression. A deletion analysis of the *nifHDK* promoter failed to implicate any particular region in binding of a repressor molecule.

A negative effect has been observed of the presence of lactose in the growth medium on the expression of both *dctA* and *nifA*. Catabolism of the carbon compound is not required and the effect is not mediated through *fixLJ*.

In order to facilitate genetic manipulation of the various components and to study *dctA* expression in a well defined genetic background, the Dct system has been reconstituted in the heterologous *E. coli*. Expression of the *dctA* gene was found to be absolutely dependent on the presence of the homologous *dctBD* genes and to respond to the presence of dCA in the medium.

3. Nitrogen metabolism and symbiotic nitrogen fixation

3.1. Bacterial nitrogen metabolism (objectives 7, 8, 9 and 10 ; Dublin and Naples)

In order to understand the nature of the shift from free living toward symbiotic bacterial nitrogen metabolism, we characterized components of nitrogen assimilation pathway in *R. leguminosarum* such as

GSII and GSIII as well as its genetic regulatory elements, *glnB* which encodes the PII regulatory protein and the nitrogen general activator *ntrC*. In particular we have shown that the *glnT* gene codes for a newly recognized glutamine synthetase GSIII of *R. leguminosarum* which is expressed in *K. pneumoniae* where it can complement a *glnA* mutant. We purified this protein, determined the aminoterminal sequence and obtained an antiserum. We sequenced the corresponding DNA and identified an ORF coding for GSIII (Chiurazzi *et al.*, in press).

We found that expression of the *R. leguminosarum* gene coding for GSII requires in *K. pneumoniae* the *ntrC* gene. In this way we identified the *glnII* gene, sequenced it and studied its promoter. We have shown that it is a sigma 54-dependent promoter requiring a UAS for NtrC activation (Patriarca *et al.*, in press).

We have also characterized GSII. We find that addition of NH4Cl to a derepressed culture causes a specific decrease in transferase activity that may only be interpreted as a post-translational modification (Manco *et al.*, in press). Previous authors reported that GSII transferase activity is not present in bacteroids. If the presumed post-translational modification is present in bacteroids it is possible that the enzyme is present, but inactive when transferase activity is assayed. We find the presence of GSII in the nodule by immunofluorescence and immunogold. When analyzed by immunogold we find a positive signal at the periphery of the bacteria, suggesting that GSII might be associated with the membrane(s).

Using an appropriate DNA construction we obtained, by translational fusion, a pure protein containing most of PII, which we used to raise an antiserum. An immunoblot after SDS-PAGE of *R. leguminosarum* extracts shows a single band of the expected molecular weight.

We found that the *glnB* promoter is of the sigma 54 type and does not require an upstream activating sequence in *K. pneumoniae*. In collaboration with Dr Merrick we have shown that the *glnB* promoter may be activated also by a heterologous activator, the product of the *nifA* gene. More recently we began studying the *glnB* promoter in *R. leguminosarum*. We have shown the presence of a high NtrC-independent level of expression and a partial positive role for NtrC interacting with a sequence located between 511 and 203 bp upstream of the transcription initiation site. This sequence does not contain canonical NtrC binding site sequences.

We characterized a *ntrC* mutant. More recently we began sequencing the upstream region of *ntrC*, containing the *ntrC* gene. Preliminary results suggest that the promoter of the *ntrBC* genes may be located more than 600 bp upstream of the *ntrB* initiation codon and that this region might be untranslated.

The study of bacterial ammonium transport is crucial for the understanding of the interaction between nitrogen fixation and plant nitrogen metabolism. Preliminary studies have focussed on the mechanism by which ammonium is actively taken up by living cells and the conditions that influence the expression of active transport. The underlying hypothesis is that some of the mechanisms involved in ammonium uptake could also play a role in the transport to the plant during nitrogen fixing symbiosis. Work has been concentrated on (i) assays to monitor ammonium transport (ii) isolation of mutants altered in ammonium uptake ability (iii) analysis of membrane proteins induced by alterations in nitrogen status.

(i) Transport assays : Radiolabelled methylammonium has been used as a model substrate to demonstrate the presence of a high affinity ammonium transport system in *R. meliloti* 2011 when methylammonium is the sole nitrogen source. The competitive inhibition of methylammonium uptake observed when ammonium is added provides evidence that this system operates for ammonium uptake also. (ii) Mutant isolation : Following transposon mutagenesis cells were exposed to penicillin and lysozyme while growing in 0.5 mM NH4Cl to enrich for mutants defective in ammonium uptake ability. Surviving cells were plated on rich medium and replicated to media having a sole nitrogen source of 0.5mM NH4Cl or 20mM NH4Cl. Mutants that are unable to grow on low ammonium are being tested to determine whether the mutation is associated with regulation or ammonium transport. (iii) Analysis of membrane proteins : Cells were grown in minimal medium containing a variety of nitrogen sources. Cell envelope material was isolated following sonication and treated with sarkosyl to selectively dissolve the inner membrane. The outer membrane protein patterns were analysed by SDS-PAGE. Proteins present in the outer membranes of cells grown in 0.5mM NH4Cl but not in cells grown in 20mM NH4Cl were reproducibly detected. One such protein was also detected in cells grown in 0.1 % glutamate. Optimisation of the production and detection of these proteins has been carried out to permit further characterisation.

3.2. Plant nitrogen metabolism and nitrogen fixation (objectives 10, 11 and 12 ; Toulouse and Cork)
Two approaches have been taken in an effort to understand how plant nitrogen metabolism inteferes with symbiotic nitrogen fixation.
Because the first step in the assimilation of fixed nitrogen by the plant partner is catalysed by plant glutamine synthetase, identification of *Medicago truncatula* GS genes and study of their expression has been undertaken. Three members of the GS gene family have been identified ; the expression of two of them Mtgs16 and Mtgs8 is significantly enhanced in the nodule, simultaneously with the appearance of Leghemoglobin mRNA.
Several constructions have been achieved which should allow either an increase or a decrease of GS expression in the plant nodule. Several *Medicago sativa* transgenic plants are currently under study. If indeed these plants show an altered level of GS expression in the nodule compared with control plants, nitrogen fixing efficiency as well as expression of *nif* and *fix R. meliloti* genes will be measured.
In parallel with this approach, the influence of various nitrogen sources on *nif* and *fix* gene expression in *R. meliloti* has been studied. We have shown that ammonia and nitrate depress the level of *nif* gene expression and that this effect is mediated by FixL (Noonan *et al.*, in press).
In conclusion, it can be stated that a coherent view of the influence of major physiological parameters i.e. oxygen concentraton, carbon and nitrogen metabolism on the regulation of nitrogen fixation gene expression is emerging through the integrated approach conducted in the participating laboratories. In addition comparison of two symbiotic systems leads to the view that response to a given regulatory signal can result from different combinations of related but non identical regulatory proteins.

HIGHLIGHTS/MILESTONES
Influence of nitrogen source on *nif* gene expression. This finding could provide a link between plant nitrogen metabolism and symbiotic efficiency.

WIDER CONSIDERATIONS
Symbiotic nitrogen fixation is a finely tuned biochemical process which interferes with major metabolic pathways of both symbiotic partners. Therefore its improvement ultimately depends on a better understanding of regulatory circuits in both partners and of their mutual interactions. Such complex systems are particulary suited to the integrative approach allowed by European programmes.

COOPERATIVE ACTIVITIES
One meeting in Paris 8-9 April 1991
One meeting in Naples 28 October 1991.
Visit of M. Iaccarino to Toulouse 29 January-1 February 1992

LIST OF JOINT PUBLICATIONS
De Philip, P., Boistard, P., Schlüther, A., Patschkowski, T., Pühler, A., Priefer, U., O'Gara, F., Boesten, B. and Noonan, B. Developmental and metabolic regulation of nitrogen fixation gene expression in *Rhizobium meliloti*. Canadian Journal of Microbiology (In press).

OTHER PUBLICATIONS
Noonan, B., Moherway, M. and O'Gara, F. (1992). Ammonia regulation of the *R. meliloti* nitrogenase structural and regulatory genes under free-living conditions : Involvement of the *fixL* gene product. Molecular and General Genetics. (In press).
Manco, G., Rossi, M., Defez, R., Lamberti, A., Percuoco, G. and Iaccarino, M. (1992). Dissociation of enzymatic activities of glutamine synthetase II after NH4Cl treatment. Journal of General Microbiology (In press).
Chiurazzi, M., Meza, R., Lara, M. Lahm, A., Defez, R., Iaccarino, M., and Espin, G. (1992). The *Rhizobium leguminosarum* biovar *phaseoli glnT* gene. Gene (In press).
Patriaraca, E.J., Chiurazzi M., Manco, G., Riccio, A., Lamberti, A., De Paolis, A., Rossi, M., Defez, R. and Iaccarino, M. (1992). Activation of the *Rhizobium leguminosarum glnII* gene by NtrC is dependent on upstream DNA sequences. Molecular and General Genetics (In press).
Waelkens, F., Foglia, A., Morel, ., Fourment, J., Batut, J. and Boistard, P. Molecular genetic analysis of the *Rhizobium meliloti fixK* promoter : identification of sequences involved in positive and negative regulation. Molecular Microbiology (In press).

Title	Tomato transposon tagging : isolation of genes involved in disease resistance, hormone biosynthesis and plant cell development.
Contract number	BIOT-900192
Official starting date	01-01-91
Coordinator	Dr. J. Hille, VU Amsterdam, NL.
Participants	Dr. K. Theres / Prof. P. Starlinger, Universität zu Köln, DE. Dr. J. Jones, Sainsbury lab, Norwich, GB. Dr. I. Taylor, University of Nottingham, GB. Ir. R. Dankert, RZ Research, Metslawier, NL.

Objectives set for the reporting period

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 first fifteen months it was aimed to (1) construct suitable T-DNA based vectors, (2) obtain transgenic tomato genotypes and (3) to initiate mapping of the T-DNA insertions in a RFLP analysis.

Major problems encountered

For routine practical purposes the inverted polymerase chain reaction (IPCR) technique, to obtain T-DNA flanking plant DNA for RFLP analysis, showed to be problematic in several labs. The main problems have been solved, but still to obtain reliable IPCR products takes more time than anticipated. Cloning and sequencing of IPCR products remains necessary to be able to subsequently use them in a RFLP analysis.

Results

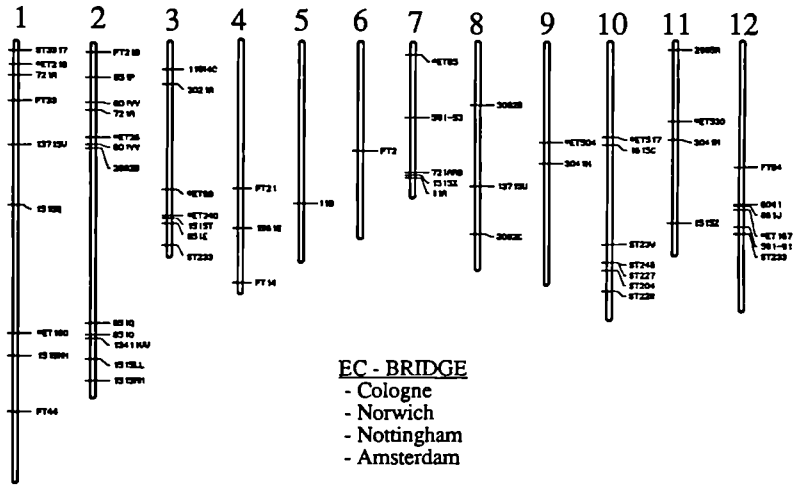
Constructs have been designed to enable *Agrobacterium* strains to carry transposable elements into the tomato genome on T-

DNA. Both the autonomous Ac-element and non-autonomous Ac-derivatives(Ds) have been located 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.

All groups have introduced the T-DNA vectors into tomato by *Agrobacterium* mediated gene transfer, selecting for more then fifty independent transformants per group. These transgenic tomato lines are being analyzed for integrity and copy number of the introduced T-DNA's, for ploidy level and for fertility in crosses.

The chromosomal positions of T-DNA inserts have mainly been determined using a molecular approach. In most cases the IPCR technique was used to amplify a DNA fragment that borders the site of T-DNA insertion and can serve as a hybridization probe for the subsequent RFLP analysis. In some cases, when an origin for replication in *E. coli* was present within the T-DNA, plasmid rescue was used to directly clone flanking plant DNA sequences. Alltogether, 60 locations for transposable elements have been established in tomato (see figure). On all the twelve different chromosomes of tomato inserts have been mapped. Although on some chromosomes(like 2) many inserts have been mapped and on some (like 6) only one, it is too early to draw conclusions about T-DNA insertion specificity in tomato.

Transposon positions



Measurements have been made of Ac germinal excision frequency for six different loci. There is marked between transformant variation in transposition frequency. Typical germinal excision frequencies range from 2% - 25%, but 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. 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. Both excision of Ds from the original position and integration on new chromosomal locations could be established.

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 a programme of transposon mutagenesis. First experiments have been initiated in all groups.

Amsterdam

Up to now 9 transposable element inserts were mapped on chromosome 3, on which also the fungal disease resistance locus *Asc* is located. 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, was crossed on a large scale with a sensitive male sterile tomato line (*asc, asc*). Currently, this seed population, heterozygous for the *Asc* locus is analyzed for Alternaria susceptible mutants in order to screen for transposon induced mutations at *Asc*.

Cologne

One Ds insertion maps on chromosome 7 in proximity to the *Lanceolate* locus. This is our gene of interest because it plays an important role in the process of plant development. Plants containing the Ds element on chromosome 7 and an Ac element were pollinated with pollen from heterozygous *La*/+ plants with the goal of tagging the *Lanceolate* locus. About 10000 seeds were obtained and will be screened for "reduced" phenotypes, the phenotype of the homozygous mutant.

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). Currently, a programme of transposon mutagenesis of these genes has been initiated.

Norwich

The locations of our target genes *Cf-2*, *Cf-4*, *Cf-5* and *Cf-9* have been remapped. To our surprise, an extensive mapping effort revealed that they all were to various degrees incorrectly assigned. *Cf-2* and *Cf-5* lie at the same locus, about 2 cM proximal to *yellow virescent* on chromosome 6. *Cf-4* and *Cf-9* also map to a very similar or identical position, about 20 cM proximal to *aurea* on chromosome 1. This very interesting result is consistent with data from other systems in which resistance gene loci exhibit extensive allelic series with different biological specificities, suggesting they are complex loci.
Tagging *Cf*-genes with a linked transposon requires several backcrosses prior to being able to initiate crosses. Currently we are doing these backcrosses, but in order to accelerate the programme a "brute force" approach has been adopted involving crossing stocks homozygous for *Cf-2* or *Cf-5*, and that carry active Ac at either unlinked or unknown locations, to *Cf-0*, and looking for mutations in the *Cf*-gene. We crossed to a *Cf-0* line that was also mutant at other loci, such as *yellow virescent* (*yv*) or *anthocyanin absent* (*aa*), so that mutations at both these loci and the *Cf*-genes could be screened simultaneously. In a large scale crossing programme over 300000 seeds have been produced. The resulting seedlings are being screened and several promising mutations have already been identified. Conceivably, there will be a signal to noise problem, in that some mutations will be caused by mechanisms other than insertional inactivation of the gene, but if enough mutations are characterized, at least one should be tagged.

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.

The first transposon tagging experiments in tomato have been initiated, both using linked and unlinked transposons. A number of putative mutants has been identified and awaits further characterization.

Wider considerations

Excellent progress has been made towards our objectives. We are now in a position to tag genes of interest, such as genes involved in disease resistance, hormone biosynthesis and plant cell development.

Cooperative activities The annual meeting exchanging the results of the participants obtained sofar, was held for the third time, now in Cologne, from 25 - 27 november 1991.

A post doc from Cologne was working during 10 days in Amsterdam (7-17 october 1991) and a post doc from Nottingham worked for six weeks in Norwich.

All groups have exchanged on a very regular basis both information, plasmid constructs and mapped tomato genotypes. This made it possible for each group to use the most suitable materials for their further research.

List of joint publications/patents with trans-national authorship

Haring, M.A., Scofield, S., Teeuwen-de Vroomen, M.J., Leuring, G.S., Nijkamp, H.J.J., and Hille, J. (1991). Novel DNA structures resulting from dTam3 excision in tobacco. Plant Mol. Biol. 17 : 995 - 1004.

Other publications/patents

Dean, C., Sjodin, C., Bancroft, I., Lawson, E., Lister, C., Scofield, S., and Jones, J.(1991). Development of an efficient transposon tagging system in Arabidopsis thaliana. In : Molecular Biology of Plant Development (eds. : G.I. Jenkins and W. Schuch) Company of Biologists pp. 63-76.

Dean, C., Sjodin, C., Page, T., Jones, J.D.G., and Lister, C. (1992). Behaviour of the maize transposable element Ac in Arabidopsis thaliana L. Plant J. 2 : 69-81.

Duckham, S.C., Linforth, R.S.T., and Taylor, I.B. (1991). Abscisic-acid deficient mutants at the aba gene locus of Arabidopsis thaliana are impaired in the epoxidation of zeaxanthin. Plant, Cell and Environment 14 : 601-606.

Haring, M.A., Teeuwen-de Vroomen, M.J., Nijkamp, H.J.J., and Hille, J. (1991). Trans-activation of an artificial dTam3 transposable element in transgenic tobacco plants. Plant Mol. Biol. 16 : 39-47.

Haring, M.A., Rommens, C.M.T., Nijkamp, H.J.J., and Hille, J. (1991). The use of transgenic plants to understand transposition mechanisms and to develop transposon tagging strategies. Plant Mol. Biol. 16 : 449-461.

Hille, J., Zabel, P., and Koornneef, M. (1991). Genetic transformation of tomato and prospects for gene transfer. In : "Monographs on Theoretical and Applied Genetics 14 : Genetic improvement of tomato" (Kalloo, ed.) Springer Verlag Heidelberg, pp283-291.

Rommens, C.M.T., Kneppers, T.J.A., Haring, M.A., Nijkamp, H.J.J., and Hille, J. (1991). A transposon tagging strategy with Ac on plant cell level in heterologous plant species. Plant Science 74 : 99-106.

Rommens, C.M.T., Biezen, E.A. van der, Ouwerkerk, P.B.F., Nijkamp, H.J.J., and Hille, J. (1991). Ac-induced disruption of the double Ds structure in tomato. Mol. Gen. Genet., 228 : 453-458.

Rommens, C.M.T., Haaren, M.J.J. van, Buchel, A.S., Mol, J.N.M., Tunen, A.J. van, Nijkamp, H.J.J., and Hille, J.(1992). Transactivation of Ds by Ac-transposase fusions in tobacco. Mol. Gen. Genet. 231 : 433-441.

Witsenboer, H.M.A., Kloosterziel, K.M., Hateboer, G., Nijkamp, H.J.J., and Hille, J. (1992). Tomato susceptibility to *Alternaria* stem canker : parameters involved in host-specific toxin induced leaf necrosis. Plant Science, 81 : 127-134.

Haaren, M.J.J. van, Nijkamp, H.J.J., and Hille, J. (1991). Werkwijze voor het aanbrengen van een deletie- of inversie- mutatie in een plantengenoom ; daarvoor bruikbaar recombinant DNA ; gemuteerde plant. Nederlandse octrooiaanvrage nr. 9101620.

Rudenko, G.N., Nijkamp, H.J.J. and Hille, J. (1991). Supported PCR : A novel PCR method with a single primer for nucleic acid analysis. European patent application number 91403181.0

TITLE :IDENTIFICATION OF REGULATORY GENES CONTROLLING MAJOR METABOLIC PATHWAYS

CONTRACT NUMBER : **BIOT 0164 C (EDB)**

OFFICIAL STARTING DATE : **01/02/1991**

COORDINATOR : **Michel CABOCHE - Laboratoire de Biologie Cellulaire INRA Versailles (France)**

PARTICIPANTS :

1 - Chiara TONELLI - Universita degli studi di Milano - Milan (Italy)
2 - G. GIULIANO - ENEA - Roma (Italy)
3 - Cathie MARTIN - John Innes Institute - Norwich (U.K)
4 - William D.O. HAMILTON - A.G.C- Cambridge (U.K)
5 - Javier PAZ-ARES - CSIC - Madrid (Spain)
6 - R. M. WALLSGROVE - Rothamsted Experimental Station -(U.K)
7 - M. JOIGNY - SEITA - Bergerac (France)

OBJECTIVES SET FOR THE REPORTING PERIOD :

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 :

The regulation of the nitrate assimilatory pathway has been studied at Rothamsted and Versailles. The nitrate assimilatory pathway has been shown to be regulated by N-metabolites in *Nicotiana*. At Rothamsted, where mutants affected in glutamine synthetase have been obtained, a start has been made on examining the effect of endogenous glutamate and glutamine concentrations on the expression of NR in barley leaves. At Rothamsted, conditions have been established for the seletion of constitutive nitrate reductase-producing mutants of *Arabidopsis*. Considerable difficulties were encountered in finding the right conditions for the plants, such that adequate and even growth could be obtained in the absence of nitrate, an essential requirement for the selection procedure. As a back-up for the *Arabidopsis* screen, mutants are also selected from chemically mutagenised barley seed, and this screen is already producing positive results.

In Versailles, a *nia* promoter has been linked to a GUS reporter sequence and found to be expressed in transient experiments performed on protoplasts. However none of the regulations known to affect *nia* gene expression was detectable under these conditions.

The regulation of the reporter gene will be studied in stable transformants and in transient expression by particule gun bombardment in leaves of plantlets grown in vitro on nitrate or ammonium. The recognition of the *nia* promoter by transcription factors has been studied in Versailles in collaboration with the Roma group. In Roma, transcription factors involved in light regulation of plant genes are being characterized. The tomato light-regulated *Rbc*S, *Cab* and *nia* promoters all show a conserved GATAA motif at variable distances upstream from the TATA box. Oligonucleotides containing this region from the three promoters were synthetized and used in gel retardation assays with tomato nuclear extracts. All three oligonucleotides bound one or more factors present in the extract. The binding was specific, as judged from competition experiments with homologous, heterologous and mutated sequences. Cross-competition experiments also show that promoters bind at least one common factor.

In Versailles gel retardation experiments were performed with the nit-2 transcription factor regulating N- metabolism in *Neurospora*. Selective and specific gel retardation was detected in two domains of the nia promoters from tomato. Nit-2 is a member of the zinc finger type transcription factor family. Oligonucleotides derived from consensus sequences shared by the zinc finger domains of several members of the family were synthesized with the help of the Madrid group and used for PCR amplifications. A clone showing 52 % homology to Nit-2 was identified after cloning and sequencing of the amplification products. The obtained clone is used to identify the corresponding single copy gene from *N. plumbaginifolia*. In parallel, a yeast mutant defective for the gln-3 gene homologous to nit-2 and also involved in the regulation of N-metabolism has been used for screening of cDNAs from *Arabidopsis thaliana* able to complement the metabolic deficiency resulting from the mutation affecting gln-3. Two clones have been identified which reproducibly complement the mutation. They will be sequenced to study their eventual homology to nit-2 and gln-3. In Roma, the screening of an *Arabidopsis* genomic library using a mouse zing finger probe of the Cys_2-His_2 type has been proven, up to now, unsuccessful.

Nitrate reductase expression is highly integrated in the metabolic activity of higher plants. Tobacco plants in which nitrate reductase is constitutively expressed have been obtained by transformation of a nitrate reductase cDNA put under the control of transcription signals derived from the caMV. Once characterized for the expression of the transgene, these plants will be evaluated for their physiological characteristics by SEITA.

At the John Innes, the characterisation of regulatory mutants affecting anthocyanin production in *Antirrhinum majus* has been performed. Several new interesting mutants have been identified :

a) *Mixta*. In the backcrossing procedure the *mixta* mutation that affects the shade of colour in the flower was found to be somatically and germinally unstable.The transposon, Tam 4, has been shown to co-segregate with the mutant phenotype.

b)*Extenta*. This mutation gives rise to seedlings with extended hypocotyls and pale

cotyledons similar to the *aurea* mutations of tomato. The itensity of floral pigmentation is also affected. The similarity of phenotype to *aurea* and *hy* 3 in *Arabidopsis* suggests that *extenta* may be a mutation affecting phytochrome production or action.

c) *Delila.* In further analysis of a stable allele of *delila* (line JI:8), a novel instability, allowing gene expression in cells of particular tube, has been activated. *Del* has now been cloned and shown to encode a *myc* homologous transcription factor.

d) *Lavata, violacea.* Two more mutations affecting pigment distribution and intensity have been established as near isogenic lines.

The role of *Del* as both an activator and a repressor of transcription has been established. It has also been shown that a coordinated expression of flavanone-3-hydroylase (F3H), dihydroflavanol-4- reductase (DFR), *Candi*, and UDP glucose-flavonoid 3-0- glucosyl transferase (UDGT) is taking place during flower development and across the cells of the flower. Chalcone synthase (CHS), chalcone isomerase (CHI) and phenylalanime ammonia lyase (PAL) appear to be regulated separately.The genomic sequences encoding seven of the anthocyanin biosynthetic genes are being characterized by sequencing. Common motifs are contained amongst these different 5' promoter sequences which fit the consensus motifs for *myb* and *myc* transcription factors.

Six *myb*-homologous genes from *Antirrhinum* flowers (two of which are flowers specific) have been cloned. The expression patterns of each clone has been studied in a range of organs, during flower development and in light and dark. None of them, by theses criteria, are particularly good candidates for regulating anthocyanin biosynthesis. None of the *myb* genes appears to be the *El*, *del* or *ros* gene product, as tested through RNA analysis of the mutants and through RFLP analysis. The expression of one *myb* gene, 340, has been studied by *in situ* hybridisation and found to be primarily expressed in the nectary and transmitting tract of the style. The 308 promoter is active in the petals particularly the coloured lobe area, the sepals, the stigmatic surface and the anther filaments. The 305 promoter is active in flower petals, sepals, the style and the nectary.

To complement the *in vitro* analysis of *myb* gene function described above, tobacco plants overexpressing (double 35S enhancer), antisense and truncated constructs for each *myb* gene are being obtained.To date we have observed no phenotypic influence using the truncated *myb* gene constructs, expressing only the DNA-binding domain. The antisense constructs for *myb* 340 have given plants of reduced stature. Two of the transformants were male sterile. One had white flowers. It is therefore possible that in this plant the longer antisense 340 can interfere with the *myb* gene controlling floral anthocyanin production in tobacco. Overexpression of one constitutive *myb* gene, *myb*-308 gives pale wilting leaves with darkers veins. Production of *myb*-315 antisense appears to be lethal in that no transgenetics of tobacco have been produced and root growth in *Antirrhinum* is severely inhibited. Complementary DNA clones homologous to the Bzip class of transcription factors shown to bind to the CHS promoter from parsley are being screeened. A number of cDNA clones containing this specific motif have been identified and they show a close correlation in their expression to the anthocyanin biosynthetic

genes. There are some data to support the view that members of this gene family may be mediators of the light signal controlling expression of anthocyanin biosynthetic genes.

The work undertaken by AGC has been focused in the isolation of *myb* related genes from the tomato, *Lycopersicon escelentum* using the *Antirrhinum myb*- related clones isolated at the JICPSR as probes. A cDNA library was made from tomato hypocotyl mRNA and screened using a mixture of degenerate oligonucleotides showing homology to the conserved DNA binding region of *myb*-related genes. The probe composition was derived from the sequences of the original maize C1 probe and the 6 genes that were subsequently isolated from *Antirrhinum majus* at the JICPSR. A total of 30 positive plaques were identified after primary screening. Preliminary analysis revealed the presence of regions similar to those areas conserved in the 51 to 53 amino acid imperfect repeats found in *myb*-related genes. More encouragingly, 6 of the clones show amino acid homology to the N-termini of the *myb*-related genes isolated at the JICPSR. Having isolated a number of different *myb*-related clones from tomato, experiments have been stared to determine tissue specificity and expression levels. The expression patterns for all the clones are currently being investigated by probing northern blots containing polyA$^+$ RNA isolated from hypocotyl, leaf, root, green and red fruits, immature and mature flowers. With a view to future antisense/sense expression of the isolated genes *in planta*, transformation experiments using constructs containing the GUS marker gene have been carried out in order to develop a tomato transformation system.

In Madrid, anthocyanin biosynthesis has been studied in *Petunia*. Three cDNA clones corresponding to almost full copy *myb* genes and one which represented a 3' incomplete cDNA for a third *myb* gene had previously been isolated. To search for a putative C1 homolog from *Petunia*, PCR amplification of flower cDNAs were preformed with two mixtures oligonucleotides corresponding to two 5 aa stretches highly conserved in all plant *myb* proteins. Five additional *myb* genes could be identified. One of them showed high identity to C1 (90 % aa identity).Work is in progress to establish whether or not this C1-like *myb* gene is able to substitute for the C1 function in tansient expression experiments. In addition RFLP mapping of the 8 *myb* genes from *Petunia* is underway. DNA binding studies has been performed with *myb* Ph3 proteins showing that it binds to a sequence very similar to that recognized by the animal *myb* oncoprotein (CA/CGTTA/G) Work is in progress to establish whether or not the relative binding affinity for different versions of the consensus binding sequence is the same for different *myb* Ph proteins. Several in vitro mutagenized *myb* cDNAs have been prepared to evaluate the role of conserved residues in DNA binding properties of *myb* proteins. Transgenic Petunia plants expressing a *myb* Ph3 antisense RNA have been obtained. One plant and its progeny showed reduced flower colouration. The molecular basis of this effect is under study.

The regulation of anthocyanin accumulation is studied in maize by the Milano group. The genetic system under investigation is defined by two major components : a gene, *Sn*,

conferring tissue specific anthocyanin accumulation in different plant regions and another gene, *R*, lying two units *Sn* proximal, required for pigment accumulation in tissue not of *Sn* competence.The analysis of a recently cloned *Sn* cDNA sequence, a transcription factor of anthocyanin pathways in maize has been performed. The whole cDNA sequence of the *Sn:bol 3* allele has been completed and compared with the cDNA sequences of *Lc* and of the *S* component of the *R* complex, referred as *R-S*. The putative protein encoded by the 616-amino acid open reading frame has features to those of transcriptional activators. It contains a large acidic domain (aminoacids 188-324) with 35 acidic and 8 basic aminoacids for a cumulative negative charge of -27 and a basic region (aminoacids 421-514) part of which was found to have similarity to the *myc* family of oncogene proteins. A comparison between the three cDNA sequences of *Sn*, *Lc* and the *S* component of *R* (R-S) disclosed a very high degree of homology particularly in the translated region. These findings support the hypothesis that *Sn*, *Lc* and *R* genes encode functionally related proteins that act as transcriptional activators of the genes encoding the enzymes of the anthocyanin biosynthetic pathway. A Sn genomic clone has been isolated and sequences 1.5 kb of the region upstream the transcription start site performed. Besides the canonical regulation sites (TATA box and CAAT-like box), the presence of several putative sites involved in the control of transcription was detected.Other promoter regions of different *Sn* and *R* alleles are being currently characterized in order to understand by comparison the basis of tissue specificity and light inducibility.

Pericarp and aleurone anthocyanin accumulation is a process controlled by two homologous genes, *Sn* and *R* that allow a dramatic increase in pigmentation in response to white light irradiation. Irradiation of these two issues at succeding developmental times during seed formation and in different genotypes has disclosed the following points :
1) The two tissues differ in their response to light during their development.
2) Irradiation of *Rsc Sn* developping seeds with light of different quality, but equal fluence rate, indicaes that both red and blue light elicit a positive response in the aleurone while in the pericarp only blue light is effective.
3) Presence of *R-se* and *Sn* together in the genome leads to a drastic reduction in pericarp pigmentation in comparison to that observed in *r Sn* genotypes. White and blue light treatments lead to an enhanced transient accumulation on *Sn* mRNA in the pericarps with a maximum level of induction after 18-24 h of illumination. C2 and A1 transcripts increased in a coordinated manner after 24 h of illumination as expected, considering the regulatory role played by *Sn*. Different results were obtained when aleurone were analysed. Following white, blue and red light treatments the structural genes mRNA levels increased following a kinetic similar to that one observed in the pericarps, white *R* transcript was not affected by light treatment suggesting that *R* is controlled at the post-transcriptional level. Tissue specific expression was studied by northern analysis. Results obtained correlate perfectly with the phenotypic expression. Presence of *R* or *Sn* transcripts correlated precisely with the induction of A1 and C2 messengers and with the accumulation of the anthocyanin pigment. Absence of the a gene of the *R* family, as in the

r-g genotype, shows, in all the tissues examined, a lack of *R*, *A1* and *C2* transcripts correlating with colorless phenotypes. Labelled antisense and sense RNA probes were hybridized to serial sections of developping seeds of different genotypes. As expected, a specific localization (aleurone or pericarp) of regulatory and structural genes hybridization signal is correlated to the presence of the *R* or *Sn* genes respectively.

HIGHLIGHTS/MILESTONES

During the first year of this program several new regulatory mutants affecting anthocyanin biosynthesis have been identified. Simultaneously different genes coding for transcription factors are being cloned in the contracting laboratories. DNA retardation experiments, RFLP analysis, mutant complementation and antisense strategies will be used as tools to identify the fuctions of these newly identified genes in the coming years.

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, 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 Rothamsted Experimental Station in March 1991 to start the collaboratoins.A second meeting of all participants will be organized next May at ENEA in Roma.

Materiels (vectors, clones) and sequence information have been exchanged between Norwich and the group of Dr Paz-Ares and a meeting in Madrid (November, 1991) was organized to discuss results and plan future collaborative projects including a joint effort on RFLP mapping of *myb* genes in *Petunia*. Dr Paz-Ares also visited Versailles last March to further discuss strategies of cloning transcription factors. Francesca Sparvoli from the Milano group is in Norwich since October 1991 for a joint project to isolate a number of clones encoding anthocyanins biosynthetic enzymes and regulatory genes encoding *myb* and *myc* related transcription factor from grape using maize and *Antirrhinum* clones as heterologous probes.

The collaboration between INRA and SEITA has not started yet. Authorizations for field experiments involving transgenic plants have been requested, and we are waiting for these authorizations to start these field experiments.The industrial-academic collaboration between AGC and the JICPSR group is working well. Meeting have taken place a regular intervals between the two laboratories, four to date, and have proved a good forum for the exchange of information and materials. The JICPSR group has supplied probes and sequence data and AGC personel have visited the John Innes Institute to obtain details of tomato transformation.

JOINT PUBLICATIONS

- Ceccarelli E., Giuliano G. (1991) Preparation of nuclear extracts, gel retardation and DNAase footprinting. In Negrutiu I., Gharti Chetri G. (eds) : A laboratory guide for cellular and molecular plant biology. Birkhauser, Basel, pp 256-266.

- Consonni G., Viotti A., Dellaporta S., Tonelli C. (1992) cDNA nucleotide sequence of *Sn*, a regulatory gene in maize. Nucleic Acids. Res. 20. 373.

- Dorbe M. F., Caboche M., Daniel-Vedele F. (1992) The tomato *nia* gene complements a *Nicotiana plumbaginifolia* nitrate reductase-deficient mutant and is properly regulated. Plant Molecular Biology. 18 : 362-375.

- Jackson D., Culianez-Macia F., Prescott A., Roberts K., Martin C. (1991) Expression patterns of *myb* genes from *Antirrhinum* flowers. The Plant Cell 3 : 115-125.

- Jackson D., Roberts K., Martin C. (1992) Temporal and spatial control of anthocyanin biosynthetic genes during flower development in *Antirrhinum majus*. Plant J.. (in Press).

- Jarai G., Truong H.N., Daniel-Vedele F., Marzluf G. A. (1992) NIT 2, the nitrogen regulatory protein of *Neurospora crassa*, binds upstream of nia, the tomato nitrate reductase gene, in vitro. Curr. Genet. 21 : 37-41.

- Martin C., Prescott A., Mackay S., Bartlett J., Vrijlandt E. (1991) The control of anthocyanin biosynthetis in flowers of *Antirrhinum majus*. The Plant Journal, 1, 39-45.

- Mereghetti M., Tonelli C. (1991) Developmental expression of light inducible genes of the *R* family in immature seeds of maize. Maydica 36. 337-342.

- Tonelli C., Consonni G., Dolfini S., Dellaporta S., Viotti A., Gavazzi (1991) Genetic and molecular analysis of *Sn*, a light inducible, tissue specific regulatory gene in maize. Mol. Gen. Genet. Berlin, 225: 401-410.

Title: The plasma membrane and the tonoplast of plant cells as targets to increase plant productivity

Contract number: BIOT-0175

Official starting date: 01/01/91

Coordinator: Serge Delrot, Plant Physiol.and Biochem., Univ. of Poitiers, F-86000 Poitiers

Participants:
- H. Canut, UA CNRS 1457, Université Paul Sabatier, F- 31062 Toulouse Cédex, France
- S. Delrot, UA CNRS 574, Univ. of Poitiers, F-86000 Poitiers, France
- B. Forde, Rothamsted Experimental Station, Harpenden, Herts, AL5 2 JQ, U.K.
- W. Frommer, Institut für Genbiol. Forsch., Ihnestrasse 63, D-1000 Berlin 33, Germany
- E. Marré, Dipartimento di Biologia, Via Celoria 26, I- 20133 Milano, Italy
- G. Silvestri, COSPAG, 93 via Cabriolo, I- 43036, Fidenza, Italy
- J.A.C. Smith, Dep. of Plant Sciences, Univ. of Oxford, Oxford, OX1 3RB, U. K.

Objectives set for the reporting period

Crop productivity depends on the ability of the plant to transport the assimilates from the leaf blade to the receiving organs harvested for human consumption. This transport is controlled by the distribution and the activity of specific membrane transporters. The general aim of the project is therefore to characterize and to identify plant plasmalemma transporters (sucrose, amino acids, ions) and tonoplast transporters (malate), and to clone the corresponding genes. Sucrose is the major mobile carbohydrate in the plant and may be finally stored as sucrose or as starch in fruits. Amino acid transport is necessary for the synthesis of storage proteins found in cereals and legumes. Malate is one of the major solutes accumulated in vacuoles of higher plants, being involved in transient storage of carbon, charge balance, cytoplasmic homeostasis and turgor maintenance. Finally, ion transport across the plasmalemma is necessary for the maintenance of cell metabolism.

The objectives set for the reporting period consisted mainly in designing the techniques and the tools that were necessary to achieve the general objective. From a general standpoint, it was planned to compare and to adapt several methods of preparation of purified plasmalemma and tonoplast since very large amounts of membranes are necessary to run the experiments planned. For the sucrose carrier, already putatively identified as a 42 kD polypeptide in previous work, it was planned to prepare polyclonal and monoclonal antibodies directed to the 42 kD polypeptide, to screen cDNA libraries in sugar beet , and to start reconstitution experiments in proteoliposomes. In parallel, it was planned to develop a oocyte expression assay in order to check the identity of genes putatively identified as genes coding for transporters by the molecular biology approaches. For the amino acid carriers, the objective was to design differential labeling procedures, and to start PCR (polymerase chain reaction) with heterologous primers from bacteria. To develop a mutational approach towards the ion carriers in *Arabidopsis*, it was necessary to characterize the physiology of transport in this species, and to start the selection of mutants. Concerning the malate carrier, the objective set consisted in the comparison of translation products from induced and non-induced plants, and in the development of strategies based on monoclonal antibodies and differential labeling .

Major problems encountered

Most of the problems appearing during the work were expected, and none of them really impaired the achievement of intermediary or final objectives. General problems include the very large amounts of membranes that must be prepared to characterize the carriers, and the difficulty to work with membrane proteins (vs soluble proteins). Initial strategies have been improved to take these problems into account. An additional strategy, not initially included in the proposal (heterologous complementation of yeasts), which has appeared recently and has proven very powerful to identify membrane carriers (Sentenac et al., 1992) is now developed in the project.

Results

I. Plasma membrane

1. Preparation of plasma membranes. The groups of Poitiers and Toulouse have compared the yield, purity and functional status of plasma membrane fractions prepared by phase partitioning or by free flow electrophoresis. Both methods yield highly purified plasma membranes. Yet, the phase partitioning technique was faster and gave higher yields. Moreover, upon storage, the membranes prepared by free flow electrophoresis rapidly lost much of their transport activity (H^+ and sucrose). Phase partitioning, although expensive and somewhat tedious, is therefore routinely used to prepare the plasma membranes used for the biochemical studies on transporters.

2.The sucrose carrier

2a. Biochemistry. Several polyclonal sera and ascitic fluids directed against the 42 kD region of the plasmalemma have been raised in Poitiers. These sera selectively inhibited proton-driven uptake of sucrose into purified plasma membrane vesicles, while they had no effect on proton-driven valine uptake. This confirmed, on a functional basis, the previous hypothesis that a 42 kD polypeptide is involved in sucrose transport across the plasmalemma. The 42 kD polypeptide has been identified after denaturating gel electrophoresis. Successful attempts were made to purify the sucrose carrier in a functional state, using solubilisation of the plasma membrane proteins by non-denaturating detergents and high performance liquid chromatography. The solubilized proteins were separated by gel filtration and by ion-exchange (Mono-Q) chromatography. Two batches of membrane proteins were used. One batch was labeled by [^{3}H]/[^{14}C]N-ethylmaleimide in the presence of sucrose, according to procedures which differentially label the sucrose carrier. The other batch was unlabeled, and the fractions eluted from the column were monitored for their ability to be recognized by the anti-42 kD sera (ELISA test). Under non-denaturating conditions, a peak of differential label coincides with an ELISA reactive peak at 120 kD upon gel filtration. When the 120 kD peak is recovered and denatured by SDS, the 120 kD cluster yields a peak of differential label at 42 kD, which coincides with an ELISA reactive peak at 42 kD. Therefore, at least under these in vitro conditions, the 42 kD polypeptide is part of a higher molecular weight cluster (120 kD). The 120 kD fraction has been used to design a procedure for the reconstitution of proton-driven transport activity into proteoliposomes. The 120 kD fraction has been further separated on a Mono-Q column eluted by a NaCl gradient, and the proteins eluted were tested both for their ability to be recognized by the anti-42 kD sera, and for their ability to transport sucrose after reconstitution in proteoliposomes. A peak eluted at 0.31 M NaCl was found to react with the anti-42 kD sera, exhibited a high activity of sucrose transport, but did not transport valine. Gel electrophoresis showed that this fraction was strongly enriched in two very close bands at 42 kD . Further improvements to the purification (affinity chromatography) and the reconstitution steps are presently made.

Unlike the plasma membrane vesicles (PMV) prepared from mature exporting sugar beet leaves, the PMV prepared from young importing leaves are devoid of proton-driven sucrose transport activity. Monodimensional gel electrophoresis showed that PMVs from young leaves are depleted in a 42 kD polypeptide. These data provide another line of functional evidence for the identity of this polypeptide as a component of the sucrose carrier. Furthermore, the Berlin group has separated the proteins by two-dimensional electrophoresis (2D-PAGE) using immobilized pH gradients in the first dimension. Both in potato tubers and in sugar beet leaves, a whole set of proteins mainly in the 40 kD range are absent from the importing stage but are apparent at the exporting stage. The 2D-PAGE system has been scaled up and proteins have been transferred to PVDF membranes with the intention to sequence individual polypeptides. Purified fractions of the 42 kD protein prepared by non- denaturating high performance liquid chromatography in Poitiers are being analysed by 2D-PAGE to further narrow down the number of candidates to be characterized

Initial attempts to prepare monoclonal antibodies against the sucrose carrier have not been successful, possibly because the antigen used was the denatured 42 kD region of the plasma membrane. Further attempts will therefore be made with the non denatured fraction prepared by high performance liquid chromatography.

2b. Molecular biology. The Berlin group has constructed a directional expression

cDNA library in λ gt 11-Not from mature sugarbeet leaves. Approx. 5 x 10^5 plaques were screened using a biotinylated second antibody and streptavidin/peroxidase complex to detect the binding of the primary anti-42 kD antiserum (prepared in Poitiers). Ten positive plaques were obtained, purified and further analysed after subcloning. Complete DNA-sequencing showed that three clones, the biggest being 1.6 kb in size, are highly homologous to the propionyl CoA carboxylase gene from vertebrates and seem to encode a biotin binding protein. Related genes have been previously isolated as cloning artefacts due to direct interaction of streptavidin with the protein. No transcript was detectable in Northern blots of RNA isolated from different organs of sugarbeet. For the other clones of which substantial parts are sequenced, no homology was found in the data banks. The clones are still under investigation. Northern blot analysis showed differential expression in different organs of sugarbeet.

To identify a sucrose transporter in yeast by complementation, we needed a strain unable to transport, but able to metabolise sucrose if taken up via a functional plant carrier protein. The Berlin group showed that compared to wild type strains, a yeast strain deficient in invertase (suc) is retarded in growth on media containing sucrose as the sole carbon source. This strain, which does not transport sucrose, has been stably transformed with a gene coding for a sucrose hydrolysing activity. This strain still is not able to grow efficiently on sucrose. A cDNA expression library from mature spinach leaves has been constructed in a yeast expression vector. The cDNA library was transformed into the modified yeast strain and the recombinant clones were selected on media containing sucrose as the sole carbon source. We are currently characterizing clones that regained their ability to grow on sucrose. Other cDNA libraries presently prepared will be tested.

Our project also plans to use the oocyte expression assay to test the identity of genes coding for transport proteins. At the outset of this project, no plant transporter had been functionally expressed in *Xenopus* oocytes. The group in Rothamsted has used a full-length cDNA clone for the STP1 H^+/hexose cotransporter from *Arabidopsis* (kindly provided by Dr. N. Sauer). This cDNA has been transcribed *in vitro* and the mRNA injected into oocytes. Under optimized conditions, oocytes injected with the STP1 mRNA accumulated 3-O-methyl glucose at rates up to 3000 times greater than water-injected controls. These results demonstrate for the first time that a plant membrane transporter can be functionally expressed in oocytes, and therefore that the strategy planned is valid.

3.The amino acid carrier

3a.Biochemistry. The initial objective assigned to the Poitiers group for this period was to develop differential labeling methods for the amino acid carrier, similar to what had been done before for the sucrose carrier. Proton-driven valine transport into purified PMV from sugar beet leaves was characterized. A full range of inhibitors and conditions was tested to find a substrate-protectable inhibition of valine transport. Amino acid uptake was relatively insensitive to a variety of chemicals: DIDS , SITS , iodoacetamide , iodoacetate, phenylglyoxal (all tested at 2 mM). A variety of conditions was tested to try to find some inhibition by NEM, but without success. PCMBS, mersalyl or DEPC inhibited valine uptake significantly. Valine has no protective effect against PCMBS inhibition. L-Valine, D-valine and glycine are able to protect partially the carrier against mersalyle inhibition, while sucrose exerts no such protection. Unfortunately, mersalyl, which might be used to label differentially the amino acid carriers, is not available as a labelled compound. Therefore, attempts will be made to separate the membrane proteins on a mersalyl-column in the presence or in the absence of amino acids in the elution buffer. Since good progress is also currently made by affinity chromatography of the sucrose carrier, a similar strategy will also be developed for the amino acid carriers with valine-substituted columns.

Importantly, after several modifications of the procedure initially designed for the sucrose carrier, we are now able to reconstitute "routinely" amino acid transporters into proteoliposomes. This reconstitution procedure is necessary to identify plasma membrane fractions that are competent in amino acid transport after separation of the proteins on either of the column used (mersalyl, or valine-substituted columns).

3b.Molecular biology. A PCR (polymerase chain reaction) approach was successfully used in Rothamsted to isolate plant homologues of the ABC superfamily of ATP-dependent transport proteins. These transporters have diverse substrate specificities but some

bacterial members of the family are amino acid carriers. Two highly conserved amino acid sequences have been used to design two sets of mixed primers. DNA fragments of the expected size (ca 300 bp) were amplified from barley root mRNA and these were cloned and sequenced. Two related barley genes (45 % identical at the amino acid level) with significant homology to the mammalian P-glycoproteins were identified. A third clone also encoded a sequence with homologies to the ABC superfamily. The function of these three putative ATP-dependent transporters in barley roots has still to be determined. The success of these experiments has established the feasibility of the PCR approach to cloning plant homologues of well characterized families of transport proteins.

All eukaryotic amino acid permeases so far sequenced (five fungal and one human) belong to a single gene family, and members of the same family occur in bacteria. A set of four inosine containing primers based on conserved sequences in this amino acid permease family was designed. These primers were tested on a model template consisting of a fragment of the *Aspergillus nidulans* prnB (proline permease) gene and it was shown that they are able to amplify fragments of the expected size. These primers are now being used in Rothamsted to try to amplify amino acid permease sequences from plant genomic DNA.

Several unsuccessful attempts have been made in Rothamsted, using different batches of oocytes and different preparations of barley polyA$^+$ RNA, to detect the expression of a plant amino acid carrier by monitoring uptake of radiolabelled amino acids into the injected cells. Other sources of polyA$^+$ RNA will be tried, such as castor bean cotyledons, which should be enriched for amino acid permease mRNAs.

In *Saccharomyces cerevisiae*, several amino acid transport mutants are known (Cooper, 1982). Several of the respective genes have been isolated by complementation of the mutants. We intend to use the well established selection systems to isolate amino acid transporters from plants.

4.The ion carriers

4a.Physiology. The initial objective was to characterize important physiological features of transport in *Arabidopsis* at various levels (seedlings, isolated cells, protoplasts, membrane vesicles). Satisfactory conditions have been set up for the growth of *Arabidopsis* in liquid medium and for the establishment of cell suspension cultures. H^+ transport and the uptake and the effect on membrane potential of several ions (K^+, Na^+, NH_4^+, Cs^+, Cl^-, SO_4^-, NO_3^-) were studied in *Arabidopsis* seedlings. The reciprocal interactions (for uptake) among K^+, Na^+, NH_4^+, Cs^+, Cl^- and SO_4^- were also determined.

The sensitivity of ion transport to physical and chemical factors was studied. Using 3 day-germinated seedlings, it was shown that the stimulation (by K^+ or K^+ plus FC) or the inhibition of proton extrusion (by vanadate or by erythrosin B) is associated with an alkalinization or, respectively, an acidification of both cytosol and vacuole. In cultured cells, low water potential media induce a strong stimulation of H^+ extrusion. The effects of FC and of the osmotic pressure on H^+ extrusion are synergistic.

In protoplasts, two kinds of channels were identified: a) hyperpolarization-activated cationic channels, permeable to K^+, inhibited by triethylammonium ; b) depolarization-activated channels, permeable to K^+ or to Cl^- and also to polyamines (putrescine, spermidine and spermine).

In microsomes from 72-h-old seedlings and purified plasma membranes, specific activities of H^+-ATPase and of Ca^{2+}-ATPase were measured and their sensitivity to various effectors was studied. The H^+-ATPase of *Arabidopsis* vesicles was stimulated by fusicoccin, and the Ca^{2+}-ATPase was stimulated by calmodulin.

4b.Mutagenesis. Mutants putatively altered in K^+ uptake have been screened for in M_2 progeny from EMS-mutagenized M_1 plants by selecting slow-growing seedlings in a medium containing NH_4^+ and sub-optimal K^+ concentration, or by selecting for resistance to toxic concentrations of compounds such as Cs^+ or tetraethylammonium which interfere with K^+ uptake.

Mutants altered in the proton pump activity or sensitivity to fusicoccin have been

selected by resistance to hygromycin B or to toxic cations (paraquat) whose uptake is promoted by fusicoccin. A recessive mutant, resistant to Cs^+ when heterozygote and slow-growing at sub-optimal K^+ concentration when homozygous together with two independent mutants insensitive to fusicoccin, have been isolated. Future work will complete the characterization of ion transport in the wild type, extending it to sugar and aminoacids symports with H^+, and initiate the characterization of transport in the mutants already at our disposal (isolated by us or by other workers).

II. Tonoplast

1. Malate transport at the tonoplast of CAM plants (*Kalanchoe daigremontiana*)

Malate influx has been studied in Oxford, using the patch-clamp technique. Whole-vacuole experiments characterized an electrophoretic, inwardly directed transport system highly selective for malate and certain other four- and five-carbon dicarboxylates. The malate currents show relatively slow activation kinetics and are strongly rectifying, favouring malate transport into the vacuole at inside-positive vacuolar potentials. Recordings in isolated membrane patches revealed a small channel with a unitary conductance of 3.4 pS and high open probability. Thus, the malate-influx system at the tonoplast in CAM plants appears to be a novel anion-selective ion channel (submitted).

Tonoplast vesicles were used for further transport studies and for the production of monoclonal antibodies. A membrane-filtration technique allowed characterization of the inhibitor sensitivity of the malate transporter. Malate transport is sensitive to sulphydryl reagents and to reagents that covalently react with positively charged amino-acid residues (i.e. His, Lys, Arg). In particular, inhibition of malate transport by the lysyl reagent pyridoxal phosphate was almost completely substrate-protectable, suggesting that a lysine residue might form part of the dicarboxylate recognition site of the malate transport system. However, the differential labelling approach to identify the malate channel protein will not be pursued as a priority because the malate channel is believed to be a low-abundance membrane protein that might be well below the limits of detection.

Monoclonal antibodies have been raised in mice against native tonoplast membrane. Single-cell lines have been selected by the limiting dilution method for subsequent cloning, and the supernatants are being screened for their possible ability to inhibit malate transport at the tonoplast. Preliminary screening has been performed in assays of malate-dependent H^+ transport, and those supernatants with an inhibitory effect subsequently tested in the membrane-filtration assay. Recently, three monoclonal antibodies have been identified that significantly inhibit malate transport. These antibodies will now be further characterized by western blotting and by cross-reaction with translation products from membrane-bound polysomal RNA .

We are also attempting to identify transcripts associated with synthesis of the proteins at particular stages of CAM induction. Essential for this approach is the isolation and purification of RNA and DNA from *Kalanchoë* leaf tissue, a task that has not previously been attempted. A successful protocol was developed that yields RNA and DNA free from contamination by the acidic polysaccharides characteristically present in species of the Crassulaceae. Further, we have succeeded in isolating membrane-bound polysomal mRNA from *Kalanchoë* mesophyll cells, which we anticipate will be enriched in transcripts for membrane proteins. These protocols will now be used to compare transcript levels at different stages of CAM induction, and also for immunoprecipitation of tonoplast proteins using the monoclonal antibodies raised above. Additionally, PCR primers have been designed against conserved regions of genes from three known superfamilies of membrane transport proteins (the ABC proteins, *Nod 26* family, and the sugar transport family), and these will be tested for hybridization to cDNA from CAM and non-CAM tissue using the approach developed by the Rothamsted group.

2. Malate transport in C3 plants

Inhibitor studies performed in Toulouse with vacuoles from *Catharanthus* showed that a histidyl residue(s) is involved either in the binding or the translocation of malate. The histidine-specific reagents diethylpyrocarbonate and Rose Bengal efficiently inhibited the activity of the malate carrier in a substrate-protectable manner. A photoactivable analog of

malate has been synthesized with the co-operation of a chemistry group. The probe, radiolabelled with ^{125}I, will be used to identify the carrier.

Highlights/milestones. Reconstitution procedures for the sucrose carrier and the amino acid carriers. Complementation studies with yeasts. First demonstration that a plant membrane protein can be successfully synthesized and targeted to the oocyte plasma membrane. Isolation of *Arabidopsis* mutants for K^+ uptake. Identification of a novel malate-selective ion channel in the tonoplast of CAM plants. Library of monoclonal antibodies against the native tonoplast membrane of *Kalanchoë*. Development of a protocol for successful isolation of RNA and DNA from *Kalanchoë* mesophyll cells.

Wider considerations. From current progress in this project and in other groups, it may be foreseen that genetic engineering of transport proteins in plants will be possible in the near future. This open new insights on possible manipulation of the plant content, and on its stress tolerance, for example.

Cooperative activities. Two 2-days internal plenary meetings were held in Poitiers (07/91) and in Milan (01/92). A general meeting with external participants will be held in Oxford (June 25, 26 1992). Short duration bidirectional exchanges (2-3 weeks) of scientists occurred between Poitiers and Berlin to share techniques. Membranes are exchanged between Poitiers and Toulouse. Membranes and antibodies are supplied to the Berlin group by Poitiers. Other exchanges between Poitiers/Milan, Berlin/Rothamsted, Oxford/Toulouse and Rothamsted/Oxford are planned in the near future.

Joint publications

- Lemoine R., Frommer W.B., Gallet O., Hummel S., Gaillard C., Delrot S. Development of sucrose transport in plasma membrane vesicles from leaf tissues during the sink/source transition, submitted.

Other publications

- Colombo R. and Cerana R.,1991 - Inward rectifying K^+ channels in the plasma membrane of *Arabidopsis thaliana*. Plant Physiol. 97, 1130-1135.
- Colombo, R., Cerana R., Bagni N., 1992. Evidence for polyamine channels in protoplasts and vacuoles of *Arabidopsis thaliana* cells. Biochem. Biophys. Res. Comm. 182 No 3, 1187-1192.
- Delrot S., 1992. Assimilate translocation and membrane transport as limiting factors for plant growth. In : "Biochemical Mechanism Involved in Growth Regulation", D. Chiatante, J. Gallon, C. Smith and G. Zocchi (eds.). Phytochemical Society of Europe, Oxford Universy Press, in press.
- Gallet,O., Lemoine , R., Gaillard, C., Larsson, C., Delrot, S., 1992. Selective inhibition of active uptake of sucrose into plasma membrane vesicles by polyclonal sera directed against a 42 kD plasma membrane polypeptide. Plant Physiol., 98, 17-23.
- Li Z-S, Gallet O., Gaillard, C., Lemoine, R., Delrot, S., 1991. Reconstitution of active sucrose transport in plant proteoliposomes. FEBS Lett, 286, 117-120.
- Li Z-S., Gallet, O., Gaillard, C., Lemoine, R. ,Delrot, S., 1992. The sucrose carrier of the plant plasmalemma. Partial purification and reconstitution into proteoliposomes. Biochim. Biophys. Acta, 1103, 259-266.
- Rasi-Caldogno F., De Michelis M.I., 1992. The plasma membrane Ca^{2+} pump: biochemical characteristics and regulatory properties. In : "Biochemical Mechanism Involved in Growth Regulation", D. Chiatante, J. Gallon, C. Smith and G. Zocchi (eds.). Phytochemical Society of Europe, Oxford Universy Press, in press.
- Soave C., Vannini C., Gomarasca S., 1992. Selection for mutants of *Arabidopsis thaliana* altered in solute uptake. In "Biochemical Mechanism Involved in Growth Regulation", D. Chiatante, J. Gallon, C. Smith and G. Zocchi (eds.). Phytochemical Society of Europe, Oxford Universy Press, in press.

TITLE: CONSTRUCTION OF ARTIFICIAL CHROMOSOMES FOR HIGHER EUCARYOTIC CELLS

CONTRACT NUMBER:	BIOT-CT91-0259
OFFICIAL STARTING DATE:	1/4/91
COORDINATOR:	H. J. Lipps, MNF, Univ. of Tübingen, 7400 Tübingen, FRG P. Donini, Univ. of Rome, Rome, Italy
PARTICIPANTS:	(1) H. J. Lipps, Univ. of Tübingen, Tübingen, FRG (2) F. Azorin, Consejo Superior de Investigaciones Cientificas, Barcelona, Spain (3) H. Cooke, Medical Research Conncil, Edingburgh, UK (4) F. Grummt, Univ. of Würzburg, Würzburg, FRG (5) P. Donini, Univ. degli Studi di Roma, Rome, Italy (6) M. Shirley, Agricultural and Food Research Council, Pirbright, UK

OBJECTIVES:

The general objective of the proposed project is the construction of artifical chromosomes for mammalian cells by assembling biologically relevant DNA sequences from chromosomes and naturally occurring 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 mole-

cules, will help to unterstand 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 in higher organisms 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:

There are three functional elements necessary to turn a segment of DNA into an artificial chromosome: Telomeres, replicators and centromeres or other stabilizing sequences. The major effort therefore was made to identify and isolate such sequences from different sources as well as to assemble the different elements and test them in several organisms (number in parenthesis indicate the groups performing the experiments).

TELOMERES: Telomeres are the best characterized elements of the eucaryotic chromosome. While all telomeric repeats function in yeast, this is not the case for higher eucaryotic cells. If mammalian cells are transfected with YACs (yeast artifical chromosomes), the YAC integrates into the genome. Therefore, mammalian telomeres were ligated to a YAC carrying

the neomycin resistance gene under the control of the TK promoter. Cells transfected with this construct became G418 resistant, i. e. the neomycin resistance gene is expressed. Further experiments will show whether this construct is extrachromosomally stable (3). Experiments to study the requirements to keep a linear DNA molecular physically stable were performed by injecting constructs with different telomeric structures in Xenopus oocytes. It clearly could be shown that the native DNA structure is sufficient to prevent a linear DNA molecule from exonucleolytic digestion (1, 5). Telomeric sequences adopt a special DNA structure. A number of vectors containing different telomeric repeats were constructed and the conformation adopted by these sequences analyzed (1, 2, 5). Further work concentrated on the characterization of telomeres from the parasite Eimeria and from Xenopus laevis. While Eimeria telomeres are identical to those of Plasmodium and the plant Arabidopsis (6), the Xenopus telomeres are homologous the human telomeric repeat (1, 3). A protein that binds specifically to the single stranded protrusian of yeast telomeres was identified. This protein interacts with the well known RAP 1 protein (5).

REPLICATION ORIGINS: In contrast to the ARS (autonomous replicating sequences) in yeast nothing is known about mammalian origins of replication. However, cytological data indicate that in S phase initiation of replication occurs about every 100 kb apart. Since a YAC easily can span this length, a mammalian YAC library was constructed and is now tested for replication capacity (3). Using a different approach, mammalian DNA was restricted to about 20 kb and ligated to the pMAMneo vector. After introduction into mammalian cells, they were selected for G418 resistance. None of the cells survived for longer than one generation. These data are consistent with the notion that the information for replication initiation is present on long DNA fragments but that it is not sufficient for maintenance of replication (5). A simple and efficient method for the mapping of eucaryotic origins of replication was established and, using SV40 and polyoma virus as model

system, the feasibility of this method was demonstrated (4). In an attempt to isolate sequences involved in copy number control, the mouse amplification promoting sequences (APS) were inserted in a BPV-1 containing vector. Insertion of these sequences results in a drastic reduction of plasmid copy number. As working hypothesis we assumed, that the amplification promoting sequences act with the aid of BPV coded trans-acting factors as an origin of replication. Such constructs then would carry two origins of replication (from BPV and APS) and replication would be badly disturbed. This hypothesis is now experimentally tested (1, 4).

CENTROMERES: Centromeres seem to be the most complicated sequences, so far they are mapped down to the megabase level. Conversion vectors carrying large mammalian DNA fragments are constructed and will be tested for mitotic stability (3). However, it may turn out that for the construction of an artificial chromosome other stabilizing sequences may be easier to obtain and handle.

FURTHER EXPERIMENTS concentrated on the study of the structural organization of pericentromeric satellite DNA sequences and the construction of an extrachromosomally replicating expression vector for Xenopus. The structural organization of mouse and Drosophila satellite DNA was studied and analyzed (2). From mouse nuclear extracts proteins were isolated that bind specifically to these sequences. In this context the structural analysis of a $d(GA.CT)_{11}$ sequences was completed. This sequence adopts a special structure and increases the rate of recombination about 5-10 fold (2).

Based on the observation that a BPV-1 containing vector replicates extrachromosomally in Xenopus embryos an expression vector containing actin sequences in antisense orientation was constructed and injected into Xenopus embryos. It could be shown that transcripts produced by this vector specifically inhibit actin gene expression. This kind of vector may prove usefull for the identification of as yet uncharacterized sequences (1).

MILESTONES: All the experiments described above are important prerequisites for the construction of an artificial chromosome for higher eucaryotes. 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.
WIDER CONSIDERATIONS: An artifical chromosome provides the chance for safe and specific genetic manipulation of higher organisms. Since these constructs never will integrate into the genome and not disturb gene balance in the organisms they may be the ideal vector for somatic gene therapy.
COOPERATIVE ACTIVITIES: There are strong interactions between all participating groups as well as exchange of material and staff between the different laboratories. Two group meetings were held in Tübingen and one took place in Lisbon in conjunction with another group of the BRIDGE programm.

LIST OF JOINT PUBLICATIONS

Schmid, M., Steinbeisser, H., Ascenzioni, F., Trendelenburg, M.F. and Lipps, H. J. (1991): Native yeast telomeres are sufficient to stabilize linear DNA in Xenopus oocytes. Gene 106, 121-124.

OTHER PUPLICATIONS

Bernues, J., Beltran, R. and Azorin, F. (1991): SV40 recombinents carrying a d $(CT.GA)_n$ sequence show increabed genomic instability. Gene 108, 269-274.

Cooke, H. J. (1992): Using chromosome features in genome mapping. TIBTECH 10, 23-27.

Kipling, D. and Cooke, H.J.: Mammalian telomeres? beginning or end. Human Molecular Genetics, in press.

McKay, S-J. and Cooke: A mammalian single stranded DNA binding protein with specifity for telomeric sequences. Nucl. Acids. Res., in press.

Schmid, M., Steinbeisser, H., Epperlein, H. H., Trendelenburg, M. F. and Lipps, H. J.: An expression vector inhibits gene expression in Xenopus embryos by antisense RNA. Submitted.

TITLE: *Improved techniques for establishing a high expression production system for recombinant proteins from animal cells*

CONTRACT NUMBER: *BIOT-CT 90-0185*

OFFICIAL STARTING DATE: *01-04-1991*

COORDINATOR:

Dr.H.Hauser, GBF, 3300 Braunschweig, F.R.G.

PARTICIPANTS:

Dr.H.Hauser, GBF, 3300 Braunschweig, F.R.G.

Prof.Dr.M.J.T.Carrondo, IBET, 2780 Oeiras, Portugal

Prof.Dr.B.Griffiths, PHLS CAMR, Porton, Salisbury, Wiltshire SP4, OJG, UK

Manfred Mistler (Subcontractor of Prof. Griffiths), Schott Engineering, 6500 Mainz 1, F.R.G.

Dr.U.Weidle, Boehringer/Mannheim, 8122 Penzberg, F.R.G.

OBJECTIVES SET FOR THE REPORTING PERIOD:

For the genetic work in the initial year of the project the evaluation of feasibility of selection principles, genetic manipulation and gene targeting was planned. Cell biology work intended to evaluate the chosen cell lines and the base line assessment of cell culture properties and methods. This should include the establishment of bioreactors with macroporous supports and aggregated culture systems.

MAJOR PROBLEMS ENCOUNTERED:

Stability of one type of recombinant cell clone was critical in the initial part of the work.

RESULTS: **Genetic work:** A system was established which allows the identification and isolation of highly active chromosomal loci in production cell lines. Expression levels of a reporter gene which is integrated as a single copy into the chromosome of a large number of cell clones indicate the transcriptional activity of the integration loci. The isolation of flanking DNA sequences of

these highly transcribed reporter genes involve the next step. To achieve this, inverse PCR was established. Single copy chromosomal DNA fragments which are appropriate for targeting have been identified.

For directing the genes of interest into the defined, favourable chromosomal loci, gene targeting vectors are under investigation.

Bioreaction: Two cell lines secreting recombinant antibody, based on BHK-21 and SP2-0 were established by conventional transfection procedures and used for physiological studies in bioreactors. Cultivation was carried out in reactors in suspension culture, on microcarriers and in fixed bed bioreactor systems. While batch microcarrier cultures of recombinant BHK cells show negative correlation between growth and productivity in the myeloma cell lines suspension culture the productivity is strongly correlated to cell growth.

Aggregation: In addition, a new sampling system for aggreate flocks has been developed. The influence of the variables that control aggregate size and number have been studied, namely medium composition (with different amounts of serum and a serum free medium formulation) and inoculum concentration; neither had an appreciable influence on aggregate formation and size.

Aggregate size is basically dependent on hydrodynamics. Bioreaction using different spinner and impeller geometries shows that this is a generic property, independent of those variables. The next developments of the work are: optimization of aggregates, productivity and stability evaluation, stability growth in continuous cultues during long periods of time.

A theoretical model is under development to evaluate the transport phenomena through macropouros supports with different characteristics will be performed once the model is tested against literature data.

BHK cells grown in continuous culture in **fixed bed bioreactors** show at least twofold higher volumetric activity compared to batch microcarrier cultures grown with the same available volume of media. The maximum specific productivity of the microcarrier cultures lies within the range of average specific productivities for fixed bed reacture cultures. However, while the maximum values for the microcarrier cultures are for a single time point, the

values for the fixed bed reacture cultures are the average for up to 10 days.

HIGHLIGHTS/MILESTONES:

Natural aggregation is a generic phenomenon. Aggregates size is only a function of the cell line and hydrodynamics of the system. The correlation of cell growth and productivity depends on the cell line.

WIDER CONSIDERATIONS:

The major result of the first year of this project is that all bottleneck technologies could be established. These include the methods of identifying transcriptionally active chromosomal loci in which the genes of interest have to be targeted, the establishment of aggregated cell bioreactor system and the bioreaction on a fixed bed bioreaction system. At this time bioreaction is carried out with production cell lines constitutively secreting recombinant antibody. After having established recombinant cell lines with the genes of interest at defined chromosomal loci their expression will be challenged in the established bioreaction systems.

COOPERATIVE ACTIVITIES:

During the initial year of the project, members of the Portuguese group went to *PHLS* and *GBF* to establish common laboratory procedures and protocols, namely the quantification and secondary products like glutamine and ammonium or specific products obtained from the genetically modified cells. Short course at *Boehringer/Mannheim* to learn the *modus operandi* with SP2/0 also took place. *Vice versa* a member of the *CAMR* spent one week at *IBET* in order to exchange information. ELWW-Meeting on May 1991 on "Host-vector systems for animal cell biotechnology" was held in Oeiras, Portugal.

JOINT PUBLICATIONS:

Moreira, J. L., Wirth, M., Fitzek, M. and H. Hauser: Evaluation of reporter genes in mammalian cell lines. Methods in Molecular and Cellular Biology 3 (1992), 23-29

OTHER PUBLICATIONS/PATENTS: *Nothing to report*

TITLE: Structural and functional analysis of regulatory genes controlling liver-specific proteins
CONTRACT NUMBER: BIOT-CT91 0260 (TSTS)
OFFICIAL STARTING DATE: 01/06/91
COORDINATOR: 01 Vassilis I. Zannis, Inst. Mol. Biol. and Biotech. of Crete (IMBB), Iraklion, Crete Greece

PARTICIPANTS:
02 Vincenco DeSimone, Genetic Engineering Center of Napoli, Napoli, Italy 005
03 Ricardo Cortese, Istituto di Recherdhe di Biologia Molecolare, Pomezia, Italy 005
04 Giorgio Bressan, Institute of Histology & Embrylogy, Padova, Italy 005
05 Jean Chambaz, Biochimie Lab, URA CNRS 1283, CHU St. Antoine, Paris, France 001
06 Moshe Yaniv, Pasteur Institute, 25, rue du Docteur Roux 75724 Paris, France, Cedex 15
07 Gennaro Ciliberto, Istituto di Ricerche di Biologia Molecolare, Pomezia, Italy 005
08 Mary C. Weiss, Genetic Unit, Dept. of Molec. Biol., Pasteur Institute, Paris, France 001
09 George Mignot, Lab of Cell Culture, 3 Ave. des Tropiques, Les Ulis, France 001
12 George Brownlee, Chem. Pathol., Sir Wm. Dunn School, U of Oxford, Oxford, UK 006
13 Anna Maria Rollier, Dept. Biol. Genet., Univ. degli Studi di Milano, Milano, Italy 005

OBJECTIVES SET FOR THE REPORTING PERIOD:

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 genesin advanced tissue culture systems employing different extracellular matrices.

MAJOR PROBLEMS ENCOUNTERED:

There are no major problems in pursuing our original specific aims.

RESULTS:

01 Vassilis Zannis and John Talianidis

I. Molecular Cloning of the cDNAs of Transcription Factors Modulating the ApoCIII Gene: A unidirectional cDNA library from rat liver mRNA in λgt22 has been constructed which allows the expression and detection of β-galactosidase fusion proteins from the cloned inserts. By screening with the method developed by S. McKnight, we have purified several clones putatively encoding transcription factors binding to the apoCIII elements B, C, and D. Further characterization of these clones is in progress.

II. Definition of the Binding Domains of the Various Transcription Factors on the ApoA-I Promoter and Evaluation of Their Importance for the Transcription of the ApoA-I Gene: Footprinting analysis showed that the heat stable activities NF-BA2,3 and C/EBP bind between nucleotides -175 to -155, whereas the heat labile activities 1 and 3 bind in the -168 to -148 region. The importance of the factors which recognize the regulatory region C for the transcription of the apoA-I gene was established by *in vitro* mutagenesis of this region. Mutations in the -171 to -166 region and the -158 to -153 region which

diminished the binding of the heat stable activities reduced transcription to 8 to 14% of control. In contrast, a mutation in the -164 to -159 region which abolished the binding of the heat labile activity AIC1 increased transcription 4.6 fold. The findings suggest that the heat stable NF-BA2,3 are posistive regulators and the factor AIC1 is a negative regulator of the apoA-I gene transcription.

02 Vincenco DeSimone

LFB1/HNF1 and LFB3: Sites of Synthesis and Their Role in Liver Differentiation

LFB1 and LFB3 are two transacting factors which play a central role in the expression of liver-specific genes. The developmental expression pattern of these two genes has been characterized in great detail by RNase protection, western blot and *in situ* hybridization. It was shown that LFB3 expression is not directly correlated with the liver-specific phenotype. LFB3 and LFB1 are expressed in the epithelial component of many organs of endodermal and mesodermal origin. suggesting that they may play a more general role associated with the differentiation of specialized epithelia. A more detailed analysis of the expression pattern during the development of the kidney shows that LFB3 transcription starts with the induction of the presumptive mesenchymal cells, while LFB1 appears only when the proximal and the distal tubules start to be detectable. The induction pattern of LFB3 can be reproduced in the transfilter organ culture system, in which the presumptive kidney mesenchymal tissue can be grown *in vitro* and induced to differentiate by cocultivation with spinal cord explants. In this system, LFB3 expression "bursts" between 24 and 48 hours after induction, when the first morphologic differentiation of the nephrogenic vesicles occur.

03 Ricardo Cortese

I. Cloning of two zinc finger proteins which bind to the α1-antitrypsin promoter

Two partial cDNAs coding for DNA-binding proteins (AT-BP1 and AT-BP2) have been isolated. Both proteins bind to the B-domain of the α1-antitrypsin promoter, an element which is important for the liver-specific expression of α1-antitrypsin. Analysis of the cDNA sequences encoding these proteins reveal that both contain two zinc fingers of the Cys2-His2 type followed by a highly acidic stretch of 20 amino acids. AT-BP1 contains a second putative DNA binding domain consisting of an 8-fold repeat of a SPKK (Ser Pro Lys/Arg-Lys/Arg) motif. Both proteins bind to the NF-kappa B recognition site in the MHC gene enhancer with significantly higher affinity than to the kappa immunoglobulin gene enhancer, or to the B-domain of the α1-antitrypsin gene promoter. Analysis of mRNA expression shows that AT-BP1 and AT-BP2 are expressed in all the tissues examined.

II. Secondary structure and conformational stability of the dimerization domain of the transcription factor LFB1: The structure of a 32 residue dimerization domain of LFB1 solution has been determined by nuclear magnetic resonance. Three structurally distinct regions can be distinguished. The N-terminal region from residues 1 to 6 is extended. Two helical regions span from residues 7 to 18 and from 23 to 32. The absence of dipolar effects involving residues more than four positions apart in the sequence excludes the possibilities both of a four-helix bundle formed by two hairpins and of an antiparallel dimer; the domain must therefore be arranged as a parallel dimer formed by kinked monomers. This structural solution presents important differences from the leucine zipper-type structure observed in other transcriptional activators. The thermodynamic parameters associated with the unfolding of the 32 residue long dimerization domain were determined under the two-state assumption by the van't Hoff procedure. The enthalpy of unfolding increases linearly with temperature, and the corresponding value of delta Cp, the different in heat capacity between the unfolded and the folded forms of the peptide, is estimated to be ca. 0.7 kcal mol^{-1}. These results indicate that the dimerization domain of LF-B1 can fold and dimerize independently of the rest of the protein, with a thermodynamic stability comparable to that of a small globular protein.

diminished the binding of the heat stable activities reduced transcription to 8 to 14% of control. In contrast, a mutation in the -164 to -159 region which abolished the binding of the heat labile activity AIC1 increased transcription 4.6 fold. The findings suggest that the heat stable NF-BA2,3 are posistive regulators and the factor AIC1 is a negative regulator of the apoA-I gene transcription.

02 Vincenco DeSimone

LFB1/HNF1 and LFB3: Sites of Synthesis and Their Role in Liver Differentiation

LFB1 and LFB3 are two transacting factors which play a central role in the expression of liver-specific genes. The developmental expression pattern of these two genes has been characterized in great detail by RNase protection, western blot and *in situ* hybridization. It was shown that LFB3 expression is not directly correlated with the liver-specific phenotype. LFB3 and LFB1 are expressed in the epithelial component of many organs of endodermal and mesodermal origin. suggesting that they may play a more general role associated with the differentiation of specialized epithelia. A more detailed analysis of the expression pattern during the development of the kidney shows that LFB3 transcription starts with the induction of the presumptive mesenchymal cells, while LFB1 appears only when the proximal and the distal tubules start to be detectable. The induction pattern of LFB3 can be reproduced in the transfilter organ culture system, in which the presumptive kidney mesenchymal tissue can be grown *in vitro* and induced to differentiate by cocultivation with spinal cord explants. In this system, LFB3 expression "bursts" between 24 and 48 hours after induction, when the first morphologic differentiation of the nephrogenic vesicles occur.

03 Ricardo Cortese

I. Cloning of two zinc finger proteins which bind to the α1-antitrypsin promoter

Two partial cDNAs coding for DNA-binding proteins (AT-BP1 and AT-BP2) have been isolated. Both proteins bind to the B-domain of the α1-antitrypsin promoter, an element which is important for the liver-specific expression of α1-antitrypsin. Analysis of the cDNA sequences encoding these proteins reveal that both contain two zinc fingers of the Cys2-His2 type followed by a highly acidic stretch of 20 amino acids. AT-BP1 contains a second putative DNA binding domain consisting of an 8-fold repeat of a SPKK (Ser Pro Lys/Arg-Lys/Arg) motif. Both proteins bind to the NF-kappa B recognition site in the MHC gene enhancer with significantly higher affinity than to the kappa immunoglobulin gene enhancer, or to the B-domain of the α1-antitrypsin gene promoter. Analysis of mRNA expression shows that AT-BP1 and AT-BP2 are expressed in all the tissues examined.

II. Secondary structure and conformational stability of the dimerization domain of the transcription factor LFB1: The structure of a 32 residue dimerization domain of LFB1 solution has been determined by nuclear magnetic resonance. Three structurally distinct regions can be distinguished. The N-terminal region from residues 1 to 6 is extended. Two helical regions span from residues 7 to 18 and from 23 to 32. The absence of dipolar effects involving residues more than four positions apart in the sequence excludes the possibilities both of a four-helix bundle formed by two hairpins and of an antiparallel dimer; the domain must therefore be arranged as a parallel dimer formed by kinked monomers. This structural solution presents important differences from the leucine zipper-type structure observed in other transcriptional activators. The thermodynamic parameters associated with the unfolding of the 32 residue long dimerization domain were determined under the two-state assumption by the van't Hoff procedure. The enthalpy of unfolding increases linearly with temperature, and the corresponding value of delta Cp, the different in heat capacity between the unfolded and the folded forms of the peptide, is estimated to be ca. 0.7 kcal mol^{-1}. These results indicate that the dimerization domain of LF-B1 can fold and dimerize independently of the rest of the protein, with a thermodynamic stability comparable to that of a small globular protein.

04 Giorgio Bressan

The Extracellular Matrix in Liver Function and Morphogenesis. Targeted Disruption of the Alfa1 Type VI Collagen Gene in Embryonic Stem Cells

The biological function of type VI collagen has been undertaken following the targeted gene disruption approach in the mouse. A 9 kb genomic clone of the alfa1 (VI) gene was isolated and was used for further constructions. A neo cassette was inserted into an Eco47III site within the second exon and an Herpes simplex tk cassette was placed at the 3' end of the fragment and the construct was electroporated into embryonic stem (ES) cells of the D3 strain at passage 11 and colonies resistant either to G418 or G418+gancyclovir or G418+FIAU were selected. Clones with the expected recombination event were confirmed by Southern blotting employing probes external to the construct and various restriction enzymes. The frequency of targeted disruption of the alfa1 (VI) collagen gene was 1 in 90 G418 resistant clones. Cells of the selected clones are being injected into mouse blastocysts in order to derive alfa1 (VI)-deficient animals.

05 Jean Chambaz

Regulation of the Human ApoA-II Gene by the Synergistic Action of Factors Binding to the Proximal and Distal Regulatory Elements

It has been shown that the human apoA-II promoter contains a set of 14 regulatory elements (A to N). Deletion or nucleotide substitution analysis in elements L (nucleotides -803 to -773) and K (nucleotides -760 to -743) reduced hepatic transcription to 25 and 20% and intestinal transcription to 8 and 4% of control, respectively, as measured by CAT assays, indicating that these elements play an important regulatory role. Nucleotide substitutions in element AB (nucleotides -65 to -33) reduced hepatic and intestinal transcription to 60 and 36% of control, respectively. DNA binding and competition assays have shown that elements AB, K, and L bind with different affinities to a newly characterized heat-stable factor, CIIIB1, which is a transcription activator of the human apoCIII gene. In addition, elements AB and K bind a heat-labile activity, designated AIIAB1, and element L binds to several CCAAT box binding activities. Mutations in domain L that prevented the binding of CCAAT box binding activities reduced both hepatic and intestinal transcription to 30% of control, indicating the importance of these factors in transcription. Simultaneous nucleotide substitutions that prevented the binding of CIIIB1 activity in elements AB, K, and L reduced hepatic and intestinal transcription to 7 and 6% of control, respectively, suggesting that the synergistic interaction of CIIIB1 (bound to the proximal and distal regulatory elements) with CCAAT box proteins (bound to element L) can modulate the level of transcription of the human apoA-II gene.

06 Moshe Yaniv

I. Transcriptional Regulation of the HNF1/LFB1 Gene: A 3 kb DNA fragment containing the HNF1 promoter when linked to β-galactosidase, confers enzymatic activity both after transfection in HepG2 hepatoma cells or in transgenic animals. The profile of expression in the mice closely resembles the expression of the endogenous gene, suggesting that tissue specificity is conferred by this fragment. Transfection experiments in hepatic cells have shown that a minimal promoter of 80 bp was still active. This segment contains a binding site for another liver enriched transcription factor, HNF4. The role of HNF4 in the control of HNF1 was further confirmed in transfection of non hepatic cells. While the HNF1 promoter was inactive in these cells, it was activated by cotransfection with a clone coding for HNF4.

II. Structure of the Transactivation Domain of HNF1/LFB1

Deletion and domain swap experiments have shown that the C-terminal part of HNF1 or vHNF1 are responsible for transcriptional activation. Further experiments were undertaken to localize more precisely the residues involved in this activity and the potential targets for the action of HNF1 in the general transcriptional machinery.

III. Chromosomal Mapping of HNF1 and vHNF1 Genes in Man and Mouse

HNF1 is found on human 12 q 24.3 and mouse 5 E4-G1. vHNF1 was found on human 17 q 11.2-q 21.1 and mouse 11 B4-D. The mouse gene was mapped more precisely relative to other known genes on mouse chromosome 11 by inter subspecific mouse backcross to give the order centromer Edp-1-5.8cM-vHNF1-16.3cM-Erba. vHNF1 is clearly separated from the Hox-1 cluster of homoetic genes that was mapped 0.7 ± 0.7 cM proximal to Erba.

07 Gennaro Ciliberto

Signal Transduction by Il-6 in Hepatic Cells: The ability of various recombinant IL-6DBP derivatives, under the control of strong eukaryotic promoter, to stimulate transcription from the IL-6RE-CAT reporter gene in the presence and absence of IL-6 in HepG2 cells was analyzed. The reporter plasmid contained four copies of the CRPα IL-6RE fused to the TATA box and transcriptional initiation site of the CRP gene. The results from an initial set of domain-swap experiments showed that whilst C/EBP transactivates in anIL-a6 independent manner, activation by IL-6DBP is dramatically induced by IL-6. In contrast, a truncated form of IL-6DBP, coding for a smaller protein containing the leucine zipper and basic amino acid region, fails to transactivate despite being expressed at comparable level as full length IL-6DBP and also efficiently translocated to the nucleus. This suggests that the activation domain is located in the amino terminus of the protein, a conclusion supported by the observation that the amino terminal amino acids of IL-6DBP can impart the same level of constitutive and IL-6 induced activity of the reporter gene when fused to the DNA binding domain of C/EBP.

A second member of the C/EBP family of transcription factors is involved in the IL-6 signal transduction pathway in hepatic cells. Differently from IL-6DBP, this protein, named C/EBPδ, is under transcriptional control by IL-6: mRNA and protein are undetectable is unstimulated cells and their levels are strongly stimulated by IL-6. Basically, activation of C/EBPδ temporally follows IL-6DBP activation. Therefore at least two members of the same family of transcription factors is controlled by IL-6 through different mechanisms.

08 Mary Weiss

Establishing the Transcription Rate of Liver Specific Genes: Cell Phenotype, Promoter Structure and Binding Affinity Modulate Transactivation by HNF1/LFB1 and LAP.

Co-transfection of expression vectors encoding transcription factors as well as of reporter CAT plasmids containing binding sites for the transcription factors into cells of different phenotypes provides a means of evaluating transactivation potential in different contexts. For this work, a panel of hepatoma lines and their variants, expressing different levels of the endogenous factors, have been employed.

Well differentiated hepatoma cells produce abundant HNF1 and show high levels of expression of a reporter CAT gene whose expression is directed by the albumin promoter. Addition of exogenous HNF1 does not enhance the expression of the albumin promoter-reporter gene constructs. Dedifferentiated variants do not produce endogenous HNF1, nor do they express the albumin-CAT gene. Here strong transactivation of the CAT gene by exogenous HNF1 is observed. When the albumin HNF-1 binding site is substituted by a sequence of weaker binding affinity, exogenous HNF1 possesses transactivation potential in all cell lines, whether or not endogenous HNF1 is abundant. It can be concluded that the threshold of activity of a given transcription factor on a particular promoter can vary depending upon the affinity of the factor for the cognate binding site.

LAP is a member of the leucine zipper C/EBP family of transcription factors. It has been shown to be an activator of transcription using an artificial promoter composed of a binding site linked to the albumin TATA sequence. However, when used in a transactivation test employing the authentic albumin promoter, which contains the same sequences as the artificial promoter but combined with other binding sites (HNF1 and a CCAAT site), exogenous LAP represses expression. A likely interpretation is that exogenous LAP

displaces C/EBP, the latter being a more powerful activator of transcription.

09 George Mignot

T.M. Innovation is currently optimizing the culture condition required for culture of new types of hepatic cell lines, and will scale up hepatocyte cultures using macroporous carriers (gelatin or glass spheres) to achieve high cell densities with a combination of high microcarrier concentration and perfusion system. Expression of recombinant factor IX will include study of the quality of the molecule expressed in hepatocyte lines and analysis of specific activity.

12 George Brownlee

Characterization of the Clotting Factor IX Promoter

In this last year the discovery of the mutation in the patient Haemophilia B Brandenburg in Germany (a G→C mutation at -26) has given new insight into further liver-specific and ubiquitous transcription factors, involved in the regulation of the factor IX gene. A remarkable property of the Haemophilia B promoter mutants is that patients have a more-or-less severe bleeding disorder, requiring treatment during childhood, but that shortly after puberty the patients improve and no longer need treatment. Brandenburg proved to be different from all the other promoter mutants in that he failed to improve after puberty. The reason for this turns out to be that there is an androgen responsive element (ARE) in the factor IX promoter, which the mutation in Brandenburg disrupts. By using conventional transient assay systems in HepG2 cells, we have been able to show that this ARE functions in these cells, requiring the androgen receptor and testosterone for activity. When this ARE is mutated as in Brandenburg it fails to respond. These functional assays have been confirmed by gel shifts with the DNA binding domain of a recombinant androgen receptor. The reason for the reduced expression of factor IX before puberty is that there is a LF-A1/HNF4 binding site partly overlapping with the ARE. This was demonstrated in gel shifts with purified LF-A1, comparing the wild-type promoter and the Brandenburg mutants as competitors. Competition with the classic factor IX promoter mutant, Haemophilia B Leyden, which has a mutation at -20, also fails to bind LF-A1.

In summary then, it has been shown that the -20 promoter mutants interfere with the binding to the liver-specific transcription factor LF-A1/HNF4, while the -26 Brandenburg mutant interferes with the binding of both LF-A1/HNF4 and an androgen responsive element. The molecular details of how the binding of the androgen receptor "rescues" the effect of nearby mutations in the binding sites of other transcription factors remains obscure.

13 Anna Maria Rollier

Analysis of the Expression of Albumin Promoter in Human and Murine Hepatoma Cells

HepG2 and BW1J are albumin producing hepatoma cells which fail to show transient expression of a reporter gene driven by the rat albumin proximal promoter. HepG2 and BW1J cells have been examined for the presence of HNF1 and C/EBP mRNA. A significant signal has been observed when polyA+ RNA from both cell lines is used to prepare Northern blots probed with HNF1 and C/EBP cDNA. Gel shift assays using PE element as a probe have been therefore performed to test the binding properties of the critical transcription factor HNF1. Bands equivalent to those obtained with rat liver have been observed with the human and murine hepatoma cells. Two versions of rat albumin promoter spanning respectively from -151 to +16 and from -68 to + 4 were co-transfected with different amounts of HNF1 expression vector. Both fragments of the promoter are activated in HepG2 cells and the factor of transactivation obtained is 15-fold for the -68/+4 version and 8-fold for the -151/+16 version. In BW1J cells a weaker level (2 to 4-fold) of transactivation was observed only for the -151/+16 albumin promoter as not only the activity of the shorter promoter fragment alone is undetectable, but often also its activity in the presence of HNF1 is too weak to be measured.

HIGHLIGHTS/MILESTONES:

1) Characterization of the regulatory proteins which control the transcription of the human apolipoprotein CIII and A-II, the albumin and α1 antitrypsin genes, and clotting factor IX gene and cloning of cDNAs encoding new regulatory proteins.
2) Analysis of the sites of synthesis of previously cloned factors such as LFB1/HNF1, LFB3, C/EBP, and HNF4, IL-6DBP, and LAP, their role in the regulation of transcription of liver-specific genes and in liver differentiation.
3) Analysis by NMR of the secondary structure in solution and the conformational stability of the dimerization domain of the transcription factor HNF1/LFB1. Localization of the transactivation domain of HNF1/LFB1 in the C-terminal region.
4) Transcriptional regulation of the regulatory protein HNF1/LFB1 by another regulatory protein HNF4 *in vitro*.
5) Documentation that interaction of IL-6, IL-6 receptor mediates signal transduction in HepG2 cells via IL-6DBP and other members of the C/EBP gene family.

WIDER CONSIDERATIONS:

The transnational BRIDGE project entitled "Structural and Functional Analysis of Regulatory Genes Controlling Liver-specific Expression" involves 12 laboratories from 4 countries. This vast undertaking includes research projects of both fundamental and applied interest: interactions between the two types of projects is expected to open new avenues of approach to the development of products of pharmaceutical interest and to the treatment of disease, such as hemophilia, atherosclerosis, and liver diseases. Focus on the liver is dictated by the fact that this organ is the site of synthesis of many proteins that are crucial in the normal functioning of the organism as well as in diverse pathological states. Some representative vital functions of the liver are: a) synthesis and degradation of sugars and fats that are required as the source of energy (fuel) in our body; b) synthesis of nucleotides and amino acids which are the building blocks of our genetic material (DNA) and of proteins respectively; c) synthesis of most of the proteins found in plasma, several of which, such as factors IX, XII, apoA-I, are of pharmacological importance; d) synthesis and secretion of bile acids required for the solubilization and digestion of fats; e) synthesis and catabolism of the macromolecules (lipoproteins, LDL, HDL, etc.) which carry cholesterol and fats in our blood stream and redistribute it to different cells; f) detoxification of the body and catabolism of drugs.

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, acute phase proteins that are produced in response to inflammation, etc. While each of these proteins is essential for normal function, pathological states may result from the production not only of not enough protein, but in some cases from too much. This explains why an understanding of the gene controlling processes is essential.

The basic knowledge that will emerge from this biotechnology project has numerous industrial and pharmaceutical applications as follows:
1. Specific regulatory genes and target genes can be introduced into hepatocytes in order to generate a uniquely tailored type of hepatocyte which produces large quantities of a liver-specific protein of pharmaceutical importance such as factor IX, VIII or XII.
2. Drugs can also be designed based on a) the precise knowledge of the three dimensional interaction of the regulatory proteins with its recognition site on the DNA, b) the interaction of the different regulatory factors (protein-protein interaction), and c) the interaction of a regulatory protein with an activating hormone. Such drugs may selectively promote or inhibit the attachment of a factor to its target gene and, thus, may increase or decrease the transcription of the gene.

3. Improved hepatocyte cultures can also be used for toxicology tests that are currently performed using livers of animals. Such cultures can be generated by introducing the required regulatory genes or by changing the culture medium or the extracellular matrix to which cells attach and grow.
4. Some of the 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) All the participants of the BRIDGE project met in Sorento, Italy on June 1, 1991. In the context of the meeting, gene expression during liver differentiation and disease hald on June 2-6, 1992.
2) Dr. Yaniv visited the laboratory of Dr. Zannis October 14-20, 1992.
3) Dr. Zannis visited the laboratories of Dr. Chambaz, Dr. Yaniv, Dr. Weiss on October 25-27, 1991 and the laboratories of Dr. Cortese and Dr. Ciliberto on October 28 and 29, 1991. Work was done towards writing the blue booklet report.
4) Dr. Zannis visited the laboratory of Dr. Chambaz on March 18, 1992.
5) A meeting has been planned for all the participants at Iraklion, Crete on September 10-12, 1992.

cDNA clones, purified proteins and antibodies for IL-6DBB, LFB3, C/EBPγ, C/EBPδ, NF1 have been given by Drs. Cortese, Yaniv, Ciliberto, and DeSimone to the participating laboratories. Various hepatocyte cell lines have also been made available by Dr. Weiss to the other participants. Various other biological materials and techniques have been transferred among the participating laboratories.

LIST OF JOINT PUBLICATIONS/PATENTS WITH TRANS-NATIONAL AUTHORSHIP:

1. Papazafiri,, P., Ogami, K., Ramji, D.P., Nicosia, A., Monaci, P., Cladaras, C., andnnis, V.I. (1991) Promoter elements and factors involved in hepatic transcription of the human apoA-I gene. Positive and negative regulators bind to overlapping sites. J. Biol. Chem. 266:5790-5797.
2. Cardot, P., Chambaz, J., Cladaras, C., and Zannis, V.I. (1991) ApoA-II gene regulation by the cooperation of factors binding to the proximal and distal regulatory elements. J. Biol. Chem. 266:24460-24470.
3. Morrone, G., Ciliberto, G., Oliviero, S., Arcone, R., Dente, L., Content, J., and Cortese, R. (1988) recombinant interleukin 6 regulates the transcriptional activation of a set of human acute phase gene. J. Biol. Chem. 263, 12554-12558.
4. Rees, D.J.G., Jones, L.M., Harndford, P.A., Walter, S., Esnouf, M.P., Smith, K.J., and Brownlee, G.G. (1988) The role of hydroxyaspartate and adjacent carboxylate residues in the first EGF domain of human factor IV. EMBO J. 7, 20253-2061.
5. Herbomel, P., Rollier, A., Tronche, F., Ott, M.O., Yaniv, M., and Weiss, M.C. (1989) The rat albumin promoter is composed of six distinct and positive elements within 130 nucleotides. Mol. Cell. Biol. 9P, 4750-4758.
6. Cereghini, S., Yaniv, M., and Cortese, R. (1990) Hepatocyte dedifferentiation and extinction is accompanied by a block in the synthesis of mRNA coding for the transcription factor HNF1/LFB1. EMBO J. 7, 2257-2263.

OTHER PUBLICATIONS/PATENTS:

1. DeSimone, V., DeMagistris, L., Lazzaro, D., Gerstner, J., Monaci, P., Nicosia, A., and Cortese, R. (1991) LF-B3, a heterodimer-forming homeoprotein of the LFB1 family, is expressed in specialized epithelia. The EMBO J. 10:1435-1443.
2. DeSimone, V., Cortese, R. (1991) Transcriptional regulation of liver-specific gene expression. Current Opin. Cell Biol. 3:960-965.
3. Bulla, G.A., DeSimone, V., Cortese, R., and Fournier, R.E.K. (1992) Extinction of α1-antitrypsin gene expression in somatic cell hybrids: Evidence for multiple controls. Genes and Development 6:316-327.
4. Lazzaro, D., DeSimone, V., DeMagistris, L., Lehronen, E., and Cortese, R. (1992) LFB1 and LFB3 homeoproteins are sequentially expressed during kidney development. Development (In Press).
5. Xanthopoulos, K.G., Prezioso, V.R., Chen, W.S., Sladek, F.M., Cortese, R., and Darnell, J.E., Jr. (1991) The different tissue transcription patterns of genes for HNF-1, C/EBP, HNF-3, and HNF-4 protein factors that govern liver-specific transcription. Proc. Natl. Acad. Sci. USA 88:3807-3811.
6. Mitchelmore, C., Traboni, C., and Cortese, R. (1991) Isolation of two cDNAs encoding zinc finger proteins which bind to the α1-antitrypsin promoter and to the major histocompatibility complex class I enhancer. Nucleic Acids Res. 19:141-147.
7. Pastore, A., DeFrancesco, R., Barbato, G., Castiglione-Morelli, M.A., Motta, A., and Cortese, R. (1991) 1H resonance assignment and secondary structure determination of the dimerization domain of transcription factor LFB1. Biochemistry 30:148-153.
8. DeFrancesco, R., Pastore, A., Vecchio, G., and Cortese, R. (1991) Circular dichroism study on the conformational stability of the dimerization of transcription factor LFB1. Biochemistry 30:143-147.
9. Poli, V., Mancini, F.P., and Cortese, R. (1990) IL-6DBP, a nuclear protein involved in interleukin-6 signal transduction, defines a new family of leucine zipper proteins related to C/EBP. Cell 63:643-653.
10. Selmin, O., Volpin, D., and Bressan, G.M. (1991) Changes of cellular expression of mRNA for tropoelastin in the intraembryonic arterial vessels of developing chick revealed by *in situ* hybridization. Matrix 11:347-358.
11. Marigo, V., Sitta, A., Volpin, D., and Bressan, G.M. (1992) Mapping of binding sites for monoclonal antibodies to chick tropoelastin by recombinant DNA techniques. Connective Tissue Res. 27:1-16.
12. Marigo, V., Daga-Gordini, D., Sitta, A., Volpin, D., and Bressan, G.M. (1992) Effect of monoclonal antibodies to defined regions of tropoelastin on elastogenesis *in vitro*. Eur. J. Cell Biol. In Press.
13. Rey-Campos, J., Chouard, T., Yaniv, M., and Cereghini, S. (1991) vHNF1 is a homeoprotein that activates transcription and forms heterodimers with HNF1. EMBO J. 10:1445-1457.
14. Bach, I., Mattei, M.G., Cereghini, S., and Yaniv, M. (1991) Two members of an HNF1 homeoprotein family are expressed in human liver. Nucl. Acids Res. 19:3553-3559.
15. Tronche, F., Rollier, A., Sourdive, D., Cereghini, S., and Yaniv, M. (1991) NFY or a related CCAAT binding factor can be replaced by other transcriptional activators for cooperation with HNF1 in driving the rat albumin promoter *in vivo*. J. Mol. Biol. 221:31-43.
16. Blumenfeld, M., Maury, M., Chouard, T., Yaniv, M., and Condamine, H. (1991) Hepatic nuclear factor 1 (HNF1) shows a wider distribution than products of its known target genes in developing mouse. Development 113:589-599.

17. Ott, M.O., Rey-Campos, J., Cereghini, S., and Yaniv, M. (1991) vHNF1 is expressed in epithelial cells of distinct embryonic origin during development and precedes HNF1 expression. Mechanisms of Development 36:47-58.
19. Karolyi, J., Guenet, J.L., Rey-Campos, J., and Camper, S.A. (1992) The gene coding for variant hepatic nuclear factor 1 (Tcf-2) maps between the Edp-1 and Erba genes on mouse chromosome 11. Mammalian Genome (In Press).
20. Giannelli, F., et al. (1992) Haemophilia B: database of point mutations and short additions and deletions-third edition. Nucl. Acid Res. (In Press).

TITLE: THE DEVELOPMENT OF A GENETIC AND PHYSICAL MAP OF THE PORCINE GENOME (Pig Gene Mapping Project - PiGMaP)

CONTRACT NUMBER: BIOT-0187-C(EDB)
OFFICIAL STARTING DATE: 1st February 1991

COORDINATOR(S):
Drs. A.L. Archibald and C.S. Haley, AFRC IAPGR Edinburgh Research Station, Roslin, G.B.
PARTICIPANTS:
2. Dr. E.M. Tucker, AFRC IAPGR Cambridge Research Station, Cambridge, GB
3. Dr. A. van de Weghe, State University of Ghent, Merelbeke, BE
4. Dr. M. Fredholm, The Royal Veterinary and Agricultural University, Copenhagen, DK.
   Dr. V. Nielsen, National Institute of Animal Science, Foulum, DK.
5. Prof. Dr. H. Geldermann, Universität Hohenheim, Stuttgart, DE.
6. Dr. L.Ollivier, INRA Station de Génétique Quantitative et Appliquée, Jouy-en-Josas, FR.
   Dr. J. Gellin, INRA Laboratorie de Génétique Cellulaire, Toulouse, FR.
   Dr. M. Vaiman, CEA INRA Laboratoire de Radiobiologie Appliquée, Jouy-en-Josas, FR.
   Dr. P. Popescu, INRA Laboratoire de Cytogénétique, Jouy-en-Josas, FR.
7. Dr. A.A. Bosma, State University of Utrecht, Utrecht, NL.
8. Dr. M. Groenen, Wageningen Agricultural University, Wageningen, NL.
9. Dr. W. Davies, Norwegian College of Veterinary Medicine, Oslo, NO.
12. Dr. L. Andersson, Swedish University of Agricultural Sciences, Uppsala, SE
13. Dr. I. Gustavsson, Swedish University of Agricultural Sciences, Uppsala, SE.

also ELWW participants
   Dr. E. Signer, University of Leicester, Leicester, GB.
   Dr. R. Davoli, Università degli Studi di Bologna, Regio Emilia, IT.

OBJECTIVES SET FOR THE REPORTING PERIOD:
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 first twelve month period include -
a). Reference family foundation stock karyotyped for abnormalities.
b). 100 markers of PIC > 0.3 in reference families identified.
c). Degree of polymorphism in reference families established, comparison of information content and complementarity of different reference families.
d). Use of microsatellites with PCR genotyping established.

MAJOR PROBLEMS ENCOUNTERED:
No major problems have been encountered.

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 1, 2, 6, 7, 23, 24, 25, 32, 34].

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). The Swedish and German pedigrees have European Wild Boar and European improved breeds as their grandparents. The karyotypes of some of the founder animals were checked for abnormalities. These pedigrees were established ahead of schedule. Distribution of DNA from 100 F_2 pigs plus their respective parents and grandparents to all ten laboratories for genotyping has begun and will be completed ahead of schedule in May 1992. The 100 F_2 pigs correspond to two litters of ten pigs from each centre with four grandparents, two female parents and a single male parent.
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. Over fifty such RFLPs have been characterised by the Edinburgh, Foulum, Oslo and Uppsala groups and include polymorphisms at the *ADH*, *ALB*, *BF*, *CASAS1*, *CASAS2*, *CASB*, *FGA*, *FGB*, *FGG*, *GH*, *LPL*, *PI*, *PLAU*, *PPLA2*, *PP2ARB*, *TF*, *TSHB*, *PDGFRA*, *PDGFRB* and *UBB* loci [refs - 13, 14, 15, 26, 27, 28, 33, 35]. These expressed sequences will provide the means of integrating the genetic and physical maps as well as for exploring comparative aspects of gene mapping. Hypervariable markers based upon both minisatellite and microsatellite loci are also being developed. The Merelbeke, Hohenheim and Leicester groups have isolated and characterised sixteen new locus specific minisatellite (VNTR) loci [refs - 18, 21. The structure, number of repeat blocks and chromosomal distribution of porcine microsatellitte loci (dG-dT)n - (dC-dA)n have been found to exhibit the same desirable features as their human counterparts [ref - 38]. Already, over 200 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 several of these loci have been designed and the highly polymorphic nature of the loci confirmed [refs - 22, 37, 38]. The PCR based methods of rapid microsatellite genotyping have been established, as planned. Thus, the objectives of identifying 100 markers of PIC > 0.3 and determining the degree of polymorphism in the reference pedigrees have been fulfilled.
Protein polymorphisms and SLA genotypes have also been determined in some of the reference pedigrees.
A pilot linkage mapping experiment involving seven laboratories and 20 F_2 pigs was carried out in the six weeks prior to the second PiGMaP project meeting held in Toulouse in December 1991. A total of 35 loci were genotyped and four new linkage groups identified. One of these new linkage groups encompasses *CASAS1*, *CASAS2*, *CASB*, *FGA*, *FGB*, *FGG*, *PDGFRA*, two microsatellite loci, *ALB*, and the dominant white coat colour locus and can be assigned to chromosome 8 from knowledge of the location of *ALB*.

Physical mapping
Participants in Copenhagen, Toulouse, Uppsala and Utrecht are applying *in situ* hybridisation techniques to the assignment of genes to chromosomes. Improvements to the banding techniques used to identify the individual chromosomes have been effected [ref 40]. Amongst the loci which have been mapped by this approach are - *APOE* (chr 6), *ATPB* & *ATP1A* (chr 4), *CP* (chr 13), *CS* (chr 5), *DYZ-1* (chr Y), *ENO1* (chr 6), *GH* (chr 12), *INSR* (chr 2), *LHCGR* (chr 3), *LPL* (chr 14), *PEPN* (chr 7), *rRNA* (chr 8, 10 & 16), *PGD* (chr 6), *TGFB* (chr 6), *TF* (chr 13) [refs - 3, 5, 8, 10, 11, 12, 16, 19, 20, 29, 36, 39, 41]. This 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 is also being effected by analysis of somatic hybrid cell lines. The cell lines available at the outset did not constitute a mapping panel and therefore further hybrid cell lines are being developed by the Copenhagen, Cambridge, Toulouse and Utrecht groups

[ref 9]. A total of 50 and 130 hybrid clones in Cambridge and Utrecht respectively, are being characterised prior to their selection (or rejection) for a mapping panel of cells. A fluorescent *in situ* hybridization procedure with a porcine SINE (short interspersed elements) probe has been developed which enables the identification of pig chromosomes in pig x rodent hybrid cells [ref - 4]. Synteny mapping studies have confirmed the localization of the following loci - *GPI* (chr 6), *MPI* (chr 7), *PEPN* (chr 7), *SLA* (chr 7), and *SOD1* (chr 9).
The number of loci assigned to chromosomes either directly or through linkage to or synteny with direct assignments has doubled during the first year of the collaboration and now approaches 100. Genes have now been assigned to 15 of the 18 autosomes.
The polydisperse nature of the porcine karyotype allows the chromosomes to be sorted effectively by the use of a dual laser FACS machine. The flow sorting of pig chromosomes has been achieved by groups in France and Cambridge. The determination of the chromosomal identity of the flow sorted peaks should be completed within the next few months, well ahead of schedule (original target 24 months). Two of the French groups have combined a PCR amplification of each of the twenty flow sorted peaks with chromosome painting to effect the identification. The *CYP21* and *TNFA* loci have been assigned to chromosome 7 by PCR analysis of flow sorted chromosomes. Already the flow sorted material has been used to develop chromosome specific libraries for chromosomes 1, 6, and 13, again well ahead of schedule (original target 30 months) [ref - 31]. A novel PCR technique has been used to create biotinylated chromosome-specific probes from the flow sorted material. Two new reciprocal translocations (6/8 and 6/15) have been identified which may prove to be useful as markers for physical mapping or flow cytometry [ref - 17].
Repetitive sequences and the physical components of chromosomes - telomeres and centromeres are also being studied [refs - 4, 30]. The characterisation of porcine SINE sequences has proved to be useful in the amplification of the limited amounts of DNA produced by flow cytometry or chromosome scraping and in the characterisation of the chromosome content of somatic hybrid cell lines. The PRE-1 SINE sequences have been shown to unevenly distributed along the chromosomes, and are clustered around the centromeric regions.

HIGHLIGHTS/MILESTONES:

The first year of PiGMaP has been marked by the friendly and open collaboration between the participants. Several targets have been reached ahead of schedule. In particular, the development of a flow karyotype, the establishment of the necessary reference pedigrees and PCR based genotyping of microsatellite loci have been successfully achieved.

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 first 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 have been established. The number of genes assigned (or mapped) to chromosomes has almost doubled and approaches 100. Techniques for physical 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. Preliminary discussions have taken place with groups from the United States of America and Australia on the basis for widening the collaboration.

COOPERATIVE ACTIVITIES:

The collaboration between the participating laboratories has operated 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. A pilot linkage study involved seven groups genotyping a shared pool of animals. DNA prepared from 12 primary pig x mouse hybrid cells have been distributed by the Copenhagen group to other

participants for synteny mapping studies. 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. The development of SINE priming involved a three way collaboration [ref - 4].
Three project meetings have been held in the first reporting period. The first meeting, at which all the participating laboratories were represented, was held in Edinburgh on April 15th 1991, immediately after the 2nd European Farm Aninal Genome meeting. Five laboratories took part in a meeting held at Jouy-en-Josas, France in June 1991 to discuss the design of the experiments to map the quantitative trait loci (QTLs). The recommendations for the design of the reference mapping populations made at the June meeting have subsequently been adopted. Finally, a full project meeting was held at INRA, Toulouse on the 6th and 7th December, 1991. Not only were all participating laboratories represented but also three US groups and one Australian group attended, two with the support of EC funds.

JOINT PUBLICATIONS/PATENTS WITH TRANS-NATIONAL AUTHORSHIP:

1. Archibald, A. L., Haley, C. S., Andersson, L., Bosma, A. A., Davies, W., Fredholm, M., Geldermann, H., Gellin, J., Groenen, M., Gustavsson, I., Ollivier, L., Tucker, E. M., and Van de Weghe, A., 1991. PiGMaP - a European initiative to map the porcine genome. *Animal Genetics* 22, Suppl. 1, 82-83.
2. Bosma, A. A., de Haan, N. A. and Macdonald, A. A., 1991. The current status of cytogenetics of the Suidae: a review. *Bongo Berlin* 18, 258-272.
3. Chowdhary, B. P., Harbitz, I., Davies, W. and Gustavsson, I., 1991. Mapping of genes belonging to the halothane linkage group in pigs using in situ hybridization. *Génétique, Sélection Évolution* 23, Suppl. 1, 96s-99s.
4. Frengen, E., Thomsen, P., Kristensen, T., Kran, S., Miller, R. and Davies, W., 1991. Porcine SINEs: chracterization and use in species-specific amplication. *Genomics* 10, 949-956.
5. Gu, F., Harbitz, I., Chowdhary, B. P., Davies, W. and Gustavsson, I., 1992. Mapping of the porcine lipoprotein lipase (LPL) gene to the chromosome 14q12-q14 bands by *in situ* hybridization. *Cytogenetics and Cell Genetics* 59, 63-64.
6. Haley, C. S., Archibald, A. L., Andersson, L., Bosma, A. A., Davies, W., Fredholm, M., Geldermann, H., Groenen, M., Gustavsson, I., Ollivier, L., Tucker, E. M., and Van de Weghe, A., 1990. The Pig Gene Mapping Project - PiGMaP. *4th World Congress on Genetics Applied to Livestock Production.* XIII, 67-70.
7. Haley, C. S. and Archibald, A. L.; Andersson, L., Bosma, A. A., Davies, W., Fredholm, M., Geldermann, H., Gellin, J., Groenen, M., Gustavsson, I., Marmirolli, N., Nielsen, V. H., Ollivier, L., Popescu, P., Signer, E., Tucker, E. M., Vaiman, M., and van de Weghe, A., 1991. The pig gene mapping project (PiGMaP), ELWW leaflet, Commission of the European Communities.
8. Harbitz, I., Chowdhary, B., Thomsen, P. D., Davies, W., Kaufman, U., Kran, S., Gustavsson, I., Christensen, K. and Hauge, J. G., 1990. Assignment of the porcine calcium release channel gene, a candidate for the malignant hyperthermia locus, to the 6p11-q21 segment of chromosome 6. *Genomics* 8, 243-248.
9. Thomsen, P. D., Bosma, A. A., Kaufman, U. and Harbitz, I., 1991. The porcine *PGD* gene is preferentially lost from chromosome 6 in pig x rodent somatic cell hybrids. *Hereditas* 115, 63-67.
10. Thomsen, P. D., Christensen, K. and Davies, W., 1991. Regional localization of repetitive DNA on the porcine Y chromosome. Human Gene Mapping 11 (1991). *Cytogenetics and Cell Genetics* 58, 2131.
11. Thomsen, P. D., Poulsen, P. H., Olsen, J., Harbitz, I. and Miller, J. R., 1991. Comparative gene mapping of the chromosomes in the domestic pig. *Animal Genetics* 22, Suppl. 1, 80.
12. Yerle, M., Archibald, A. L., Dalens, M. and Gellin, J. 1990. Localization of PGD and TGFβ-1 to pig chromosome 6q. *Animal Genetics* 21, 411-417.

OTHER PUBLICATIONS/PATENTS:

13. Archibald, A. L., Brown, J. F., Couperwhite, S. and Haley, C. S. 1991. Reference families and markers for genetic mapping in the domestic pig. 11th International Workshop on Human Gene Mapping (HGM11) *Cytogenetics and Cell Genetics* 58, 2123.
14. Archibald, A. L., Couperwhite, S. and Haley, C. S. 1991. Genetic mapping in Meishan/Large White pig families. 1st Mammalian Genetics and Development Workshop, *Genetical Research* 58, 75.
15. Archibald, A. L., Couperwhite, S. and Haley, C. S. 1991. Markers for genetic mapping in the domestic pig. 7th Tenovus - Scotland Symposium "Eukaryotic genes and their expression". University of Glasgow, 20-22 March 1991.

16. Bosma, A. A., de Haan, N. A. and Mellink, C. H. M., 1991. Ribosomal RNA genes located on chromosome 16 of the domestic pig. 11th International Workshop on Human Gene Mapping (HGM11) *Cytogenetics and Cell Genetics* 58, 2124.
17. Bonneau, M., Boscher, J., Delate, J. J., Legault, C. and Popescu, C. P., 1991. Deux nouvelles translocations réciproques impliquant le chromosome 6 chez le porc domestique. *Ann. Génét.* 2, 65-69.
18. Buitkamp, J., Ammer, H. and Geldermann, H., 1991. DNA fingerprinting in domestic animals. *Electrophoresis* 12, 169-174.
19. Chaudhary, R., 1992. Physical gene mapping in pigs. Localization of the genes for PGD, ALB, TF, CP and CS using in situ hybridization. *Master of Science thesis. Publication No 99, Uppsala, 1992.*
20. Chowdhary, B. P., 1991. Chromosome mapping of some genes in farm animals. *Dissertation, Uppsala 1991.*
21. Coppieters, W., van de Weghe, A., Peelman, L. and Depicker, A., 1991. Probes for multiallelic loci from a pig library enriched for variable number of tandem repeats (VNTR). *Arch. Internat. de Physiologie de Biologie et de Biophysique* 99, 352.
22. Coppieters, W., van de Weghe, A., Peelman, L., Depicker, A., van Zeveren, A. and Bouquet, Y., 1991. Ststematic cloning of dinucleotide repeats and their use as polymorphic markers for the pig genome. *Arch. Internat. de Physiologie de Biologie et de Biophysique* 100, B36.
23. Gellin, J. and Grosclaude, F., 1991. Analyse du génome des espèces d'élevage: projet d'établissement de la carte génétique du porc et des bovins. *Prod. Anim. INRA* 4, 97-105.
24. Haley, C. S., Archibald, A., Tucker, E, M. and Miller, J. R., 1991. PiGMaP - a pan-European study to map the pig genome. In: AFRC News, July 1991. Agriculture and Food Research Council, Swindon. pp 4-6
25. Haley, C. S., Knott, S. A. and Thompson, R., 1991. Mapping quantitative trait loci in farm animals. 42nd Annual Meeting of the European Association of Animal Production, Berlin, Vol 1, 81.
26. Larsen, N. J., Nielsen, V. H. and Høj, S., 1991. Detection of a point mutation in the porcine growth hormone gene using polymerase chain reaction and restriction fragment length polymorphism. *Animal Genetics* 22, Suppl. 1, 70-71.
27. Mariani, P., Johansson, M., Ellegren, H. and Andersson, L., 1991. A *Taq*I RFLP at the porcine platelet-derived growth factor β-receptor locus (*PDGFRB*). *Animal Genetics* 22, 446.
28. Mariani, P., Johansson, M., Ellegren, H. and Andersson, L., 1991. *Taq*I and *Pvu*II RFLPs at the porcine plasminogen activator, urokinase locus (PLAU). *Animal Genetics* 22, 447.
29. Mellink, C. H. M., Bosma, A. A., de Haan, N. A. and Wiegant, J., 1991. Distribution of rRNA genes in breeds of domestic pig studied by non-radioactive in situ hybridization and selective silver-staining. *Génétique, Sélection Évolution* 23, suppl. 1, 168s-172s.
30. Miller, J. R. and Thomsen, P. D., 1991. A centromere-specific tandemly repeated DNA sequence from the domestic pig. Human Gene Mapping 11 (1991). *Cytogenetics and Cell Genetics* 58, 2130.
31. Miller, J. R., Miller, N. G. A. and Tucker, E. M., 1991. Construction of a chromosome 1-specific library from the domestic pig by universal amplification of FACS purified material. Human Gene Mapping 11 (1991). *Cytogenetics and Cell Genetics* 58, 2129-2130.
32. Müller, E., Wilke, K., Moser, G. and Geldermann, H., 1991. Mapping of quantitative trait loci by means of marker genes in F2 generations of wild, Pietrain and Meishan pigs. 42nd Annual Meeting of the European Association of Animal Production, Berlin, Vol 1, 216-217.
33. Nielsen, V. H. and Larsen, N. J., 1991. Restriction fragment length polymorphisms at the growth hormone gene in pigs. *Animal Genetics* 22, 291-294.
34. Ollivier, L., Gellin, J., Popescu, P. and Vaiman, M., 1992. La cartographie du génome porcin: une aide à la sélection. *J. Rech. Porcine en France* 24, 1-8.
35. Peelman, L., van de Weghe, A., Coppieters, W., van Zeveren, A. and Bouquet, Y., 1991. Cloning and sequencing of the porcine complement factor B. *Immunogenetics* 34, 192-195.
36. Poulsen, P. H., Thomsen, P. D. and Olsen, J., 1991. Assignment of the porcine aminopeptidase N (*PEPN*) gene to chromosome 7cen-q21. *Cytogenetics and Cell Genetics* 57, 44-46.
37. Winterø, A. K. and Fredholm, M., 1991. Towards construction of a high resolution map of the porcine genome using evolutionary conserved sequences. Human Gene Mapping 11 (1991). *Cytogenetics and Cell Genetics* 58, 2132.
38. Winterø, A. K., Fredholm, M. and Thomsen, P. D., 1992. Variable $(dG\text{-}dT)_n$- $(dC\text{-}dA)_n$ sequences in the porcine genome. *Genomics* 12, 281-288.
39. Yerle, M., Dalens, M., Galman, O., Lahbib-Mansais, Y., Archibald, A. L., and Gellin, J., 1991. Localization on pig chromosome 6 of five markers: GPI, APOE, TGFβ1, ENO1 and PGD, carried by human chromosomes 1 and 19, using *in situ* hybridization. *Animal Genetics* 22, Suppl. 1, 81.
40. Yerle, M., Galman, O. and Echard, G., 1991. The high resolution GTG-banding pattern of pig chromosomes. *Cytogenetics and Cell Genetics* 56, 45-47.
41. Yerle, M., Galman, O., Lahbib-Mansais, Y. and Gellin, J., 1992. Localisation of the pig luteinizing hormone/choriogonadotropin receptor gene (LHCGR) by radioactive and non-radioactive *in situ* hybridization. *Cytogenetics and Cell Genetics* 59, 48-51.

TITLE :

A STUDY OF FISH GENES AND THE REGULATION OF THEIR EXPRESSION

CONTRACT NUMBER :

BIOT - 0188 - C (EDB)

OFFICIAL STARTING DATE :

01 January 1991

COORDINATOR :

Prof. F. Ollevier, KU Leuven, Leuven Be

PARTICIPANTS :

Dr. A. Belayew, Univ. de Liège, Liège Be

Dr. C. Lecomte, Eurogentec S.A., Liège Be

Prof. L. Colombo, Univ. di Padova, Padova It

Dr. M. Carillo, C.S.I.C., Ribera de Cabanes Es

Mme. A. Brunel, Sepia International, Paris Fr

Dr. D. Chourrout, INRA, Jouy-en-Josas Fr

Prof. M. Schartl, Univ. Würzburg, Würzburg D

Prof. F. Gannon, Univ. College Galway, Galway Ei

Prof. L. Pilström, Univ. of Uppsala, Uppsala Sw

OBJECTIVES SET FOR THE REPORTING PERIOD :

The initial objectives set for the reporting period put special emphasis on the smooth integration of the participating groups, the introduction of new concepts and techniques, and the development of basic research tools. Starting up the project included a major effort to stimulate contacts and exchanges.
More specifically the following objectives were set : (1) ploidy manipulation (gynogenesis and triploidy) of seabass and African catfish. Cloning of fish is an ideal tool to standardise assay conditions. (2) Cloning of several genes from cDNA banks and genes with their promoters from gDNA banks (*PRL*, *Xmrk*, *MT*, *GH*, *Insulin*, *Ig*, smoltification genes) of tilapia, swordtail, seabass, rainbow trout, Atlantic cod and Atlantic salmon, and a nucleocapsid gene of the virus VHS. These genes are the focus of gene regulation studies. (3) The *in vitro* testing of selected genes (*PRL*,

Xmrk, *Ig*), including the selection of appropriate reporter genes. (4) The establishment of a small aquarium fish species model. (5) The *in vivo* testing of selected genes (*Ig*, *Xmrk*), including the optimisation of techniques for the testing of genes in embryos. (6) The adaptation of seabass to controlled conditions.

MAJOR PROBLEMS ENCOUNTERED :

In general, the groups participating in the project have not run in major problems. Progress is substantial and the satisfaction with the research effort seems to be good. Problems vary from administrative bottlenecks (funding did not arrive in time because of an administrative dispute within a university; the absence of clear administrative precedents) over practical problems (moving from one institute to another one; lack of sufficiently qualified personnel; insufficient funding to strongly reorient research interests) to scientific problems (incomplete gene library, difficult husbandry of fish, delayed progress with the biological characterisation of fish).

RESULTS :

The growing integration among BRIDGE members makes it very interesting to summarise the results.
The University of Leuven has tested 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 expression has been observed within days after fertilisation. Ploidy manipulation of African catfish (gynogenesis) resulted in the identification of the critical periods for heat, cold and pressure shocking and the verification by means of DNA fingerprinting.
The Univ. de Liège cloned and characterised successfully the *tiPRL-1* gene. Four reporter genes (*Amp*^{R}, *Luc*, *Gal* and *CAT*) coupled either to the CMV promoter or to the promoterless construct were tested in EPC cells grown *in vitro*. This resulted in the choice of the LUC reporter gene which was recommended to all BRIDGE members.
Eurogentec provided help with the cell culture and initial electroporation experiments.
The group at the Univ. di Padova has spliced chimaeric constructs of *rtGH* and *rtInsulin*. Pituitary protein interaction with the rtGH gene promoter has been studied *in vitro* in collaboration with the Liège group. The highly conservative nature through evolution of the *Pit-1* site has been invoked. Seabass GH cDNA has been cloned, the nucleotide sequence determined and the protein expressed. Collaboration with Sepia International (France) on the supply of seabass eggs is in progress.
The C.S.I.C. has conditioned seabass for spawning (photo- and hormonal induction). The optimal conditions for ploidy manipulation of seabass (gynogenesis) through heat shocks were identified. Verification of the ploidy level occurs with flow cytometry. Finally, the sex of seabass was influenced chemically.
I.N.R.A. has defined ameliorated conditions for *in vitro* and *in vivo* tranformation. Several fusion genes (RSV, SV40, and CMV promoters; *GH* and *CAT* genes) were assayed for their potential for *in vitro* expression. The same strategy was followed for *in vivo* expression (CMV and RSV promoters; *CAT*, *GH*, *Xmrk* and *Ig* genes). Finally the nucleocapsid protein gene from the VHS virus has been characterised extensively; strong promoters of the OMV Herpes virus are being screened.
The Univ. Würzburg has started using the medaka fish system to analyse the function and regulation of certain proto-oncogenes and oncogenes during vertebrate development. They showed that the high rate of tumor induction in *Xmrk*-injected medaka embryos is dependent on the presence of an inducing ligand. The *Xmrk*

proto-oncogene has been cloned.
The Univ. College Galway has cloned three genes which are differentially expressed during smoltification. Seven other genes expressed at high levels in salmon liver were isolated; six were identified. The *α globin* promoter has been successfully cloned; preliminary steps have been taken to clone other salmon promoters.
The Univ. of Uppsala has collaborated with I.N.R.A. to test a gene construct of the murine immunoglobulin promoter/enhancer for expression *in vivo*. Enhanced expression of the fusion gene has been detected in white blood cells. Gene libraries have been constructed from lymphoid tissue and from germline DNA of Atlantic cod and rainbow trout; these fish *Ig* genes have been isolated and sequenced.

HIGHLIGHTS/MILESTONES :

Highlights include :
- the first proof of a phenotypic effect of the *Xmrk* oncogene in fish.
- the potential conservation through evolution of a "*Pit-1*-like" protein and binding site on the prolactin gene.
- the evolutionary significance of several fish genes studied.
- the tissue-specific expression of a murine immunoglobulin promoter/enhancer in fish.
- a reliable method for the spawning, fertilisation and sex manipulation of seabass.
- the steady convergence of fish gene research towards one principal model fish.

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 battery of genes, the expression of these genes is highly specific. For example, the hormone prolactin is produced in the pituitary gland. 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 appeared in the field of cancer research and mid term applications are expected in the fields of disease control and selection. Spin off applications include the domestication of seabass and African catfish.

COOPERATIVE ACTIVITIES :

Two meetings were organised; a first contact among all members occured at a successful meeting in Leuven (Belgium) (11-12 April 1991, organised by Prof. F. Ollevier). At a second well-attended meeting in Padova (Italy) (5-6 December 1991, organised by Prof. L. Colombo) the latest research progress was presented. It was decided to compile a data bank on model fish, fish cell lines, gene banks and cloned genes available to the project. Meanwhile numerous informal contacts were made by the coordinator and among members. Short term staff exchanges occurred among I.N.R.A. and Univ. Würzburg, Univ. de Liège and Univ. Leuven; long term

exchanges occurred among I.N.R.A. and Univ. of Uppsala, Univ. di Padova and Univ. de Liège. Exchange of scientific material and consultation on technical aspects occurred among all partners (exchange of reporter genes LUC and ßGal, of medaka fish, of seabass eggs, of gene sequences, of specific gene constructs and of cell lines). Results were presented at the following international meetings : International Marine Biotechnology Symposium (Baltimore, USA), Biotechnology of Reproduction in Aqautic Animals (Toba, Japan), Research for Aquaculture (Antibes, France) and Scandinavian Meeting of Fish Immunology (Tromsö, Norway). Moreover, spinoff research initiated by the BRIDGE project includes DNA fingerprinting in fish, genotyping of fish with sex specific DNA probes and general fish husbandry procedures.

LIST OF JOINT PUBLICATIONS/PATENTS WITH TRANS-NATIONAL AUTHORSHIP :

Anonymous (1991) European Laboratory Without Walls in the field of "fish genes and the regulation of their expression". C.E.C. DG XII 7pp.

Bearzotti, M., E. Perrot, C. Michard-Vanhée, G. Joliver, J. Attal, M.C. Théron, C. Puissant, H. Grabowski, M. Dréano, J.J. Kopchick, R. Powell, F. Gannon, L.M. Houdebine and D. Chourrout (in press) The expression of various gene constructs transfected into fish cells. J. Biotech.

Michard-Vanhée, C., D. Chourrout, A. Thuvander and L. Pilström (submitted) Immunoglobulin promoter/enhancer from mouse induce a tissue-specific expression in transgenic rainbow trout (*Oncorhynchus mykiss* Walbaum). Partly presented at Int. Mar. Biotechn. Conf., Baltimore, USA, October '91 and Scand. Meet. Fish Immunol., Tromsö, Norway, July 1991

Nakayama, I., M. Schartl, R. Tewari and D. Chourrout (in prep.) Isolation and characterisation of sex specific DNA sequences in a teleost fish. Partly presented at Int. Mar. Biotechn. Conf., Baltimore, USA, October '91

(underlined names represent transnational authors)

OTHER PUBLICATIONS :

Adam, D., W. Mäueler and M. Schartl (1991) Activation of the melanoma inducing *Xmrk* oncogene in Xiphorus. Oncogene 6 : 73-80

Bengtén, E., T. Leanderson and L. Pilström (1991) Immunoglobulin heavy chain cDNA from the teleost Atlantic cod (*Gadus morhua* L.) : nucleotide sequences of secretory and membrane form show an unusual splicing pattern. Eur.J.Immunol. 21 : 3027 - 3033

Byrnes, L. and F. Gannon (in press) Sequence analysis of a second cDNA for Atlantic salmon (*Salmo salar*) serum albumin. Gene

Chourrout, D. and E. Perrot (in press) No transgenic rainbow trout produced by fertilisation with sperm incubated with linear DNA. Mol.Mar.Biol.Biotech.

Daggfeldt, A., E. Bengtén and L. Pilström (in prep.) Polymorphic organisation of immunoglobulin light chain locus in Atlantic cod (*Gadus morhua* L.) and rainbow trout (*Oncorhynchus mykiss* Walbaum) as indicated by nucleotide sequences of cDNA and Southern blot analysis

Epplen, et al., incl. Schartl, M., (1992) : Oligonucleotide fingerprinting using simple repeat motifs : A convenient, ubiquitously applicable method to detect hypervariability for multiple purposes. In : Jeffreys, A.J. Burke, T. Wolfe J. Dolf, G., (eds), Birkhäuser Verlag, Bern, pp. 50-69

Förnzler, D., Wittbrodt, J. and M. Schartl (1991) Analysis of an esterase linked to a locus involved in the regulation of the melanoma oncogene and isolation of polymorphic marker sequences in Xiphorus. Biochemical Genetics 29 : 509-524

Götz, R. Rauil, F. and M. Schartl (in press) Brain-derived neurotrophic factor is more highly conserved in structure and function than nerve growth factor during vertebrate evolution. J. Neurochemistry

Hannig, G., S. Ottilie and M. Schartl (1991) Conservation of structure and expression of the *c-yes* and *fyn* genes in lower vertebrates. Oncogene 6 : 361-369

Hardiman G. and F. Gannon (1991) Differential screening of a salmon liver cDNA library to isolate mRNA sequences expressed

at a greater abundance in sea salmon. The Society for General Microbiology Winter Symposium. Galway, Ireland. Jan. 1991

Hernandez Bétancourt, O., J. Attal, M. Bearzotti, M.C. Théron, C. Puissant and L.M. Houdebine (submitted) Introns in mammalian genes may not be processed efficiently in fish cells. Gene

Hong, Y. Winkler, C. Brem G. and M. Schartl (1992) : Development of a heavy metal-inducible fish specific expression vector for gene transfer in vitro and in vivo. Proc. Int. Aquaculture Congress, (in press)

Houdebine, L.M. and D. Chourrout (1992) Transgenesis in fish. Experientia 47 : 891-897

Lee, M.A., Rytting, A.-S. and L. Pilström (manuscript) The genes for the receptor and secreted forms of immunoglobulin heavy chain from rainbow trout (*Oncorhynchus mykiss* Walbaum)

Malitschek, B. and Schartl, M., (1991) : Rapid identification of recombinant baculovirus using polymerase chain reaction. Biotechnology 11 : 3-4

Nanda, I. Schartl, M. Feichtinger W. Epplen, J.T. Schmid, M., (1992) Early stages of sex chromosome differentiation in fish as analyzed by simple repetitive DNA sequences. Chromosoma (in press)

Ottilie, S., Raulf, F. Barnekow, A. Hannig, G. and M. Schartl (in press) Multiple *src*-related genes, *srk-4*, in the freshwater sponge *Spongilla lacustris*. Oncogene

Schartl, M., Schlupp, I. Schartl, A. Meyer, M. Nanda I., Schmid M., Epplen J. and Parzefall, J. (1991) On the stability of dispensable constituents of the eukaryotic genome. Stability of coding sequences versus truly hypervariable sequences in a clonal vertebrate, in the amazon molly, *Poecilia formosa*. Proc. Natl. Acad. Sci. USA 88 : 8759-8763

Schartl, M. Erbelding-Denk, C. Nanda, I. Schmid, M. Schröder, J.H. Epplen, J.T. (1991) Mating success of subordinate males in a poeciliid fish species, *Limia perugiae*. Fingerprint News 3 : 16-18

Schartl, M. Wittbrodt, J. Adam, D. Mäueler, W. Raulf, F. Hannig, G. Telling, A. Storch, F. Andexinger, S. Robertson, S.P. (1991) Oncogenes and melanoma formation in Xiphoporus (Teleostei : Poeciliidae). In : Schröder, J.H. Bauer, J. Schartl, M. (eds), New Trends in Ichthyology, Parey Verlag

Schartl, M. and D. Adam (1992) Molecular cloning, structural characterization, and analysis of transcription of the melanoma oncogene of Xiphorus. Pigment Cell (in press)

Schlupp, I., Parzefall, J. and M. Schartl (1991) Male mate choice in mixed bisexual/unisexual breeding complexes of Poecilia (Pisces: Poeciliidae) : Male choice thwarted by female attractiveness. Ethology 88 : 215-222

Schlupp, I. Parzefall, J. Epplen, J.T. Nandan, I. Schmid, M. and M. Schartl (submitted) Spontaneous masculinization and pseudomale behavior in the all-female teleost *Poecilia formosa* (Girard, 1859), (Teleostei: Poeciliidae)

Schröder, J.H. Bauer, J. and M. Schartl (1991) New Trends in Ichthyology. Parey Verlag

Swennen, D., B. Sekkali, A.C. Poncelet, F. Rentier-Delrue, J.A. Martial and A. Belayew (in press) Molecular biology of tilapia prolactins. In : Coastal and estuarine studies. New trends in basic and applied research in aquaculture, B. Lahlou (ed.), Springer Verlag, Heidelberg.

Swennen, D., A.C. Poncelet, B. Sekkali, F. Rentier-Delrue, J.A. Martial and A. Belayew (submitted) Structure of the tilapia (*Oreochromis mossambicus*) prolactin I gene. DNA

Tewari, R., C. Michard-Vanhée, E. Perrot and D. Chourrout (submitted) Mendelian transmission, structure and expression of transgenes following their injection into the cytoplasm of trout eggs. Transg. Res.

Volckaert, F., B. Hellemans, A. Belayew, E. Daemen and F. Ollevier (1991) Gene transfer and expression of the *CMV-β Gal* fusion gene in embryos of the African catfish (*Clarias gariepinus*) and the zebrafish (*Brachydanio rerio*). Int. Meeting "Research for Aquaculture", Antibes France, 8-10.10.91, p. 209

Volckaert, F., B. Hellemans, E. Daemen, F. Ollevier and A. Belayew (1991) Gene transfer and expression of the *CMV-β Gal* fusion gene in embryos of the African catfish (*Clarias gariepinus*) and the zebrafish (*Brachydanio rerio*). Int. Mar. Biotechn. Conf., Baltimore, USA, 13-16.10.91, p. 91

Wagner, A. Deryckere, F., Hardiman, G., Byrnes, L. and F. Gannon (submitted) The isolation and structure of liver and globin genes from Atlantic salmon

Wagner, A. and F. Gannon (in preparation) Genomic organisation of salmon globin genes

Winkler, C., J.R. Vielkind and M. Schartl (1991) Transient expression of foreign DNA during embryonic and larval development of the Medaka fish (*Oryzias latipes*). Mol. Gen. Genetics 226 : 129-140

Winkler, C. Hong, Y. Wittbrodt, J. and M. Schartl (submitted) Analysis of heterologous and homologous promoters and enhancers in vitro and in vivo by gene transfer into Japanese Medaka (*Oryzias latipes*) and Xiphophorus.

Wittbrodt, J. Lammers, R. Malitschek, B. Ullrich, A. and M. Schartl (submitted) The melanoma-inducing oncogene *Xmrk* of Xiphorus encodes a functional receptor tyrosine kinase that is highly active in malignant melanoma

TITLE:

Study of the avian herpesvirus genome Marek's disease virus

CONTRACT NUMBER:

BIOT CT90-0173

OFFICIAL STARTING DATE:

1.8.91

COORDINATOR:

Dr. L.J.N. Ross, AFRC IAH, Houghton Laboratory, Houghton, UK

PARTICIPANTS:

Mrs. A. Rey-Senelonge, IFFA Laboratory, Rhône- Mérieux, Lyon, F
Prof. E.F. Kaleta, Justus Liebig Universitat, Giessen, FRG

OBJECTIVES:

The objectives for the first 7 months were (1) To identify by partial sequencing the Marek's disease virus (MDV) homologues of gH, gD of herpes simplex virus (HSV). (2) To prepare probes to identify homologues of gD, gH and non-essential genes of herpesvirus of turkeys (HVT). (3) To prepare HVT DNA for construction of vectors, cloning and partial sequencing of non-essential genes.

MAJOR PROBLEMS ENCOUNTERED:

None

RESULTS:

Cosmid libraries of overlapping DNA fragments spanning the entire genome of MDV and HVT have been prepared. Several genes of MDV and HVT have been identified by random sequencing of selected regions of genomic DNA and comparison of the deduced proteins to the amino acid sequence of known herpesvirus proteins. The genes identified include the MDV homologues of gH, gD, protein kinase (PK) and gI of HSV and the HVT homologues of gB, gD, PK and gI of HSV. Sequencing of MDV gH and of a 3.7 kbp fragment mapping in the unique short region (U_s) of HVT which comprises PK, gD and gI is in progress.

The potential of using the thymidine kinase (TK) gene of HVT as an insertion site for expressing foreign genes has been investigated. A 3.9 kbp fragment of MDV DNA

containing the gB gene and 5' non-coding sequences has been inserted into the TK gene using a synthetic oligonucleotide adaptor containing an EcoRV cloning site. Expression of authentic MDV gB was demonstrated *in vitro* using an MDV-specific anti-peptide serum. The HVT recombinant was genetically stable *in vitro* and *in vivo* as shown by Southern blot analysis of viral DNA recovered from infected cells. Work is in progress to study the replication of the recombinant *in vivo* and its efficacy as a vaccine.

Rhône-Mérieux laboratory has cloned and sequenced the HVT homologue of the ribonucleotide reductase (RR) gene of HSV and has investigated its potential as an insertion site for expressing foreign genes using the marker gene beta-galactosidase.

Houghton and Rhône-Mérieux laboratories have worked in close contact and have exchanged cloned materials and information on transfection techniques and the generation of virus recombinants. According to plan, Houghton has concentrated on MDV glycoprotein genes and HVT TK and PK genes whilst Rhône-Mérieux has worked on the RR gene of HVT. It is envisaged that work at Giessen University will involve an investigation of the safety and efficacy of the recombinant vaccines generated as soon as they are available.

HIGHLIGHTS/MILESTONES:

MDV and HVT homologues of gH, gD, PK, gI genes of HSV have been identified. The RR gene of HVT has been sequenced. It has been demonstrated that HVT can tolerate the insertion of a 3.9 kbp MDV DNA at the TK locus and that authentic MDV gB is synthesised by the recombinant under the control of the MDV gB promoter.

WIDER CONSIDERATIONS:

Much progress has been made in identifying genes of MDV and HVT by comparison of the predicted amino acid sequence of random DNA fragments to known herpesvirus proteins. The results have shown that HVT, a vaccine widely used in chickens, has potential as a vector and that it can accommodate at least 3.9 kbp of foreign DNA. The ability of the recombinants to replicate *in vivo* remains to be determined and the search for insertion sites compatible with maximum replication *in vivo* is continuing.

COOPERATIVE ACTIVITIES:

Houghton and Rhône-Mérieux laboratories have exchanged cloned DNAs, immunological reagents and information on production of infectious DNA and recombinant virus. A meeting between the two laboratories took place at IAH, Compton Laboratory on 5th November, 1991 to discuss progress and decide on priorities for the following 6 months. Exchange of information takes place regularly by telephone.

LIST OF JOINT PUBLICATIONS/PATENTS:

OTHER PUBLICATIONS/PATENTS:

Ross, L.J.N., Binns, M.M and Pastorek, J. (1991). DNA sequence and organization of genes in a 5.5 Kbp EcoRI fragment mapping in the short unique segment of Marek's disease virus (strain RB1B). J.Gen.Virol. 72, 949-954

Ross, L.J.N. and Binns, M.M. (1991). Properties and evolutionary relationships of the Marek's disease virus homologues of protein kinase, glycoprotein D and glycoprotein I of herpes simplex virus. J. Gen. Virol. 72, 939-947

Ross, L.J.N., Binns, M.M., Pastorek, J. and Scott, S. (1991). Construction and properties of a herpesvirus of turkeys recombinant which expresses glycoprotein B of Marek's disease virus (part of this work was presented at the International Herpesvirus Workshop, Asilomar, California, July 1991.

TITLE: "Development of second generation vaccines against parvoviruses"

CONTRACT NUMBER: BIOT - CT91-0256

OFFICIAL STARTING DATE: 1.February.1991

COORDINATOR: José Ignacio Casal. INGENASA. Madrid. ES.

PARTICIPANTS: Rob Meloen. Central Veterinary Institute. Lelystad. NL.
Kristian Dalsgaard. State Veterinary Institute for Virus Research. Lindholm. DK.

OBJECTIVES SET FOR THE REPORTING PERIOD: Production of PPV antigens. Production and maintenance of specific monoclonal antibodies. Recombinant polypeptide expression. Optimization of the recombinant protein purification. Field trials. Fine mapping of PPV.

MAJOR PROBLEMS ENCOUNTERED: Nothing to report

RESULTS: Porcine parvovirus (PPV) is one of the major causes of reproductive failure in pigs. The control of the disease is usually carried out with conventional vaccines made of inactivated viruses.

In order to develop a more cost effective (for PPV) and safer (in the case of canine parvovirus [CPV]) subunit vaccine, the first strategy was the identification of the proteins containing the key epitopes involved in neutralization. From previous studies made with CPV, we know that the neutralizing MAbs reacted simultaneously with VP1 and VP2. This means that VP2 is the protein which, contains the neutralizing epitopes of parvovirus.

Ingenasa's group was the first in reporting the synthesis and sequencing of an infectious clone containing the complete genome of PPV. Using this clone as starting material we isolated the VP2 gene. Before the initiation of this BRIDGE project we cloned this gene and several fragments in different *E.coli* expression systems. The products obtained were immunologically poor, probably due to an unproper folding of the expressed polypeptides. To overcome these shortcomings, we changed to the baculovirus expression system. The VP2 gene was cloned into the baculovirus transfer vector pJVP10Z, under control of the polyhedrin promoter, we use this vector where the *lacZ* gene is expressed under control of the p10 promoter of baculovirus. The resulting vector containing the PPV VP2 gene was named pPPVEx8. After transfection of *Spodoptera frugiperda* cells with pPPVEx8 and wild-type AcNPV DNA, recombinant viruses were easily detected by the expression

of ß-galactosidase. Most of the work realized during the past 12 months was dedicated to the characterization of the product obtained by the baculovirus expression. Other aspects covered this year were related to the production of PPV antigens and the production and maintenance of specific monoclonal antibodies. Finally, we have started a detailed mapping study by PEPSCAN.

Production of PPV antigens (SVIV)

PPV virus has been grown to high titre in cell cultures of primary pig kidney cells. The virus has been inactivated by binary ethylene imine, and the product distributed to the participants: for the production of PPV-specific monoclonal antibodies at SVIV and INGENASA, for the production of rabbit polyclonal antisera and, finally, as a reference for morphological studies by electron microscopy.

Production and maintenance of specific monoclonal antibodies (INGENASA, SVIV)

Production of hybrid cell secreting monoclonal antibodies specific for PPV was performed according to well know procedures, using spleen cells from PPV immunized mice. After, at least, three cloning steps, hybridoma supernatants were screened by ELISA and IHA assay. The MAbs isotype was determined by immunodifusion with antisera specific for murine antibodies heavy and light chain.

The general properties of PPV-specific MAbs obtained at Ingenasa are shown in the Table. Four out of six MAbs are able to inhibit virus haemagglutination, all of them are useful for antigenic studies of PPV virions and recombinant proteins by immunoelectromicroscopy, immunobloting and for Pepscan studies.

		TITRE		BIOLOGICAL ACTIVITY		
HYBRIDOMA	ISOTYPE[a]	SUPERNATANT	Ig(1mg/ml)	SPECIFICITY[b]	HA INHIBITION	NEUTRALIZATION[c] PPV
13C4	IgG1	3125	10^6	-	+	+
13C6	IgG1	15625	10^6	-	+	+
15C5	IgG1	15625	10^6	-	+	+
15G4	IgG1	15625	10^6	-	+	+
11D1	IgG2a	3125	10^5	VP1, VP2	-	-
17G9	IgG1	125	10^3	VP1, VP2	-	-

A panel of 16 monoclonal antibodies against porcine parvovirus were produced at SVIV and have been purified in suitable amounts for further analysis. These monoclonals have been tested in a number of assays against PPV, including ELISA, virus neutralization, staining techniques, immunoelectrophoresis, western blot, dot blot and an indirect affinity determination assay. The affinity assay used has revealed that the conformational changes induced in infective virus *vs.* empty virus particles by the chaotropic ion isothiocyanate, make it possible to discriminate between the two. This assay may help in characterizing the recombinant virus-like particles.

The antibodies are currently being tested for their ability to specifically introduce gold labels onto the surface of PPV particles; this is a very powerful technique for electron microscopy. A number of the monoclonals have already proved useful in this respect, and it is anticipated that the technique will contribute considerably to the mapping of surface epitopes on the virus and the recombinant capsids produced in the baculovirus system.

Recombinant polypeptide expression (INGENASA)

Proteins were isolated from uninfected and recombinant AcNPV-infected Sf9 cells at 72 h post-infection and analyzed in SDS-polyacrylamide gels. Coomassie-blue staining of the gels showed the presence of a protein of 64 KDa, the expected molecular weight of the PPV VP2, in the recombinant viruses. Identity of this protein as VP2 was confirmed in immunoblot analysis. Recombinant virus also expressed ß-galactosidase. This protein was identified by immunoblot using a specific rabbit polyclonal serum.

Also, cells infected with recombinant virus were fixed and subjected to indirect immunofluorescence. Infected cells gave a clear positive signal characterized by the presence of diffuse fluorescence in the cytoplasm of the cells, not in the nucleus. Uninfected or wild type infected cells yielded a negative signal.

Optimization of the recombinant protein purification (INGENASA)

The expressed VP2 was found to be present in the cytoplasmic extract of *S.frugiperda* cells infected with AcPPV8. This fraction was treated with DNAse and RNAse to remove nucleic acids. Subsequent precipitation with 20% ammonium sulphate gave a protein fraction that contained mostly VP2 and ß-gal. To confirm the identity of the purified proteins, aliquots of this material were analyzed by western blot using rabbit anti-PPV antisera. A 64 K band of both crude and purified samples reacted strongly with anti-PPV antisera. Further purification, to selectively remove the ß-gal contamination, was done by affinity chromatography, using anti-ß-gal IgG conjugated to Sepharose. The eluted material gave a single, major band corresponding to 64 K on SDS-PAGE, with only very little contamination of ß-gal.

Electron micrographs of purified preparations of VP2 showed a large number of virus-like particles (VLPs), with a morphology very similar to the original virus PPV (Fig. 1). The diameter of the particles was estimated to be in the order of 20 nm, i.e., comparable to those of PPV. The particles were layered on self-forming CsCl gradients, banding at a density of 1.33 g/cm^3. This density is similar to that corresponding to empty viral capsids (1.33) and lower than full DNA-containing viral capsids (1.38). The estimated amount of VP2 particles produced was between 5-10 $mg/10^9$ cells, based on the comparison with standard amounts of a reference protein included in the polyacrylamide gels.

Methods for quantification of the recombinant VP2 capsids have been established, i.e. ELISA, HA and rocket electrophoresis. These particles exhibited the same or higher hemagglutinating titers (up to 10^6 UHA/ml) as observed with authentic virus. The hemagglutination titer of these VP2 preparations is a good indication of the amount of particles with respect to soluble protein. Another method to characterize the amount of particles is the use of a quantitative immunoelectrophoresis in agarose gel, rocket electrophoresis. This technique is especially useful for quantification, because it only measures the amount of VP2 present as actual particles. When these results are related to the ELISA and HA values, estimates of the degree of assembly of the recombinant products can be calculated. In this way, it was possible to standardize the antigenic mass of VLPs and adjust the amount of antigen in a pig vaccine dose to the same amount as usually included in a conventional inactivated vaccine (approximately 3 μg of virus).

Animal experiments (SVIV, INGENASA)

As in a standard potency test for the commercial vaccine, two pigs were immunized subcutaneously twice with three weeks interval, the second dose being half the volume of the initial dose (2 ml - 1 ml). In the preliminary experiments the vaccine has been formulated with the adjuvant QuilA and aluminium hydroxide. Serum samples were taken prior to and 10 days after the second vaccination. Serum antibodies against PPV were assayed by three different methods: 1. Anti PPV-virion ELISA test. 2. Hemagglutination inhibition. 3. Neutralization of PPV. All antibody titres obtained by the recombinant VLP vaccine (data not shown) were of the same magnitude as usually obtained by the commercial inactivated vaccine (Fig. 2), indicating that VLPs are highly immunogenic and may replace inactivated virions. Despite one pig had residual maternal antibody, the response to the vaccine was not inhibited and reached similar levels to that from the seronegative pig.

Fine mapping of parvovirus epitopes with PEPSCAN (CDI, INGENASA, SVIV)

These experiments were performed by using antibody samples (monoclonals from INGENASA, anti-CPV antisera from both INGENASA and SVIV, sera from infected dogs) directed against canine parvovirus in a PEPSCAN-ELISA on 740 overlapping nonapeptides covering the whole sequence of the 748 residues long capsid protein VP1 of CPV immobilized to polyethylene rods. Anti-PPV antisera and monoclonals are now under investigation in a PEPSCAN with overlapping dodecapeptides.

The PEPSCAN has been successfully (and currently) employed for the delineation of sequential epitopes on the capsid protein of parvovirus. The efforts have already resulted in the delineation of ten sites in canine parvovirus of which some are at strategic locations around potential receptor binding sites on the viral surface. The surface location of six of these epitopes was shown in PEPSCAN in

adsorption experiments with whole virions. Furthermore, in these experiments neutralizing capacity was lost in antisera from infected animals showing the significance of a few select sequences in the VP2 protein. The results of this work are corroborated and emphasized by detailed structural information on the canine parvovirus which came available during the report period (Tsa et al., 1991, Science 251: 1456-1464). Based on these data on epitopes, homology and capsid structure, peptides have been selected and synthesized, which could represent neutralizing epitopes for both porcine and canine parvovirus. Rabbits and mice are now under investigation for eliciting antibodies to these peptides to find candidates for neutralization and protection studies.

HIGHLIGHTS/MILESTONES: The major achievement up to now has been the production of porcine parvo-like capsids, similar to the original virus. These particles have shown a high immunogenic activity, eliciting an immune response in pigs indistinguishable from that obtained with a commercial inactivated vaccine. This is a promising feature for the commercial application of these particles as a vaccine. The baculovirus/insect cell line system is already in its present for capable of producing attractive amounts of antigen at a low price. Another interesting point is the efficacy of this putative subunit vaccine when combined with standard adjuvants, such as alumina and QuilA, probably due to the high immunogenicity of the capsid structure.

WIDER CONSIDERATIONS: Due to the rather simple structure of the parvoviruses, they constitute a good model for the preparation of recombinant subunit vaccines. So far, procaryotic (*E.coli*) expression has failed to give useful products. It is therefore of major importance that the eucaryotic insect cell/baculovirus expression system has been able to produce parvovirus VP2 protein in a conformation which seems to be indistinguishable from native capsids and carrying all immunodominant epitopes. It will facilitate the development of large scale fermentor techniques leading to a more cost effective and nucleic acid-free 2nd generation parvovirus vaccines in a foreseable future. This vaccines will be extremely safe, innocuous for the animal and devoid of any residual infectivity. They should constitute the technologically improved alternative to the "classical" vaccines.

COOPERATIVE ACTIVITIES: During the last year there has been three meetings of the research teams in Madrid, Lindholm and Lelystad (in February, August and December, respectively). There has been a continuous flow of information between the three laboratories with exchange of virus, particles, monoclonal antibodies and different polyclonal antisera from dog, pig and rabbit.

LIST OF JOINT PUBLICATIONS/PATENTS WITH TRANS-NATIONAL AUTHORS SHIP: C. Martínez, K. Dalsgaard, J.A. López de Turiso, E. Cortés, C. Vela and J.I. Casal. (1992). Production of porcine parvovirus empty capsids with high immunogenic activity. Vaccine. In press.

C. Martínez, K. Dalsgaard, J.A. López de Turiso, E. Cortés, C. Vela y J.I. Casal. (1991). Procedimiento para la producción de una vacuna subunidad contra el Parvovirus Porcino. Patent Application # P9100845. Spain.

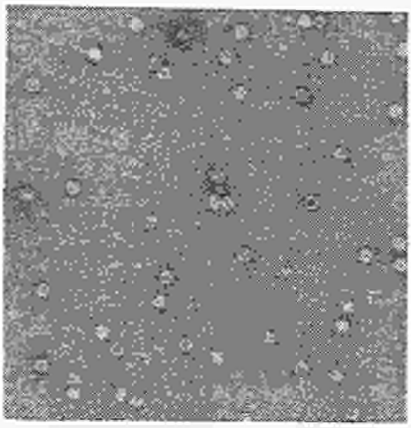


Fig. 1 - VP2 CAPSIDS (40,000 X)

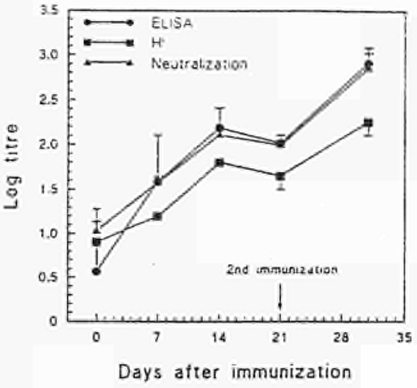


Fig. 2 - Pig Immunization

WP I. ACTIVIDAD E HITOS DE CONTROL DEL PROYECTO

INSTITUTION: INGENASA SVI - LINDHOLM CDI	BRIDGE PROGRAM	INITIAL DATE: Feb 91	DATE: March 92
	PROPOSAL: DEVELOPMENT OF SECOND GENERATION VACCINES AGAINST PARVOVIRUSES	FINAL DATE: Jan 94	REF:

MAN/MTHS ING / SVI / CDI	ACTIVITIES	INSTITUTIONS INVOLVED	1991 1 SEM	1991 2 SEM	1992 1 SEM	1992 2 SEM	1993 1 SEM	1993 2 SEM	1994 1 SEM	1994 2 SEM
- / 3 / 1	1. PRODUCTION OF PPV ANTIGENS	SVIV-LINDHOLM								
50 / 4 / -	2. PRODUCTION AND MAINTENANCE OF SPECIFIC MONOCLONAL ANTIBODIES	INGENASA SVIV-LINDHOLM								
30 / - / -	3. RECOMBINANT POLYPEPTIDE EXPRESSION (PROCARYOTIC AND EUKARIOTIC)	INGENASA								
18 / 6 / 41	4. FINE MAPPING OF PPV	INGENASA SVIV-LINDHOLM CDI								
17 / - / -	5. OPTIMIZATION OF THE RECOMBINANT PROTEIN PURIFICATION	INGENASA								
18 / - / -	6. SCALING-UP RECOMBINANT PROTEIN PRODUCTION BY FERMENTATION	INGENASA								
19 / 24 / 27	7. VACCINE PREPARATION OPTIMIZATION	INGENASA SVIV-LINDHOLM CDI								
14 / 17 / 8	8. FIELD TRIALS	INGENASA SVIV-LINDHOLM								
144 / 54 / 78										

▲ REVIEW DATES — PROGRESS STATUS

Title: ENGINEERING AND IMMUNOGENICITY OF FOOT-AND-MOUTH DISEASE VIRUS PROCAPSIDS IN INSECT CELLS

Contract number: BIOT-CT90-0190

Official starting date: 01/07/91

Coordinator: Dr GJ Belsham, AFRC IAH, Pirbright, GB

Participants: Dr J Vlak, Agricultural Univ., Wageningen, NL
Dr E Domingo, CSIC, Madrid, ES
Dr M Lombard, Rhone-Merieux, Lyon, FR

Objectives set for the reporting period:

a) construction of foot-and-mouth disease virus (FMDV) cDNA cassettes encoding the structural protein precursor P1-2A and the 3C protease which will produce the procapsid components 1AB, 1C and 1D. Preliminary characterization of the properties of the cassettes to ensure correct protein production and processing should also be undertaken.

b) insertion of cassettes into baculovirus transfer vectors and production of recombinant baculoviruses.

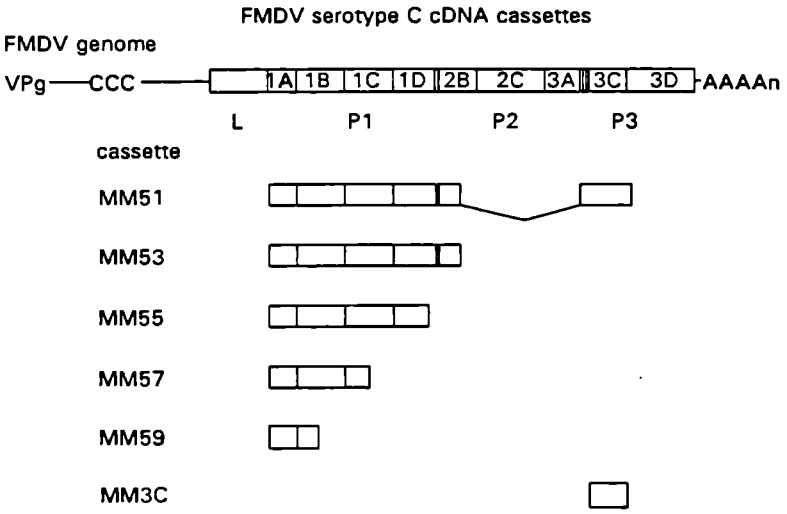
c) construction of deletion mutants for analysis of gene toxicity accounting for low level expression from previous constructs. Analysis of transcription and translation levels.
d) structural analysis of expressed procapsids.

Major problems encountered:

i) There appears to be some instability of some of the cDNA cassettes when introduced into baculovirus transfer vectors and recombined into the virus. This may be due to the inverted duplication of a short sequence specifying termination and polyadenylation signals. In new constructs this duplication has been avoided and no problems have been encountered in the construction of the recombinant transfer vectors.

Results:
a) cDNA cassettes for all three European serotypes A,O and C of FMDV have been synthesized in the form ATG-P1-2A+3C (see figure for the type C construct (pMM51) as an example) by workers in Pirbright and Madrid. Each of these cassettes has been shown to synthesize correctly processed 1AB, 1C and 1D when analysed in a transient expression system.

b) Each of these cassettes have been introduced into baculovirus transfer vectors and have now been transferred to Wageningen and transfected into insect cells to allow the isolation of recombinant viruses.



c) Other studies in Wageningen on the level of RNA transcripts being produced from the polyhedrin promoter in baculovirus/FMDV recombinants indicate that the rate of transcription is reduced by the presence of the 3C FMDV sequences. Translation of the P1-2A precursor in the absence of 3C is also low however. This suggests that the low level of FMDV protein production seen with existing baculovirus/FMDV recombinants is due to effect of 3C on mRNA transcription superimposed on a low level efficiency of translation. In order to analyse which section of the FMDV genome is adversely affecting the level of translation within baculovirus infected cells specific regions of the cassette are being deleted. The latest cassettes all lack the L protein. Further truncated versions of the L deleted cassette MM51 (see figure) have been isolated and are being introduced into transfer vectors for analysis. The 3C protease has also been constructed individually which may be useful for independent expression of the structural protein precursor and the protease. Constructs aimed at expressing reduced yields of 3C are also being produced in Pirbright.

d) Characterization of FMDV procapsid proteins expressed in mammalian cells using vaccinia virus vectors containing the type O and type A cassettes has been performed in Pirbright and indicates that assembly to particles sedimenting at 70S in sucrose gradients occurs (this material cosediments with empty capsid particles from FMDV infected cells). Furthermore empty 30nm particles have been observed when extracts have been examined using electron microscopy.

e) An aspect of work which was not anticipated has been the determination of the internal Ph of insect cells infected with baculovirus. This has been a highly cooperative expt performed in Madrid using a variety of materials supplied from Wageningen. The importance of this study is due to the lability of FMDV at low Ph. Measurements of the distribution of radiolabelled markers have shown that the internal Ph of uninfected Spodoptera frugiperda cells is maintained close to pH7 when the external Ph is varied over the range of 6.25 (standard conditions) to 6.8. Furthermore within baculovirus infected cells the Ph appears to be very similar to that found within uninfected cells. Thus it seems that the internal Ph of insect cells should not preclude the efficient assembly of FMDV empty particles.

f) new transfer vectors allowing the ready production of baculovirus recombinants containing foreign genes at two separate sites have been produced in Wageningen.

Highlights/milestones:
1) Production within cells of FMDV empty capsid particles visible by electron microscopy and biochemical analyses from self processing precursors expressed from cDNA.
2) The intracellular Ph of Spodoptera frugiperda cells has been determined, this will be useful in designing experiments with a range of different proteins whose properties are acutely pH sensitive.
3) The successful construction of self processing cDNA cassettes of FMDV for all European serotypes.
4) Development of vectors allowing coexpression of multiple foreign genes from a single recombinant virus.

Wider considerations:
Recombinant DNA techniques have been used to construct vectors containing regions of cDNA (termed cassettes) which can express proteins from foot-and-mouth disease virus which self-assemble into virus-like particles but which are completely non-infectious. This has been achieved for each of the three different serotypes of foot-and-mouth disease virus which threaten Europe. It has been established that the intracellular environment of insect cells should not adversely affect the synthesis of these particles. Hence current work on the isolation of recombinant baculoviruses (which only grow in insect cells) carrying the cassettes mentioned above will proceed, these viruses are capable of expressing foreign proteins at high level. If successful the potential of these non-infectious virus-like particles as vaccines will be assessed.

Cooperative activities:
All participants met for a two day meeting in Madrid in June 1991 to discuss the state of the work. Dr Miguel Medina from Madrid spent several weeks in Wageningen at the end of 1991 constructing baculovirus transfer vectors containing the type C cDNA. Extensive transfer of clones occurs especially between Pirbright, Wageningen and Madrid in all directions and regular telephone and FAX communication occurs. Furthermore a range of materials have been supplied to Rhone-Merieux from Wageningen for the production of analytical antisera. A further meeting of participants occurred during the ELWW on 'Veterinary viral vaccines' organized by Dr J Vlak and held in Wageningen in November 1991 which brought together participants from each of the BRIDGE programme projects

in this subject area. A second meeting of this ELWW has been planned for 1992.

List of joint publications/patents with trans-national authorship:

Belsham GJ, Abrams CC, King AMQ, Roosien J & Vlak JM (1991) Myristoylation of foot-and-mouth disease virus capsid protein precursors is independent of other viral proteins and occurs in both mammalian and insect cells. J Gen Virol 72 747-751.

Other relevant publications:

Kool M, Voncken JW, van Lier FLJ, Tramper J & Vlak JM (1991) Detection and analysis of Autographa californica polyhedrosis virus mutants with defective interfering properties. Virology 183 739-746.

Vlak JM, Schouten A, Roelvink PW, Martens JWM & Zuidema D (1991) Engineering of novel baculovirus vectors for the expression of foreign genes in insects. In: Insect Molecular Science (HH Hagedorn, JG Hildebrand, MG Kidwell and J Law, Eds) Plenum Press p87ff.

Kitson JDA, Burke KL, Pullen LA, Belsham GJ & Almond JWA (1991) Chimeric polioviruses that include sequences derived from two independent antigenic sites of foot-and-mouth disease virus (FMDV) induce neutralizing antibodies against FMDV in guinea pigs. J Virol 65 3068-3075.

Belsham GJ (1992) Dual initiation sites of protein synthesis on foot-and-mouth disease virus RNA are selected following internal entry and scanning of ribosomes in vivo. EMBO J 11 1105-1110.

TITLE:
Towards a second generation vaccine against bovine herpesvirus type 1 (BHV-1): Immunological characterization of herpesviral glycoproteins and construction of BHV-1 deletion mutants

CONTRACT NUMBER: BIOT-0191-C(EDB)

OFFICIAL STARTING DATE: 01. 02. 91

COORDINATOR: G. M. Keil, BFAV, D-7400 Tübingen, Germany

PARTICIPANTS: J. van Oirschot, CDI, 8200 AB Lelystad, Netherlands
P. P. Pastoret, Univ. of Liege, 4000 Sart Tilman-Liege, Belgium
U. H. Koszinowski, Univ. of Ulm, 7900 Ulm, Germany

OBJECTIVES SET FOR THE REPORTING PERIOD:
Objectives were 1.) to identify, characterize and, if necessary, sequence genes coding for nonessential glycoproteins of BHV-1 and 2.) glycoprotein B of murine cytomegalovirus and to express the respective genes using recombinant vaccinia viruses, 3.) to develop isotype specific ELISAs to monitor mucosal and systemic immunity in cattle vaccinated and challenged with BHV-1 and 4.) to set up methods to analyse both the activity of glycoprotein specicific cytotoxic T-lymphocytes and the proliferative response of lymphocytes isolated from BHV-1 infected cattle.

MAJOR PROBLEMS ENCOUNTERED: Nothing to report.

RESULTS:
BFAV Tübingen: Work in this laboratory concentrated on the characterization and expression of the genes coding for glycoproteins C, E, G, and I (nomenclature of herpes simplex virus glycoproteins) of BHV-1 that were integrated into vaccinia virus recombination vectors. In detail:
The sequence for gC was taken from the literature (Fitzpatrick et al. Virology 173:46-57, 1989). The gC ORF was isolated from a cloned genomic DNA fragment and recombined into vaccinia virus. Rabbits were infected with the recombinant vaccinia virus VacgC. Antibodies induced in the animals were used for radioimmunoprecipitations and reacted with the gC of all BHV-1 strains tested. VacgC was given to Liege.
The gene coding for gG of BHV-1 strain N569 was characterized. It is located upstream of gD in the unique short region of the viral genome. Using antibodies raised in rabbits by VacgG it could be shown that gG is an early glycoprotein and that it is secreted from infected cells. The sera against N569 VacgG did not react with gG of other BHV-1 strains in radioimmunoprecipitations and immunoblots. Comparison of the N569 sequence with partial sequences of gG of BHV-1 strain Los Angeles revealed a less than 80% identity of the corresponding aminoacids. Thus gG seems to differ considerably among different BHV-1 strains.
The ORF for gI is located downstream of gD and contained within the mRNA coding for gD. A less abundant mRNA is transcribed only from sequences containing the gI ORF. To prove that gI is expressed under control of an own promoter, sequences upstream of the gI ORF were fused to the ORFs of firefly luciferase and ß-galactosidase. Transient expression assays in transfected cells showed that the sequences between the gD ORF and the gI ORF have

promoter activity with the characteristics of early regulated herpesviral transcription elements. The mRNAs for gD and gi use the same polyadenylation signal downstream the gI ORF. Generation of gI deletion mutants must consider this gene organization to avoid inactivation of the essential gD gene e.g. by removing the polyadenylation signal. The gI ORF was integrated into vaccinia virus. Sera of infected rabbits (and also sera raised against selected oligopeptides) did not react specifically with proteins of BHV-1 infected cells and sera from BHV-1 infected cattle did not react with VacgI induced infected cell proteins. Thus the nature of gI remains obscure.
The sequence of gE and a plasmid containing the gE gene was provided by J. van Oirschot. RNA studies showed that transcription of gE is also early regulated and that the approximately 3.3kb transcripts terminate about 1 kb downstream the gE ORF. This indicates that the gE mRNAs might coterminate with downstream located transcripts because, as for gD, no polyA-signal is located directly 3' to the ORF. The gE ORF was integrated into a recombination vector for the insertion into vaccinia virus.

CDI Lelystad: Because a relation may exist between vaccine-induced immunity and the level of mucosal immunity, we have developed tests to measure IgA, IgM, IgG_1 and IgG_2 responses against BHV-1.
Isotype-specific ELISAs for detecting antibodies against BHV-1
For demonstrating BHV-1 specific immunoglobulin isotypes, two types of assays were developed: an antibody capture assay (ACA) for the detection of IgA and IgM, and an indirect double antibody sandwich assay (IDAS) for the detection of IgG_1 and IgG_2. In the ACA, micro-ELISA plates are coated with monoclonal antibodies against bovine IgA or IgM and then incubated with the test sample, antigen, enzyme labelled monoclonal antibody directed against gB of BHV-1 and substrate solution. In the IDAS, plates are coated with the monoclonal antibody against gB of BHV-1 and then incubated with the antigen, test sample, enzyme labeled monoclonal antibody against IgG_1 or IgG_2 and substrate solution.

Table 1. Isotype specificity and sensitivity of isotype specific ELISAs compared with a 24 h neutralization test with and without complement.

Sample	ELISA				Neutralization test	
	IgG_1	IgG_2	IgM	IgA	+ C	- C
serum[a]	2.4[b]	-	-	-	1.2	0.9
serum	1.9	-	3.4	-	2.1	0.9
serum	4.3	3.7	-	-	>3.6	>3.6
serum	3.1	2.5	3.2	1.3	2.4	2.1
serum[a]	-	2.1	-	-	1.2	1.2
serum	-	-	3.4	-	1.5	0.6
serum[a]	-	-	1.8	-	0.3	-
nasal secretion	-	-	1.2	-	-	-
eye secretion	-	-	1.5	-	-	-
nasal secretion	-	-	-	2.4	1.2	0.9
eye secretion	-	-	-	2.4	1.2	0.9
nasal secretion	-	-	1.5	1.8	1.5	0.3

[a]Samples that were purified for antibodies of a particular isotype by immunoaffinitychromatography;
[b]Titre in isotype-specific ELISa and neutralization test expressed as log_{10}; -:Negative

Specificity and sensitivity: Table 1 shows the specificity of the IDAS and ACA for the detection of antibodies against BHV-1. Selected samples with a high titre for one isotype did not react in the assays for the other isotypes. BHV-1 positive sera did not react with control antigen and all BHV-1 negative sera tested were scored negative (data not shown).
To asses the relative sensitivity of the isotype specific BHV-1 ELISAs, selected samples were tested by a 24 hr neutralization assay with and without complement (Table 1). The 24 hr neutralization test is about 10 times more sensitive than the conventional 1 hr neutralization test. The titres scored in the IDAS and ACA were higher than those in neutralization test. Neutralizing antibodies were not found in two secretions that scored positive for IgM antibodies. The titres in the neutralization test were significantly increased, primarily in samples containing IgM antibodies, by adding guinea pig serum as source of complement.
Kinetics of isotype-specific antibody responses in sera and nasal secretions of experimentally infected calves: Antibody responses against BHV-1 were found in sera, in nasal and ocular secretions, but not in genital secretions, from all calves infected with either of 3 BHV-1 strains. No antibody response was detected in uninfected control calves.
Antibody responses in serum: Antibody responses were detected earlier in serum than in nasal secretions. IgM Antibodies were usually detected between post infection day (PID) 8-9 and PID 28. IgA antibodies were detected 2-3 days later than IgM antibodies. IgG_1 antibodies were first demonstrated on PID 13, while IgG_2 antibodies generally appeared some days later. The IgG_1 and IgG_2 antibodies persisted at a high level for more than 100 days.
Antibody responses in nasal secretions: Antibodies of the IgM isotype first appeared in nasal secretions on PID 9-10 and were detectable until PID 28. As in serum IgA antibodies were detected 2-3 days later than IgM antibodies. The IgA antibodies persisted at a low level in most infected calves. IgG_1 and IgG_2 antibodies were not detected. Thus, sensitive and specific assays were developed to measure IgM, IgA and IgG antibodies against BHV-1 that will be employed to monitor isotype specific antibody responses in sera and nasal secretions of calves inoculated with spefic glycoprotein deficient deletion mutants of BHV-1. It will be determined wether a correlation exists between isotype specific antibody titres in serum or nasal secretions and protective immunity against challenge infection.

University of Liege: To evaluate the cellular immune response to an antigen three different properties of T-lymphocytes can be measured in *in vitro* assays:
1.) Cytotoxicity: 51 chromium release assay to detect activity of cytotoxic T-lymphocytes (CTL). The first method used consisted of in vitro stimulation of bulk cultures of mononuclear cells in the presence of antigen and interleukine 2. These effector cells were added to infected, ^{51}Cr labeled autologous target cells and cytotoxicity was evaluated from the amount of released ^{51}Cr. This approach, however, detectet mainly non-specific natural killer-like cytotoxic activity. Therefore the limiting dilution method was prefered that allows - by statistical calculations - an estimation of the frequency of cytotoxic T-cell precursors that are present among the mononuclear cells. Advantages of this method are that quantitative results are obtained and that the problem that specific CTL are overgrown by natural killer cells is avoided. The main results obtained until now are that the way of antigenic stimulation used (UV-inactivated virus or infected fibroblasts) influences the CTL response that is identified. Thus expansion of mononuclear cells in the presence of IL2 and without antigen will be prefered for all subsequent experiments. We could furthermore demonstrate that among the major glycoproteins both gC and gD were target antigens for the CTL response in infected cattle. The method will be used to analyze the cytotoxic response to the minor glycoproteins by comparing lysis of wild type virus infected target cells to lysis of target cells infected with vaccinia virus recombinants and BHV-1 deletion mutants. 2.)

Lymphoproliferation: measurement of ^{3}H-thymidine incorporation to detect the ability of helper T-lymphocytes to proliferate in response to the antigen. The limiting dilution method has been set up for the proliferative response. The principle is similar to limiting dilution analysis of cytotoxic cells, but at the end of the culture period, ^{3H}-thymidine is added to the cultures to identify proliferating cells. This proliferation assay can be performed in the presence of IL2, but we were able to demonstrate that in the presence of IL2 probably mainly proliferating natural killer cells were detected. Thus, assays performed without addition of IL2 will be prefered. The frequency of specific proliferating T lymphocytes is the difference between frequencies measured in the presence and absence of stimulating antigen (UV-inactivated virus).
This method will be used to determine whether the deletion mutants are able to stimulate a proliferative response that is quantitatively similar to the one induced by wild type virus. Moreover, by using both deletion mutants and wild type virus as stimulating antigen, we will determine whih of the nonessential glycoproteins is recognized by helper T lymphocytes of immune cattle.
3.) Cytokine (IL2) production: detects activity of helper T-lymphocytes.
The methods to analyse the CTL activity and the proliferative response of infected cattle were established.

University of Ulm: The gene encoding glycoprotein B of murine cytomegalovirus (MCMV) strain Smith was identified, sequenced and expressed by recombinant vaccinia virus. Similar to the location in the genome of human cytomegalovirus (HCMV) the gB gene was found adjacent to the polymerase gene. The open reading frame consists of 2784 nucleotides capable of encoding a protein of 928 amino acids. Comparison with the gB homologs of other herpesviruses revealed a high degree of homology. The similarity between the MCMV gB and the HCMV gB is most prominent as 45% of the amino acids are identical. In addition, all cysteine residues are at homologous positions indicating a similar tertiary structure of the two proteins. In contrast to HCMV the MCMV gB mRNA is a true late transcript. A recombinant vaccinia virus expressing the MCMV gB gene has been constructed (Vac-gB) . Antibodies raised against the Vac-gB recombinant precpitated proteins of 130, 105, and 52 kDa from MCMV infected cells. The identity of the MCMV gB with the major envelope glycoprotein of MCMV described by Loh et al. (Virology 166:206-216, 1988) was shown. Immunization of mice with Vac-gB gave rise to neutralizing antibodies.

HIGHLIGHTS/MILESTONES: Highly specific and sensitive tests were developed to study mucosal immunity after BHV-1 vaccination and infection, to identify viral glycoproteins inducing a specific CTL response in herpesvirus infected animals and to evaluate the contribution of individual glycoproteins to the cellular immune response. The open reading frames for all known nonessential glycoproteins of BHV-1 and glycoprotein B of murine cytomegalovirus were isolated and, with the exception of the ORF for gE(BHV-1), integrated into the genome of vaccinia virus.

WIDER CONSIDERATIONS: Tests were developed to study the antibody responses against BHV-1 on the mucosae of infected or vaccinated cattle. If this mucosal immunity is correlated with the induced protection by vaccination, which is presently determined in challenge experiments, it may make the severe experimental infections in cattle no longer necessary. Concerning the design of a BHV-1 marker vaccine that contains a deletion of a nonessential glycoprotein gene, the results obtained so far with the recombinant vaccinia viruses indicate that both gG and gI are not suitable for this purpose because gG shows a

significant strain heterogeneity in the aminoacid sequence and gI appears to be at least weak immunogenic with respect to the humoral immune response.

COOPERATIVE ACTIVITIES:

Joint meetings were held in Liège (06.02.1991) and Wageningen (29.11.1991) during the ELWW meeting on veterinary viral vaccines. All partners of the project participate in this ELWW and made contributions for the "Blue Brochure". Scientists from all laboratories attended the 16th International Herpesvirus Workshop in Asilomar (07. - 12. 07. 1991) where also aspects the common project were discussed.

Exchange of material: The nucleotide sequences of gI and gG, the VacgI recombinant and monoclonal antibodies to identify gB, gC, gD were sent from G. M. Keil to J.van Oirschot who provided G.M.Keil with the sequence of gE, a plasmid containing the gE gene and monoclonal antibodies directed against gE. J. van Oirschot obtained from P. P. Pastoret monoclonal antibodies against a hitherto unidentified glycoprotein and provided P. P. Pastoret with blood from cattle inoculated with various BHV-1 strains for determining cytotoxic T-cell responses against BHV-1. G. M. Keil received the VacgB recombinant 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 vaccinia virus recombinants.

Exchange of staff: In December 1991 a scientist from Ulm was in Tübingen for two weeks for joint experiments to characterize the gene organization in the 5' region of MCMV-gB

JOINT PUBLICATIONS/PATENTS:

Rapp, M., Messerle, M., Bühler, B., Tannheimer, M., Keil, G. M. and Koszinowski, U. H. Identification of the Murine Cytomegalovirus Glycoprotein B Gene and its Expression by Recombinant Vaccinia Virus. J.Virol., in press

OTHER PUBLICATIONS/PATENTS:

Denis, M., Slaoui, P.-P. Pastoret and Thiry, E. Specific anti-Bovine Herpesvirus 1 cytotoxic T Lymphocytes: a method to identify their target antigen. Proc. XVIth Int. Herpesvirus Workshop, Pacific Grove, Ca, July 7-12, p. 246

Fehler, F., Herrmann, J. M., Saalmüller, A., Mettenleiter, T.C. and Keil, G.M. 1992. Glycoprotein IV of Bovine Herpesvirus 1-expressing Cell Line Complements and Rescues a Conditionally Lethal Viral Mutant. J.Virol. 66: 831 - 839

Madic, J., Magdalena, J., Quak, J., Veldhuis, M.A. and Van Oirschot, J.T. Epitope-specific and isotype-specific antibody responses in sera and mucosal secretions of calves experimentally infected with bovine herpes virus type 1. Proc. 2nd Congress of the European Society for Veterinary Virology, Uppsala, 23-26 sept, 1991, p.129.

Maes, R.K., Rijsewijk, F.A.M. and Van Oirschot, J.T. PCR based detection of bovine herpesvirus type 1 DNA in bovine semen. Proc. XVIth Int. Herpesvirus Workshop, Pacific Grove, Ca, July 7-12, p. 247

Rijsewijk, F.A.M. and Van Oirschot, J.T. Bovine herpesvirus type 1 deletion mutants with natural or constructed deletions in the glycoprotein gE gene, to be used as vaccines and vaccine vectors and methods based on antibody detection against gE to distinguish infected from vaccinated cattle. Patent pending.

Van Oirschot, J.T. Risk assessment of using semen from BHV-1 seropositive bulls for artificial insemination. A Holland Genetics Report, 1991.

AREA : D

PRE-NORMATIVE RESEARCH

TITLE : DEVELOPMENT OF HUMAN ENDOTHELIAL CELL LINES WITH PRESERVED MORPHOLOGICAL AND FUNCTIONAL CHARACTERISTICS UTILIZATION IN TOXICOLOGICAL AND PHARMACOLOGICAL TESTS.

CONTRACT NUMBER : BIOT -CT90-0195.

OFFICIAL STARTING DATE : 1/1/1991

COORDINATOR: E. Dejana, Mario Negri Inst. for Pharm.Res., Milan, IT, O1

PARTICIPANTS : J.Gordon, British Biotechnol. Ltd, Oxford, GB, 02
C.de Castellarnau, St.Pau Res.Fund.Biomed. Res. Inst., Barcelona, ES, 03

OBJECTIVES SET FOR THE REPORTING PERIOD: The objectives of this first period of work have been : i) immortalization of endothelial cells with retroviral vectors; ii) construction of new viral vectors; iii) establishment of functional assay sytems for endothelial cells.

MAJOR PROBLEMS ENCOUNTERED : Nothing to report

RESULTS : In general participant 01 is involved in immortalizing normal endothelial cells with retroviral vectors containing various genes and in establishing standard functional assays for endothelial cells.During this first period of work the following milestones have been achieved : 1) three different retroviral vectors have been constructed; 2) normal cultured umbilical vein endothelial cells have been infected with two of the mentioned retroviral vectors and positively selected for their acquired antibiotic resistance; 3) two different episomal vectors have been prepared; 4) infected cells have been characterized for few functional parameters.

The amphotropic retroviral vector used is a gag+ LN vector (fig 1).This vector is composed of two LTRs, the gag+ packaging site, a multiple cloning site, an internal SV40 promoter and a neomycin resistance gene.

Three different genes have been cloned downstream the SV40 promoter in the LXSN vector : an activated H-ras allele derived from Harvey sarcoma virus; ii) the coding portion of a novel SH2-containing gene (SHC) and iii) the polyoma middle T antigen.These genes have been chosen for their transforming potential. The three recombinant vectors and the LXSN vector have been tranfected in the murine, ecotropic psi2 packaging cell line by the calcium phosphate coprecipitation method. Supernatants of the transfected cells have been used to infect the amphotropic PA317 packaging cell line. Normal cultured human umbilical vein endothelial cells have been infected with Ras and SHC containing constructs by substituting their regular culture medium with supernatants of PA317 clones. Cells have been selected for their antibiotic resistance. Northern blot analysis of total RNA extracted from these cells

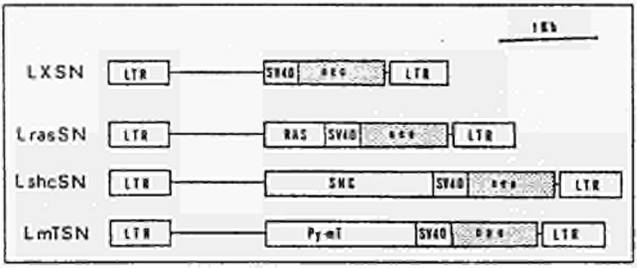


Fig. 1: The anphotropic gag+LN retroviral vector used in this study. The vector (LXSN) is composed of two LTRs, the gag+packaging site, a multiple cloning site, an internal SV40 promoter and a nomycin resistance gene. Three different genes have been cloned downstream the SV40 promoter: an activated H-ras allele (LraSN); the coding portion of a new SH2-containing gene (LshcSN); the polyoma middle T antigen (LmTSN).

showed high levels of expression of correctly spliced oncogenes.

The transfected cells have been studied for the expression of specific endothelial markers and for their morphology. Specific markers used are von Willebrand factor, PECAM/CD31 and 7B4 antigen. These proteins have been proven to be selectively expressed in endothelial cells. Indirect immunoflurescence experiments have been performed with specific monoclonal and polyclonal antibodies.

Cell morphology have been evaluated by phase contrast microscopy. Cytoskeletal organization have been also studied by indirect immunofluorescence technique.

As a second approach in transfection and expression of oncogenes we used a recently developed vector (pHEBO) which contains the hygromycin B resistance gene (hph) as a selectable marker and can replicate as a plasmid because it carries the EBV origin of replication (oriP) and the EBV nuclear antigen (EBNA).The cytoplasmic replication of pHEBO vectors leads to higher expression of the cloned genes in recipient cells, when compared to recombinant vectors that integrate in the host genome, because of the high gene copy number generated.

The coding portions of the genes to be studied have been introduced in pHEBO vectors making use of a convenient multiple cloning site.

Participant 02 has concentrated his work on planning, obtaining the necessary plasmids and strains and establishing the appropriate protocols for the handling of oncogenic DNA.Work has now started on developing conditions for the transfection of endothelial cells and on construction of oncogene expression vectors.On the basis of the results obtained above, integrative plasmids will then be used. One of the key objective is to develop an efficient and reproducible procedure for transfection of endothelial cells. Different potential reporter genes have been evluated and the best option considered was the Firefly Luciferase. Suitable luciferase containing plasmids have been obtained. Large scale DNA preparations have been made of plasmids carrying the luciferase gene under the control of the RSV-LTR and hCMV

promoters. Studies are underway to find out the most efficient way to introduce DNA contructs into endothelial cells. Different transfection procedure are under evaluation: calcium phosphate precipitation, electroporation and lipofection. The transient expression studies using luciferase as a reporter gene should provide information on the transfection efficiency of human umbilical vein endothelial cells and a comparative analysis of the two promoters hCMV and RSV.

Participant 03 established a series of functional assay systems to be used in the endothelial cell lines : i) Arachidonic acid metabolism. Different approaches have been used for studying endogenous and exogenous arachidonic acid metabolism in basal and stimulated conditions. Endogenous metabolism has been evaluated with and without cell activation with thrombin by radioimmunoassay of the two major endothelial prostaglandins: prostacyclin and prostaglandin E2. Exogenous metabolism has been studied as above after cell activation with exogenous arachidonic acid. In addition , high performance liquid chromatography has been applied for separation and analysis of different arachidonic acid metabolites. ii) Procoagluant activity. A method for studying this parameter was set up. This assay uses recalcification time evaluation and rabbit brain thromboplastin as standard. Procoagulant activity has been measured in cells activated with cytokines and endotoxin. iii) Fibrinolytic activity. This parameter has been studied on normal endothelial cells either unstimulated or activated with cytokines and heparinoids.Tissue type, urokinase type plasminogen activator and plasaminogen activator inhibitor production has been measured by antigenic and functional assays.In addition the binding of plasminogen activators and plasminogen to the cell membrane has been evaluated.

In order to develop a toxicological assay, cell lysis techniques were evaluated. Three different methods were compared: 1) lactate dehydrogenese leakage; 2) ^{3}H adenine uptake and release; 3) ^{51}chromium release. Cells have been challenged with different toxic substances such as : xanthine-xanthine oxidase, anilides and minimal modified LDL. The most sensitive and reliable test has been found to be the ^{3}H adenine uptake and release and this has been selected for further studies on endothelial cell lines.

The work in the different laboratories proceeds with good integration of results and information accompanied by exchange of materials, cells and investigators.

HIGHLIGHTS / MILESTONES: The work is proceeding as planned, the following milestones have been achieved:

1) Two human endothelial cell lines transfected with retroviral constructs and two distinct oncogenes have been developed. These cells have been characterized for specific markers and for morphological parameters.
2) Plasmid vectors have been prepared.
3) Functional assays for evaluating a series of functional activities of endothelial cells have been set up.
4) Assays for the screening of substances with potential toxicological effects have been

established.

WIDER CONSIDERATIONS : The endothelium constitutes the internal lining of the circulatory system and plays a pivotal role in the maintenance of the vessel wall integrity. The alteration of the functional properties of endothelial cells is the first step in the development of cardiovascular diseases such as atherosclerosis and myocardial infarction. The possibility to isolate and study these cells in vitro is limited by their short life span. We are working on the possibility to immortalize cultured human endothelial cells by inserting specific oncogenes . Two cell lines have been already developed during this study. They are now under evaluation for growth potential and functional properties.

COOPERATIVE ACTIVITIES :

*Three meeting among participants have been organized:
LONDON: January 28-30,1991
MILANO : June 19-20,1991
BARCELONA : November 13-15, 1991
*Participant 03 is currently spending a three months work period in Participant 01 laboratory.
* Endothelial cell lines and different constructs have been excanghed.

LIST OF PUBLICATIONS WITH TRANS-NATIONAL AUTHORSHIP :

- Lauri D., Needham L., Martin Padura I., Dejana E. Tumor cells adhesion to endothelial cells: Endothelial leukocyte adhesion molecule 1 (ELAM-1) as an inducible adhesive receptor specific for colon carcinoma cells. NCI Journal, 83: 1321-1324 (1991).
- Lampugnani,MG, Resnati, M, Raiteri, M, Pigott, R, Marchisio, PC, Pisacane, A, Ruco, LP, and Dejana, E. A novel endothelial specific memebrane protein is a marker of cell-cell contacts. J.Cell Biol.submitted

OTHER PUBLICATIONS :

- Felez,J, Chanquia,CA,Levin, EG,Miles, LA and Plow,EF. Binding of tissue plasminogen activator to human monocytes and monocytoid cells. Blood 78: 2318-2327,1991.
- Lopez,S,Vila,L, de Castellarnau,C. Interleukin-1 increases 15-hydroxy-eicosatetraenoic acid formation in cultured endothelial cells.Biochem. Biophys. Acta, submitted.
- Pich, I, Lopez,S, Vila,L, Lagunas,C, and de Castellarnau, C. Influence of fatty acid anilides present in toxic on the metabolism of exogenous arachidonic acid in cultured human endothelial cells. Toxicology, submitted.
- Bussolino F., De Rossi M., Sica A., Colotta F., Wang J.M., Alessi D., Martin Padura I., Bosia A., Dejana E., Mantovani A. Murine endothelioma cell lines transformed by polyoma middle T oncogene as targets for and producers of cytokinesJ. Immunol., 147: 2122-2129 (1991).

TITLE: Development of in vitro neural systems for the identification of agents with toxicological and pharmacological potential

CONTRACT NUMBER: BIOT-CT90-0183-C

OFFICIAL STARTING DATE: 01/01/91

COORDINATOR: D.C. Williams, Dept Biochemistry, University of Dublin, Trinity College, Dublin, IE

PARTICIPANTS: C Meredith, BIBRA Toxicology International. G.B.
A Schousboe, Dept Biology, Royal Danish School of Pharmacy, Copenhagen, DK.
R. Griffiths, Dept Biochemistry, University of St Andrews, St Andrews, GB.
E. Rodríguez-Farré, Dept. Pharmacology & Toxicology; Dept. Neurochemistry; Consejo Superior Investigaciones Cientificas, Barcelona, ES.

OBJECTIVES SET FOR THE REPORTING PERIOD: As defined in contract

MAJOR PROBLEMS ENCOUNTERED: None

RESULTS:

The objective for the group at RSDP during the first year of the contract was to further develop primary cultures of neurons and astrocytes and to study neurodifferentiative and degenerative agents in such cultures. During the first year these culture techniques have been established at USTA having multiple exchanges of personnel between RSDP and USTA.

Further the neurotoxic actions of extitatory amino acid have been analyzed with regard to characterization of mechanisms and development of strategies for protection of neurons. Moreover, differentiative agents affecting neuronal and astroglial functions such as GABA-receptor/Ca^{2+}-channel (neurons) or GABA transport (astrocytes) expression have been investigated. This will lead to a better understanding of mechanisms regulating development and differentiation in the central nervous system.

CSIC have studied the basal Ca^{2+}-dependent 25 mM K^+-evoked [^{3}H]-Noradrenaline (NA) release from rat hippocampal slices, preincubated with 100 nM [^{3}H]-NA, under exposure to hexachlorocyclohexane (HCH) isomers and some GABAergic drugs (picrotoxinin: PTX, bicuculline: BIC, pentylenetetrazol: PTZ, and diazepam: DZ) as convulsant and anticonvulsant reference compounds. γ-HCH (lindane) enhanced and δ-HCH reduced K^+-evoked [^{3}H]-NA release. The α- and β-HCH isomers did not change the [^{3}H]-NA release. The convulsant compounds - PTX (1-100 μM), BIC (10, 100 μM) and PTZ (0.1-5mM) - did not modify the K^+-evoked [^{3}H]-NA release at the concentrations studied. In contrast, the K^+-induced [^{3}H]-NA release was potently reduced by the exposure to DZ. On basal

[^{3}H]-NA release only δ-HCH and DZ induced a Ca $^{2+}$-independent, time-dependent enhancement of about 200% of the control after 1 h of exposure. The present results demonstrate that neurotransmitter release from brain slices may be a useful assay method to test the mode of action of convulsant and anticonvulsant agents on synaptic events.

Also t-[^{35}S]butylbicyclophosphorothionate (TBPS) binding was performed in neuronal primary cerebellar cultures to study the characteristics of convulsant binding sites on the γ-aminobutyric acid$_A$) receptor in intact neuronal cells. Convulsant compounds like PTX and PTZ inhibited the binding in this in vitro system. Convulsant organochlorine pesticides belonging to the group of polychlorocycloalkanes - PCCAs (γ-hexachlorocyclohexane - γ-HCH or lindane - and the cyclodienes aldrin, endrin, dieldrin and α-endosulfan) - competitively inhibited [^{35}S]TBPS binding in primary cerebellar cultures (Figure 2). Stereospecificity was also shown for HCH isomers, the non-convulsant isomers (α and δ-HCH) being 15-30 times less potent in inhibiting [^{35}S]TBPS binding than the convulsant γ-HCH, whereas the β-isomer did not inhibit the TBPS binding. A significant correlation was observed between the *in vivo* toxicity (LD_{50}) of convulsant PCCAs and their affinity for the TBPS binding site. This work demonstrates the usefulness of neuronal primary cerebellar cultures to study the interaction of convulsant drugs with $GABA_A$ receptor.

Studies of in vitro cytoxicity 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 γ-HCH showed that cytotoxicity could be prevented in cortical cultures by adding GABA and benzodiazepines to the cell culture.

The main neuroactive HCH isomers according to results obtained in the above mentioned in vitro test systems (NA release and $GABA_A$ receptor binding) are γ and δ-HCH. These were tested for their ability to interact with inositol phosphate production in cultured cerebellar granule cells. Both agents increased that production at 100 μM. Studies to characterize this effect are in progress.

USTAs progress is in line with the set timescales, current work being undertaken mainly in collaboration with RDSP. Four working exchanges of personnel have taken place to date. With help from RDSP, a brain cell culture facility has been established in USTA. Using the cytoplasmic release of lactate dehydrogenase as a marker, the screening of neuroactive agents (specifically the endogenous excitatory sulphur amino acids) for cytotoxicity has been successful and a joint manuscript is in preparation. Detailed mechanistic studies using second messenger systems as the end-points for assay of synaptic function can now be addressed. In order to follow a more logical experimental approach, certain aspects of USTAs objectives from Year 3 of the contract have been brought forward and assays for synaptic function using primary brain cell cultures of neurons and astrocytes, and isolated nerve endings, have been undertaken in relation to transmitter uptake, receptor interactions and release. This has resulted in 2 joint publications.

USTAs joint venture with BIBRA and RDSP on the screening of EAAs for pro-differentiation is set to start on time but these studies will extend into Year 3. This should not present a problem in terms of attaining objectives because (as discussed above) USTA have integrated some of Year 3 objectives into Year 1.

The PC12 cell line is typically characterised as responsive to nerve growth factor (NCF) 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. BIBRA 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 molulatory effects of drugs and chemicals.

Following treatment of PC12 cells with NCF (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 x 10^5 cells. Collaborative experiments with Paul Rumsby's group at BIBRA using competitive PCR technology have improved the sensitivity of the assay to less than 1000 cells.

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 (10-100μM) and muscarine (10-100μM) 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 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 stabilized at a level about 4-fold above baseline.

PC12 cells were also 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. Further experiments will assess the relevance of this apparent interaction between the immune and the nervous system.

Experiments in progress are investigating the effect of selected neurotoxins e.g. MPP^+ on the expression of proto-oncogenes in PC12 cultures and assessing the ability of these neurotoxicants to induce programmed cell death (apoptosis).

The objective for Dublin in Year 1 was to set up cell culture systems for primary cultures of astrocytes and neurons and to continue studies using peripheral-type benzodiazepines and their receptors and the role of these agents in controlling cell proliferation, differentiation, and regulation of convulsive activity.

Cell culture systems have been set up after initial training by other groups. Further work has been performed on studying the stabilization of the receptor by various agents including phospholipids in order to provide the receptor in an optimally native state for its use in screening agents. The role of the receptor has been further characterised in controlling mitochondrial respiration, our findings suggesting that the receptor does not play a significant role in this respect. An endogenous peptide ligand for the receptor has been purified and its interaction with the receptor studied in order to provide a 'standard' in addition to benzodiazepine drugs for studying the further development of screening methods with the receptor. A study of the influence of cytokines on the receptor in intact cells has been initiated and is well advanced. the role of the receptor as a target for HCH toxicity has been studied.

The convulsive HCH isomer - γ-HCH (lindane) is a highly potent (Ki ~ 1 μM) inhibitor of benzodiazepine binding to the receptor whereas the inactive isomers do not inhibit. studies are in progress to assess whether lindane can influence the synthesis of either convulsive or anticonvulsive steroids in cells or brain in order to establish whether the convulsant actions of

lindane could be mediated through this receptor.

HIGHLIGHTS/MILESTONES:

Competitive PCR for low level fos expression (BIBRA).In studies on HCHs, δ-HCH and DZ showed similar effects on the Na release test at similar concentrations, suggesting a relationship with their known CNS depressant activity.
Inhibition of [^{35}S]TBPS binding by convulsant PCCAs correlates with their LD_{50}values.

WIDER CONSIDERATIONS:

Neural cell culture systems are being shown to have important uses in identifying and screening compounds for toxic and beneficial properies. These methods are strengthened by emphasis on the elucidation of mechanisms of action

COOPERATIVE ACTIVITIES:

All participants - St Andrews May 1991. All participants - Puerto de la Cruz February 1992. Dublin - BIBRA planning visit Carshalton December 1991. Material exchange Dublin-BIBRA ongoing.Technology exchange BIBRA - St Andrews ongoing. Planned experiments BIBRA - St Andrews. There has been intensive collaboration between RDSP and USTA involving multiple exchanges of personnel. There also has been collaboration with CSIC on cytotoxicity studies and a joint publication is planned. Exchange of personnel and material for the 2nd year between RDSP and Dublin has been planned and confirmed.There have been 4 exchange visits between USTA and RDSP during Jan 1991-March 1992 (Feb. 1991 - 2 workers USTA → RDSP for 10 days: cell culture tuition and experiments/transfer of freeze dried materials to USTA for analysis. June 1991 - 1 worker USTA → RDSP for 6 days: manuscript discussions. October 1991 - 1 worker RDSP → USTA for 7 days: aid in establishing a brain cell culture facility. November 1991 - 1 worker USTA → RDSP for 4 days : manuscript preparation). Joint studies on lindane toxicity are in progress between Dublin, CSIC and RDSP. Training visit on cell culture has occurred (Mar 92) from Dublin to USTA.

LIST OF JOINT PUBLICATIONS/PATENTS WITH TRANS-NATIONAL AUTHORSHIP:

Grieve, A., Dunlop J., Schousboe, A. and Griffiths, R. Kinetic characterization of sulphur-containing excitatory acid uptake in primary cultures of neurons and astrocytes. Neurochem. Int. 19, 467-474 (1991).

Dunlop J., Grieve A., Schousboe A., and Griffiths R. (1991) Stimulation of γ-[^{3}H]aminobutyric acid release from cultured mouse cerebral cortex neurons by sulphur-containing excitatory amino acid transmitter candidates: receptor activation mediates two distinct mechanisms of release. J. Neurochem. 57, 1388-1397.

OTHER PUBLICATIONS/PATENTS:

Hansen, G.H., Belhage, B. and Schousboe, A. First direct electron microscopic

visualization of a tight spatial coupling between $GABA_A$-receptors and voltage sensitive calcium channels. Neurosci. Lett. 135, in press (1992).

Fransden, Aa, and Schousboe, A. Mobilization of dantrolene-sensitive intracellular calcium pools is involved in the cytotoxicity induced by quisqualate and N-methyl-D-aspartate but not by 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl)propionate and kainate in cultured cerebral cortical neurons. Proc. Natl. Acad. Sci. (USA) 89 in press (1992).

Nissen, J., Schousboe, A., Halkier, T. and Schousboe, I. Purification and characterization of an astrocyte GABA-carrier inducing protein (GABA-CIP) released from cerebellar granule cells in culture. Glia 5 in press (1992).

Grieve A., Butcher S.P., and Griffiths R. (1992) Synaptosomal plasma membrane transport of excitatory sulphur amino acid transmitter candidates: kinetic characterisation and analysis of carrier specificity. J. Neurosci. Res. 31, in press.

Griffiths R. (1992) Sulphur-containing excitatory amino acids: metabolism and function. In Schousboe A., Diemer N.H., and Kofod H. (eds) : Alfred Benzon Symposium 32, Drug Research Related to Neuroactive Amino Acids." Copenhagen, Munksgaard, in press.

R. Cristòfol & E. Rodríguez-Farré. Differential presynaptic effects of hexachlorocyclohexane isomers on noradrenaline release in cerebral cortex. Life Sci. 49:1111-1120 (1991).

A. Pomes, E. Rodríguez-Farré. and C. Sunol. Inhibition of t-[^{35}S]Butylbicyclophosphorothionate Binding at the γ-Aminobutyric $Acid_A$ Receptor by Convulsant Agents in Primary Cultures of Cerebellar Neurons. Submitted for publication.

Zisterer, D. M. Gorman, A.M.C. Williams, D.C. and Murphy, M.P. (in press) The Effects of The Peripheral-Type Benzodiazepine Receptor Ligands, Ro5-4864 and PK 11195 on Mitochondrial Respiration. *Methods Findings Exp. Clin. Pharmacol.*

Moynagh, P.N. and Williams, D.C. (in press) Stabilization of the peripheral-type benzodiazepine acceptor by specific phospholipids. *Biochem. Pharmacol.*

TITLE: *"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."*

CONTRACT NUMBER: BIOT- CT 91-0261

OFFICIAL STARTING DATE: 01/03/1991

COORDINATOR:

E. Honikman-Leban, L'Oréal Advanced Research Center, Aulnay-sous-Bois, FR.

PARTICIPANTS:

M. Balls, F.R.A.M.E., Nottingham, GB;
E. Honikman-Leban, L'Oréal, Aulnay-sous-Bois, FR;
V. Krutovskikh, I.A.R.C., Lyon, FR;
P. Martel, I.N.R.A., Jouy-en-Josas, FR;
G. Ragnotti, Universities of Brescia & Milan, IT;
L. Wärngard, Karolinska Institute, Stockholm, SE;
K. Willecke, University of Bonn, Bonn, DE.

OBJECTIVES SET FOR THE REPORTING PERIOD: Standardization of the dye transfer protocol : all participants (except Frame) have performed the microinjection on the same cell line and evaluate the activity of 5 compounds on the gap junction intercellular communication (GJIC).
Development of other cell types, depending on the activities of the laboratories;
Use of molecular tools to identify specific GJIC proteins (connexins) of those cells.

MAJOR PROBLEMS ENCOUNTERED: Nothing to report

RESULTS: *Standardization of the dye transfer assay, using IAR20 cells (rat liver epithelial cell line) to test 5 known molecules at 3 concentrations and at 4 incubation times:*
The laboratories obtained comparative results during this preliminary study: When IAR20 were treated with the highest non-cytotoxic concentrations of tumor promoters, 12-o-tetradecanoyl-phorbol-13-acetate(TPA), butylated hydroxyanisole(BHA), dichlorodiphenyl-trichloroethane (DDT) and phenobarbital (PB), a dose and time-related inhibition of GJIC was observed: 100ng/ml TPA and 20uM DDT exerted their maximal effect (90% and 75% respectively) on GJIC after 1 h treatment, but the TPA action was reverted within 24 h. PB failed to show a significative effect on IAR20 GJIC but 30ug/ml BHA exerted its maximal effect on GJIC (65%) after a 24 h treatment. A 1 h treatment with 100ng/ml of the non - promoter, 4α-phorbol-12,13-didecanoate(4α-PDD) caused no inhibition on GJIC.

After this encouraging preliminary interlaboratory evaluation of the microinjection protocol, the different laboratories performed the same protocol on their own cells depending upon their field of interest:

The **INRA group** applied the microinjection methodology to study the modulation of GJIC by different compounds (retinoids, tocopherols and flavonoids) between rat liver epithelial cells. 3 of the tested molecules appeared to increase significantly cell communication capacity. The same molecules were tested on V79 cells with the metabolic cooperation assay by **L'Oreal group**. Molecular studies are now in progress to define at which level of GJ regulation these molecules exert their stimulating action: connexin43 (cx43) expression is studied both by immunofluorescence and western-blot analysis; the expression of specific mRNA encoding cx43 is followed by northern -blot analysis. In parallel preliminary trials have been performed with the freeze-fracture analysis to detect structural effects on gap junctional complexes; new cells sy ms are being developped: rat liver epithelial cells transfected with c-fos and c-junc proto-oncogenes.

In the **Unit of Multistage Carcinogenesis of IARC**, the human hair follicule cell culture is in development to study GJIC and a possible screening system of tumor promoting agents relevant to human situation. Those cells can be routinely available from volunteering individuals by a non invasive technique. Hair follicule cells (keratinocytes) showed a reasonable level of GJIC measured by dye transfer assay. TPA inhibits GJIC and preliminary results suggest that a carcinogenic hair dye molecule (HC Blue 1) but not its non carcinogenic derivative (HC blue 2) inhibited GJIC of those cells.
It seems also essential to study in vivo GJIC from which useful information can be generated. For this purpose, a simple method to quantify GJIC in liver slices (from rat and human) was developped. Briefly, the dye is microinjected into the a liver slice, followed by cryostat sectioning and observation of the dye spread under a fluorescent microscope. Employing this method, a progressive decrease in GJIC during multistage rat liver carcinogenesis was observed. Known liver tumor promoting agents, i.e., PB, DDT, Clofibrate, PCB's and ethylene estradiol inhibit GJIC of rat liver in vivo while these chemicals are likely to act as hepatotumor promoters with different mechanisms. Further study is in progress in combination with the use of molecular probes (connexin 32 cDNA and antibodies).

At the **Karolinska Institute**, PCB-congeners were studied with the microinjection and the scrape-loading, both dye transfer techniques. The results are published in two articles which present the structure-activity relationships of these compounds. The immunostaining technique to study GJ proteins was implanted in the laboratory in collaboration with **IARC**. The cell culture technique was improved in collaboration with **INRA**. Also, the tissues from animals treated in the in vivo short-term promotion test were analysed for the amount of m-RNA in the **Bonn laboratory**.

Brescia-Milan Universities evaluate also the effect of compounds on their transformed fetal bovine aortic endothelial cells (F-BAE GM 7373) which has high basal GJIC capacity. As for the IAR20, all promoters tested induced a time-dose-related inhibition. In order to make the analysis of the results easier and more objective, a highly sensitive video-recording system was connected with the microinjector and the quantification of the extend of the dye through GJIC was performed on the video-recorded microscopic images.

Work in the **Institute of Genetics at Bonn University** is directed towards characterization of connexins (cx) gene expression in mouse tissues and cell types suitable for testing of tumor promoting compounds. For example, hepatocyte specific phenotype correlates with high expression of cx32 and cx26 but very low expression of cx43. Keratinocyte derived cell line Hel37 expresses cx31, cx31.1, cx30.3 mRNA and most likely also cx43 as well as cx26 transcripts. Since expression of these different cx seems to be regulated differently and the properties of the corresponding GJ channels may be different, it is possible that these GJ proteins represent different targets for tumor promoters.
This group succeeded in functional expression of cx40 or cx43 after transfection in human Hela cells, thereby restoring GJIC in these transformed coupling deficient cells. This system will allow to study the electrical conductance and permeability of GJ channels comprised of only one type of cx proteins - in contrast to the situation found in normal cells or tissues.

L'Oréal evaluates GJIC with the metabolic cooperation assay on V79 cells (Chinese hamster lung fibroblasts) which is based on the recovery of mutant colonies after treatment in a selective medium; this recovery is correlated with the interruption of the GJIC. The evaluation of metabolic cooperation will be carried out in association with **Karolinska Institute**. Beside this " V79 " assay, the dye transfer techniques , scrape-loading and microinjection, were adapted on IAR20. In collaboration with **INRA**, those complementary systems were used to study the activities of vitamins A and E, phenolic antioxydants (BHA and BHT) and flavonoids as possible antipromoting agents. In addition to the in vitro system on human hair follicule cells from **IARC**, we are also developping these dye transfer techniques on human skin cells (keratinocytes and dermal fibroblasts).

HIGHLIGHTS/MILESTONES:

Standardization of the dye transfer protocol is acheived and the assay system is now functional.

WIDER CONSIDERATIONS:'

Studies will be then performed with liver and skin cells to explore the mechanisms of GJIC.

COOPERATIVE ACTIVITIES:

Meetings:

L'Oréal, Aulnay, FR, 16/11/90: Standardization of dye transfer experiments; Distribution of the same batch of IAR20 cell line (obtained from IARC) and samples of the 5 selected chemicals .

IARC, Lyon, FR, 22-23/11/91: Discussion of the evolution of the GJIC concept; Presentation of the results obtained for the standardization of dye transfer experiments.

FRAME, Nottingham, GB, 27-28/03/92: Conditions for cytotoxicity and selection of chemicals; Setting the exchanges of techniques for mechanistic and molecular biology studies.

Exchanges and Collaborations:

L'Oréal - INRA: One day meeting every month to discuss results and litterature;
L'Oréal - Karolinska Institute: 2 meetings: 2 days in Stockholm (2-3/03/92) , 1 day in Aulnay (30/03/92), to discuss cytotoxicity and metabolic cooperation studies and structure-activity relationships on compounds.

Karolinska Institute - INRA: 1 week, September 1991, to practise cell culture techniques,
Karolinska Institute - IARC: 2 months, to acquire and to improve immunostaining techniques, (September 1991; March-April 1992),
Karolinska Institute - Bonn University: 4 days, for the analysis of m-RNA in the tissues of animals treated with tumor promoters.

INRA - IARC & Bonn University: Exchanges of molecular tools and protocols to characterize connexin (cx) genes expression.

Milan & Brescia Universities - IARC: 1 month, to acquire techniques for cx gene expression, (July 1991).

IARC - Bonn University: Both providing to INRA and Karolinska Institute cx cDNAs and m-RNAs to study the cx expression in the different cell types used.

Interactions with other BRIDGE groups:

ELWW proposal between our project and 2 other BRIDGE projects (PL 890177, PL 890151): workshop on "the use of human skin cells systems to predict pharmacotoxicologic effects of chemicals"; 1 day meeting of the projects coordinators to organise the proposal (26/02/92).

LIST OF JOINT PUBLICATIONS/PATENTS WITH TRANS-NATIONAL AUTHORSHIP:

MERCIER T., HONIKMAN-LEBAN E., CHAUMONTET C., MARTEL P., SHAHIN M.M., Etude de la modulation des communications intercellulaires par les retinoïdes à l'aide de deux tests in vitro. Colloque de l'Association pour la Recherche en Toxicologie, Paris, FR, 4 Mars 1992.

OTHER PUBLICATIONS /PATENTS:

HEMMING H., WÄRNGARD L., AHLBORG UG. (1991) Inhibition of dye transfer in rat liver WB cell culture by polychlorinated biphenyls, Pharmacology & Toxicology, 69, 416-420.

HEMMING H., FOLDSTRÖM S., FRANSSON-STEEN R., WÄRNGARD L., AHLBORG UG. (1992) Inhibition of intercellular communication in cell culture by polychlorinated compounds, Chemosphere(in press).

STUTENKEMPER R., GEISSE S., LOOK J., TRAUB O., NICHOLSON B.J. and WILLECKE K. (1992) The hepatocyte specific phenotype of murine liver cells correlates with high expression of connexin32 and -26, but very low expression of connexin43, Exp. Cell Res. (in press).

HENNEMANN H., SCHWARZ H-J. and WILLECKE K. (1992) Characterisation of gap junctions genes expressed in F9 embryonic carcinoma cells: molecular cloning of mouse connexin31 and -45 cDNAs, Europ. J. Cell Biol., 57, 51-58.

HENNEMANN H., DAHL E., WHITE E., 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 chromosome4 and preferentially expressed in skin, J. Biol. Chem. (submitted for publication).

DEVELOPMENT OF A PREDICTIVE IN VITRO TEST FOR DETECTION OF SENSITIZING COMPOUNDS

CONTRACT NUMBER:

BIOT-CT90-0186-C

OFFICIAL STARTING DATE:

1/6/91

COORDINATOR:

Alain BARBIER, Jean-Luc STAMPF,
SANOFI-RECHERCHE, Montpellier, FR

PARTICIPANTS:

Prof. Jan BOS, Department of Dermatology, UvA, Amsterdam, NL
Prof. Jürgen KNOP, Department of Dermatology, Mainz/Münster, DE
Dr Jean-Pierre LEPOITTEVIN, Laboratoire de Dermatochimie, ULP, Strasbourg, FR

OBJECTIVES SET FOR THE REPORTING PERIOD:

Determination of optimal conditions of epidermal cells preparation respectively from guinea pigs, human and murine skin. Cytokine production assay and/or cytokine mRNA analysis.

As a first step and as a necessary prerequisite technical procedures have to be optimized and standardized. These procedures include dermis/epidermis separation, single cell suspension preparation, expansion of keratinocyte cultures as a monolayer, haptenisation of epidermal cells. After haptenisation (contact with xenobiotics), cytokine secretion and/or surface markers expression of these epidermal cells are determined.

Preparation and/or purification of natural and synthetic allergenic, or not allergenic compounds

The contribution of the Dermatochemistry laboratory is to provide the other teams with haptens covering the whole spectrum of activity (from strong to weak allergens, including tolerogens, irritants and non allergenic products as controls) in a standardized, highly purified, and well characterized form. It is an important point that all the teams work on the same samples exempt from contaminants. This contribution for the first 6 to 12 months can be divided in two main phases.

MAJOR PROBLEMS ENCOUNTERED:

In our preliminary assays of measurement of MHC Class II expression with fluorescent antibodies by FACS, we noticed that all dinitrobenzenes-type haptens produced a very high fluorescence background which makes results unexploitable by this method. These haptens will probably be excluded from FACS analysis

The preparation of epidermal cells by itself, requiring trypsinization and epidermal sheets disruption is a strong pro-inflammatory stimulus at the cellular level. All cytokines tested so far

(IL-6 and TNFα) are induced at a high level. Specific cytokine induction after contact with haptens may therefore be overlooked due to high background noise. The methodology has to be modified.Epidermal cells will be cultured for a long time (one to four weeks) to allow return to baseline value of cytokine production. Basal keratinocytes will be propagated as adherent cells and submitted to "pulse contact " with xenobiotics when they reach confluency.

In some case subclones of B9 cells give unreproducible standard curves and contradictory interlaboratory results. We will share and exchange the cell line subclones that are reliable.

RESULTS:

UNIVERSITY LOUIS PASTEUR OF STRASBOURG

Purification of commercially available haptens. Synthesis of sensitizers and non-sensitizers

Table I: Purification of commercially available haptens

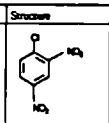
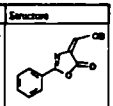
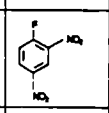
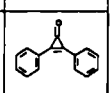
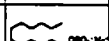
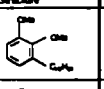
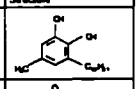
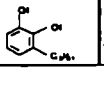
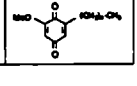
Name of hapten	Structure	Name of hapten	Structure
1-chloro-2,4-dinitrobenzene DNCB *Sensitizer*		4-ethoxymethylene-2-phenyl-2-oxazolin-5-one Oxazolone *Sensitizer*	
2,4-dinitrofluorobenzene DNFB *Sensitizer*		Diphenylcyclopropenone Diphenylcyprone *Sensitizer*	
Nickel sulfate *Sensitizer*	$NiSO_4$		
Sodium Lauryl sulfate SDS *Irritant*		Croton oil *Irritant*	Complex mixture

Table II: Preparation of synthetic sensitizers, non-sensitizers and tolerogens

Name of hapten	Structure	Name of hapten	Structure
3-n-pentadecyl-veratrole PDV *Non-sensitizer*		5-methyl-3-n-pentadecylcatechol 5-Me-PDC *Tolerogen*	
3-n-pentadecyl-catechol PDC *Sensitizer*		primin *Sensitizer*	

The compounds have been purified and distributed to all other participants.

SANOFI-RECHERCHE

Optimization of Epidermal cell preparation

Best yield and viability were obtained with cold trypsinization (4°C, overnight) using recristallised Trypsin Type IX (Sigma) at 0,1 % in PBS or HBSS. Epidermal sheets are separated easily from dermis with fine forceps. Gentle mechanical disruption of epidermal sheets separates cells from the fibrous matrix. We obtain routinely $1\text{-}1.5.10^8$ cells with a viability of 98 %, from two guinea pig flanks.

Determination of optimal conditions for "Pulse contact" of Epidermal cells with xenobiotics

The major problem with organic xenobiotics is their solubility in culture medium. Some of them (DNCB, DNFB, Oxazolone) are slightly soluble in water but others (PDC, Diphencyprone) are not. As a general methodology we have adopted after numerous different assays, a short-time (15 minutes) contact ("Pulse") of epidermal cells with haptens pre-emulsified in a soybean oil/culture medium mixture. The final concentration of oil is 2 % and has been determined as not toxic. Xenobiotics toxicity thresholds were determined (see table III). The cells are then washed thoroughly with culture medium to eliminate any remaining oil and free hapten. Lower levels of cytokine secretion were detected when haptens are not pre-emulsified.

Table III: Toxicity thresholds in vitro

Xenobiotic	conc[a]	Xenobiotic	conc
Soybean oil	2 %	PDC	100 µM
DNBS	500 µM	Diphencyprone	100 µM
DNCB	50 µM	Croton oil	0,05 %
DNFB	50 µM	Nickel sulfate	50 µM
Oxazolone	100 µM	SDS	100 µM

a Sub-toxic final concentration of xenobiotic solubilized in culture medium or emulsified in culture medium/soybean oil (2% final).

Measurement of IL-6 and TNFα secretion by bulk Epidermal cells

IL-6 assay is done with the B9 cell line which growths specifically in the presence of IL-6 and does not discriminate between human, guinea pig and mouse IL-6. The cells are cultured in RPMI supplemented with a synthetic substitute for serum to avoid stimulation by contaminating endotoxins. As mentioned in section *Problems encountered* IL-6 (and to a lesser extend TNFα) is produced by freshly prepared epidermal cells without further stimuli by xenobiotics. TNFα is measured with the L929 cell line cytolytic assay. Table IV summarizes IL-6 and TNFα production (over background) of epidermal cells stimulated with xenobiotics. Quantitative analysis and kinetic profile determination are under progress.

Table IV: Qualitative IL-6 and TNFα after stimulation with xenobiotics

Xenobiotic	IL-6	TNFα	Xenobiotic	IL-6	TNFα
Soybean oil	++	++	PDC	+/-	++
DNBS	-	+	Diphencyprone	++	-
DNCB	++	-	Croton oil	+	+
DNFB	++	-	Nickel sulfate	-	+
Oxazolone	+	+/-	SDS	++	+

So far the results show that production of IL-6 is not specific to allergenic compounds. Irritant substances also do induce IL-6 secretion by epidermal cells. This has also been shown with human epidermal cells (CR2). Striking results are obtained with TNFα. We could not demonstrate specific induction with sensitizers in the guinea pig model whereas in the mouse model TNFα seems to be preferentially induces with allergenic haptens. Further investigations are necessary to determine if the L929 cell line is able to respond to guinea pig TNF.

UNIVERSITY OF AMSTERDAN (CR2)

Epidermal cells (EC) are prepared from human foreskin. Epidermis/dermis separation, single cell suspension preparation and pulse with haptens are done as described for the guinea pig (CR1). Water-Insoluble haptens have not yet been tested.

Cytokine (IL-1, IL-6, TNFα) induction of epidermal cells "pulsed" with haptens

IL-1 is measured by using the NOB-1 cell line which possesses IL-1receptors (IL-1R). In the presence of IL-1, the NOB-1 cells produce IL-2. IL-2 is measured by the CTLL-2 cell line assay, in a direct (mixture of NOB-1 and CTLL-2) or in a two way (NOB-1 cell supernatants + CTLL-2) assay. So far this assay due to CTLL-2 unresponsiveness, did not work as expected.

Il-6 production was tested with the B9 cell line as described above (CR1). No IL-6 could be detected in supernatant of EC pulsed with water-soluble haptens. As mentionned in the section *Problems encountered* some subclones of the B9 cell line gives unreproducible responses. TNFα production was measured with the L929 cytolytic assay. We could show that DNFB and DNCB were able to induce TNFα in human epidermal cells. Inhibition assay with specific anti-TNF antibodies are under progress.

UNIVERSITY OF MAINZ (CR3)

Cytokine induction and mRNA analysis on cultured keratinocytes after contact with haptens

Production of epidermal cytokines by human keratinocytes (KC) under the influence of contact allergens was measured. Epidermal cells were prepared from human foreskin and expanded for 4 weeks by cell culture techniques. Freshly prepared epidermal cells are activated by the preparation procedures. For this reason long term culture of keratinocytes was established to circumvent this problem. The resulting keratinocytes were stimulated for 10 minutes with a subtoxic concentration (5 μM) of DNFB and cultured subsequently for 1 hour. Culture supernatants were harvested and cytokine-specific bioassays on D10-N4M (IL-1), 7TD1 (IL-6) and Wehi 164 (TNFα) were performed. In addition KC were lysed and total mRNA was prepared. Using specific cDNAs, Northern blot hybridization was used for detection of mRNA of IL-1β, IL-6 and TNFα.

Detection of IL-1 and TNFα was hampered by cytotoxic effects of keratinocyte culture supernatant. IL-1 and IL-6 are produced constitutively in untreated as well as DNFB-treated keratinocytes. This was confirmed by Northern blot analysis of mRNAs. DNFB did not alter the IL-1β and IL-6 response. Nevertheless a strong increase of TNFα was detected after stimulation with DNFB, whereas untreated cells had very low amounts of TNFα-specific mRNA.

Endocytic activity of Langerhans cells after contact with haptens

A second in vitro model was developed to study the influence of contact allergens on the internalization of MHC class II molecules by murine epidermal Langerhans cells (LC). Epidermal cell suspensions were prepared from untreated mice by standard procedures. Langerhans cells which constitute about 1-3 % of the total cell population were specifically labelled with monoclonal antibodies to MHC class II molecules and gold-labeled secondary antibodies. These complexes are internalized spontaneously by untreated LC into large intracellular aggregates, which can be monitored by light microscopy after silver enhancement

or by electron microscopy. Under the influence of contact allergens a diffuse intracellular staining pattern with a pronounced submembranous labelling in numerous endocytotic organelles was seen. This pattern was found for subtoxic concentrations of the tested contact allergens (DNFB, DNCB, diphencyprone) but not for subtoxic amounts of irritants (SDS, Benzalkonium chloride). Toxic doses of the latter compounds result in a diffuse internalization pattern in combination with irreversible cellular damage.

Taking together our present data suggest that in contrast to irritants strong contact sensitizers induce an endocytotic activation of murine Langerhans cells.

HIGHLIGHTS / MILESTONES:

Optimal conditions for *in vitro* testing of soluble and insoluble xenobiotics on bulk epidermal cells were defined.

WIDER CONSIDERATIONS:

Allergic Contact Dermatitis, a distressful disease is induced by substances present in our environment (household and industrial products, drugs, cosmetics, jewellery, plants...etc). Most new molecules, especially those that may enter in contact with skin, have to be tested as pure compounds or in the form of a manufactured product to assess their innocuity. The only available and reliable methods to determine the allergenic potential of a product are laboratory animal experimental sensitizations. These studies require a considerable amount of guinea pigs or mice. The scientific community has become aware that it is necessary to try to reduce laboratory living animals where possible by alternative *in vitro* studies. This is the aim of our investigations and some preliminary results are encouraging and there is good hope that we will soon be able to detect harmful allergenic or toxic substances in a total *in vitro* system using cultured epidermal cells.

COOPERATIVE ACTIVITIES:

Two work meetings were organized with all participants since the official start of the project. The first meeting was held at SANOFI-RECHERCHE (coordinator) in Montpellier, September 16th, 1991. The second meeting took place at SANOFI-RECHERCHE, in Paris, March 9th, 1992.

The coordinator also induced the preparation of a ELWW booklet entitled "In vitro integrated approach to skin pharmaco-toxicology", with two other groups (BRIDGE PL 890177 and BRIDGE PL 890142) working on *in vitro* models of skin biology.

JOINT PUBLICATIONS:

Nothing to report

OTHER PUBLICATIONS/PATENT:

Nothing to report

PHARMACOLOGY AND TOXICOLOGY OF DIFFERENTIATED CELL TYPES, THEIR CELL-CELL AND CELL-MATRIX INTERACTION IN AN IN VITRO RECONSTRUCTED HUMAN SKIN MODEL.
BIOT-CT90-0193-C (DSCN)
Starting date : 1 March 1991

n°1 DUBERTRET L., COULOMB B.- INSERM 312 - Hôp. H. Mondor - 94010 CRETEIL - France (Coordinator)
n°2 LAPIERE Ch.- Univ. Liège - Tour de Pathologie - B23 - 4000 SART-TILMAN - Belgium
n°3 KRIEG Th.- Lab. Dermatology - Univ. Köln - Joseph-Stelzmann St.9 - 5000 KÖLN 41 - Germany
n°4 FUSENIG N.- DKFZ - Im Neuenheimer Feld 280 - 6900 HEIDELBERG - Germany
n°5 GIACOMONI P.- L'OREAL - Recherche Fondamentale- av.E.Schueller - 93601 AULNAY sous BOIS - France

OBJECTIVES SET FOR THE REPORTING PERIOD :
The objective of this program is to develop in vitro reconstructed human living skin models reproducing physiological cellular regulations to determine the mechanisms of these processes and to use this knowledge to develop simpler, but predictive, models allowing drugs screening.
As detailed in our work planning schedule, the first step is to complete the living human skin equivalent with different cell types (normal or pathological) or by adding other components of the extracellular matrix. This step involves the definition of new parameters for analysis and the development of appropriate tools.
The second step is to identify, among the multiple interactions that the skin models allow to modulate, those of pharmacological and toxicological significance and to better understand the mechanisms that are involved. The validation of these skin models is focused on wound healing promoters, antipsoriatic drugs, pigmentogenic, antineoplastic and anti-aging substances.
The third step is the development of simpler predictive models and the transfer to industry.
During the first period of the program, each laboratory progressed mainly in the two first steps, working on its part of the program (cell types, diseases, cell-cell communications, cell-matrix interactions...) and know-how. Exchange of staff, tranfer of methodology, joined experiments were performed in accordance with specific questions of each laboratory. In parallel, the aim was also to compare cell behaviour according to the variations in the protocols of reconstruction of skin equivalents within the different laboratories using a same cell type.

MAJOR PROBLEMS ENCOUNTERED : (Nothing to report)

RESULTS :
PARTICIPANT n°1 :
1/ Fibroblasts. -a) Growth factors and cytokines have been shown to be involved in wound healing. **aFGF** stimulates cell growth and inhibits matrix contraction. Doses, frequency of treatment and cellular response according to the degree of tissue reorganisation can be evaluated in the dermal equivalent (DE), permitting determination of an appropriate treatment. **IL1** and **PDGF** both stimulate fibroblasts growth in monolayer and a matrix slows down this effect. On the contrary, IL1 inhibits matrix contraction while PDGF stimulates it. These results suggest that IL1 and PDGF contribute to the regulation of tissue organization after injury. -b) Joint experiments were made with Th. Krieg lab. (n°3) in order to make the matrix environment more complete by adding proteoglycans. The first proteoglycans studied had no effect on fibroblasts growth and matrix contraction. Various mRNAs are now studied in Th. Krieg lab. -c) We have also compared F behaviour according to the culture conditions of N. Fusenig lab (n°4) and ours.
2/ Fibroblasts and keratinocytes. -TIMP: TIMP was found to stimulate keratinocytes growth. This proliferative effect appears to result from an interaction with keratinocytes membrane and to be unrelated to the metalloproteinase inhibitor function. **-PKC:** While fibroblasts growth is inhibited by a PKC inhibitor, the growth of keratinocytes is stimulated. This difference is reinforced in the presence of EGF. These results suggest that the interaction between PKC and transduction pathway of EGF differs between the two cell types. With the help of Th. Krieg lab., we are now comparing the effect of this PKC inhibitor on mRNA expression of oncogenes, collagen and collagenase by fibroblasts and keratinocytes.

3/ Psoriatic fibroblasts. -KGF: Preliminary experiments showed that KGF could be over-expressed in psoriatic fibroblasts. The aim is now to correlate the expression of KGF with the ability of psoriatic fibroblasts to stimulate normal keratinocyte growth and to study the regulation of KGF mRNA by the collagen matrix. -**Pharmacology and predictability:** We investigate the influence of psoriatic fibroblasts and psoriatic serum on the response of normal keratinocytes to acitretin in the skin equivalent model. Acitretin in the presence of serum of psoriatic patients inhibits keratinocyte growth in vitro, as it does in vivo in the psoriatic plaques. The presence of psoriatic fibroblasts is not necessary for the expression of this effect. Acitretin acts mainly via specific serum factors rather than on the abnormality of dermal-epidermal communications.

4/ Sclerodermal fibroblasts. Comparison between normal and sclerodermal fibroblasts with respect to cell growth and IL6 mRNA expression are now studied in coll. with Th. Krieg lab.

5/ Fibroblasts from keloids. Keloids are abnormal scars anarchically present at the sites of injury. The contraction of DE by fibroblasts isolated from keloids has been shown to be faster and more pronounced than by fibrolasts from a normal skin area of a same patient. In coll. with Ch. Lapière lab. (n°2) we plan to extend these studies to a larger number of strains and to further define the phenotype of fibroblasts from keloids, by comparing their behaviour to that of normal cells, in monolayer and in DE, in response to corticoids and growth factors.

6/ Melanocytes. A collaborative work with l'Oréal (n°5) is planned for April.

7/ In vivo - in vitro correlations. To complete the skin equivalent, wound healing promoters and dressings were studied in an in vivo epidermal wound healing model (suction blister).

PARTICIPANT n°2 :

1/ Modulation of the fibroblasts phenotype by cell-matrix and cell-matrix-cell interactions. The steady-state level of mRNAs for extracellular matrix (ECM) macromolecules (collagens, fibronectin, elastin) is decreased in the retracting dermal equivalent (DE) populated by normal human skin fibroblasts (F) as compared to monolayer conditions and reactivated by the mechanical forces generated by the cells in a restrained DE. Collagenase and stromelysin are inversely regulated. The oncogenes c-fos and c-jun and the mechanisms of signal transduction by PKC are under investigation. Transfection experiments with the regulatory sequence coupled to a reporter gene are hampered by an activation of the endogenous gene of collagenase and a repression of the collagen genes by transfection procedures. Study of the regulation operated by the matrix has been extended to other types of F, gingival (HGF) and periodontal ligament (PDL). The physiological state of differentiation of skin F in the DE is further evidenced by the production of procollagen I N-proteinase undetectable in monolayer cultures. The interactions of F with the ECM also profoundly influence their response to growth factors, as EGF. Normal aging and premature aging F were also studied. In the skin equivalent, human keratinocytes modulate the biosynthetic activity of F through a paracrine secretion. Il-1 α is able to mimick this effect. Tumoral epithelial cells have also been shown to produce heparin-binding factors able to stimulate collagen synthesis by F.

2/ Establishment of cultures of human endothelial (EC) and smooth muscle (SMC) cells and analysis of their phenotypes and differentiation by ECM. EC cultures have been established from human umbilical cord vein. In monolayer they produce mRNAs for type IV and VI collagens, fibronectin, laminin, collagenases I and IV and TIMP1. Whereas they form a monolayer at the surface of a type I or III collagen gel, they organize into a microvascular network when the apical pole of the cell is in close contact with a type I or III collagen gel. This differentiation process is reversible by lifting the collagen gel, does not occur with an agarose gel, is dependent upon serum, active protein synthesis, intact cytoskeleton and is associated with a transitory increased interstitial collagenase mRNA expression. The comparison of the phenotype of human fibroblasts (F), smooth muscle cells (SMC) and myofibroblasts (MYOF) in culture allows to demonstrate that these cells retain some of their identity in vitro.

3/ Measurement of mechanical forces as a pharmacological tool. The interactions between the cell and the supporting collagen gel is expressed by the development of mechanical forces resulting in the retraction of the gel. A device based upon the use of strain gauges was designed to measure these forces and use this parameter to monitor pharmacological agents able to influence these cell-matrix interactions. All the cell types studied were able to develop mechanical forces although with variable intensity (SMC > MYOF > F; F > EC; HGF > PDL) and responded by decreased (F: corticosteroids, cytochalasin B, cycloheximid) or increasd (SMC: 5-HT, nor-epinephrin antagonized by ketanserin) mechanical activity.

PARTICIPANT n°3 :

The main topic is to study cell matrix interactions in vitro. Previous data indicate that cell matrix interactions substantially change metabolic activities of fibroblasts. So far, an extracellular matrix consisting mainly of collagens types I and III was used in our studies. When cells were embedded within this matrix fibroblast collagen type I synthesis was reduced to approximatively 10% compared to control values of cells maintained on plastic culture dishes. In contrast, collagenase synthesis and activity was significantly induced versus monolayer cultures.

1/ Extracellular matrix constituents. In order to obtain a more physiological extracellular matrix in vitro (thus resembling connective tissue in vivo) several other components have been considered in their effect on dermal cells. The objective of our current efforts is to elucidate the impact of proteoglycans added to collagen gels on modulating cellular responses compared to plain collagen gels. This interest derives from the observation that in extracellular matrix, apart from collagenous proteins, proteoglycans are major constituents of the connective tissue. The effect of modulating cell matrix interactions by this group of molecules has not been studied in detail. In addition of analyzing collagen type I and collagenase regulation in fibroblasts, several other parameters are to be examined. Regulation of extracellular matrix receptors such as the hyaluronic acid (HA) receptor (CD44), cytokine expression (IL1, IL 6) and cytokine receptors (IL1 R, PDGF R) are most interesting markers of cell activation states. This basic work should enable us to distinguish effects of added cytokine and growth factors from changes already elicited by the extracellular matrix components.

2/ Signal transduction. In initial experiments the effect of several different glycosaminoglycan side chains on collagen gel contraction were examined in joined experiments of B.Coulomb's lab. (n°1) and ours. However no striking effects could be demonstrated. Future work is now progressing to identify changes on mRNA steady-state levels for the HA receptors and IL1, IL6, IL1 R and PDGF R due to glycosaminoglycan side chains.

In parallel, work has been started to elucidate the molecular basis of cell matrix interactions and signalling mechanisms. We were able to demonstrate that integrin receptors are involved in the contraction process of collagen type I gels. However, signal transduction pathways mediated by collagen type I and integrin interactions are unknown so far. One biochemical mechanism involved in signal transduction is represented by post-translational modification of proteins. Especially, phosphorylation and dephosphorylation on tyrosine residues play a major role. Based on this knowledge, signal transduction in the contraction model is being studied by looking at tyrosine phosphorylation/dephosphorylation and tyrosine kinase/phosphatase activities in the course of contraction. Techniques required, such as western blotting with anti-phosphotyrosine antibodies, in vitro kinase assays, and co-precipitation assays are now established.

PARTICIPANT n°4 :

1/ Standardization of the basic organotypic coculture model and its comparison and adaptation with those of the other partners. By modified isolation and preparation techniques the collagen matrix was standardized in its collagen concentration, thickness and stability. After seeding fibroblasts, such matrices organized to a DE without considerable contraction when they were placed on metal grids and mounted in CRD culture chambers. Large amounts of keratinocytes and fibroblasts could be obtained from the same skin specimen and stored in liquid nitrogen to be used in large series of experiments. More important, endothelial cells from small blood vessels of the upper dermis could be isolated in good purity, characterized by functional parameters, propagated in culture to obtain large cell numbers, and stored deep-frozen.

2/ Analysis of the effect of co-cultured capillary endothelial cells in comparison with fibroblasts on growth and differentiation of normal skin keratinocytes. The effect of cocultured endothelial cells on keratinocytes was studied under different culture conditions. When used as irradiated (and thus non-proliferative) feeder layer cells, dermal capillary endothelial cells promoted clonal growth of keratinocytes at a higher rate as compared to dermal fibroblasts. After embedding in collagen gels, the effect on keratinocyte growth and differentiation was comparable to that of fibroblasts. There was first a strong stimulation of proliferation indicated by a rapid increase in cell number followed by the formation of organized epidermal strata. Within seven days a rather normal epidermal structure was formed and the cells synthesized the typical differentiation markers : keratin 1 and 10, involucrin, filaggrin. The aberrant localization of these components indicated that this reproducibly obtained in vitro epidermis is more comparable to a hyperplastic, irritated or regenerating skin. Thus, this culture

type may serve as a pharmacological model for diseased skin. By modulating mesenchymal cell number and functional state in the matrix, an equilibrium between growth and differentiation of the keratinocytes will be attempted. Results from transplantation of such cultures onto nude mice indicated a rather complete normalization of the reformed epidermis within one to two weeks.

3/ Study of the response of immortalized keratinocytes (HaCaT cell line) and tumorigenic subclones on fibroblast interaction in vivo and in vitro. The typical response of the HaCaT keratinocyte cell line to mesenchyme-mediated regulatory signals could be further substantiated in surface transplants on nude mice. In vitro, on collagen matrix HaCaT cells can also reform an epidermal structure depending on the influence of cocultured fibroblasts in the matrix. The tissue organization, however, is not as well manifested as with normal keratinocytes, so that further modifications of the model are presently underway to improve this situation.

Migration on collagen and chemotactic response are well maintained in HaCaT cells, as could be demonstrated in a cooperative study with partner 3.

Tumorigenic HaCaT-ras subclones have lost most of their dependence on cocultured fibroblast stimulation for proliferation and stratification in feeder layer and organotypic cultures. However, tissue organization seemed to be improved by cocultured fibroblasts, indicating a normalizing effect comparable to that seen in early stages of transplants. This indicates that HaCaT cells and the tumorigenic variants still respond to mesenchymal cell interaction.

PARTICIPANT n°5 :

It was established that melanocytes and keratinocytes interact in culture, but the cellular and molecular mechanisms of this interaction are unknown.

When the two cell types are cultivated separately in the same medium, growth and proliferation of melanocytes requires the supplementation of melanocyte growth factors. In the absence of these factors, melanocytes survival requires the contact with keratinocytes. We have observed that in this case the two cell types organise themselves and reconstruct the epidermal melanin unit (EMU). This EMU constituted by a melanocyte and a pool of associated keratinocytes, in which the melanocyte synthesizes melanin and melanosomes, and the latter are transfered to keratinocytes and modified within them. In vivo, the melanocytes associates with keratinocytes in a ratio of about 1:36. Preliminary results indicates that adding melanocytes to confluent keratinocytes in the ratio 1:10 allows one to observe the cohabitation of these two cell types. One function of the EMU is to protect the skin from the detrimental effects of external stimuli. Exposures to external stimuli modify the EMU (synthesis of melanin, melanosomes and number of functional melanocytes).

The chemical composition of the culture media, the state of proliferation of the two cell types at the moment of the contact and the ratio of the number of melanocytes/keratinocytes had first to be defined to permit the realisation of this coculture in vitro.

These studies were realized with homologous or heterologous cells from different ethnic origins. For each culture condition we have observed the melanocyte position during the differentiation of the keratinocytes.

In culture of melanocytes we can quantify the variation of intracellular melanin after the application of external stimulation by spectrophotometry and by the kinetic of incorporation of radioactive precursor of melanin synthesis. The evaluation of the melanocyte number can be measured using specific antibodies.

With participant n°1, we plan to cultivate melanocytes and keratinocytes on dermal equivalents and to reproduce a functional epidermal melanin unit which respond to external stimulation. By these studies we want to understand the role of dermal fibroblasts in the EMU organisation.

HIGHLIGHTS / MILESTONES :

Cell-cell communications: The importance of fibroblasts-keratinocytes interactions was already demonstrated. Capillary endothelial cells are also implicated in this dermal-epidermal dialogue.

Cell-matrix interactions: The mechanical forces developed by cells through interactions with the extracellular matrix are important biological regulators of the cell phenotype and are most significant in organogenesis in vivo and in many pathological processes.

Predictability: The skin equivalent can be used as a predictive model for antipsoriatic efficiency of retinoids when the serum used in vitro comes from psoriatic patients.

WIDER CONSIDERATIONS :

Even if some "skin equivalents" are on the market (Organogenesis, Marrow Tech), they are not

universal. A permanent research is necessary to develop, adapt and validate in vitro models, in order to obtain a predictability of the results for humans and progressively replace animal experimentation. The human in vitro reconstructed living skin equivalent can be considered as the most advance prototype of a reconstructed organ. It can serve as an example to develop blood vessels, tendons, periodontal ligaments, etc ... for studying cellular physiology, creating new pharmacological tools and building organs for replacement therapy.

COOPERATIVE ACTIVITY :

MEETINGS :

02/07/91 : n°3 and n°4 in Cologne
11/10/91 : n°1 and n°3 in Paris
17-18/12/91 : n°1 and n°3 in Cologne
25/01/92 : n°1, n°2 and n°3 in Cologne
31/01/92 : n°4 and n°5 in Aulnay
24/06/91 : n°2 and n°5 in Chevilly-Larue
12-13/09/91 : n°1 and n°2 in Reims
16/10/91 : **Plenary meeting in Créteil**
20/01/92 : n°2 and n°3 in Cologne
30/01/92 : n°1 and n°4 in Paris
28/02/92 : n°1 and n°5 in Paris

EXCHANGE OF STAFF :

-n°1 : 02-06/12/91; 16-20/12/91 and 10-14/02/92 : n°1 -> n°3 (Joint experiment in Cologne)
-n°2 : January-February 92 : n°2 -> n°5 (learning techniques in Heidelberg)
-n°3 : 25-29/11/91; 06/12/91 and 19-20/12/91 : n°3 -> n°1 (Joint experiment in Créteil)
-n°4 : 18-21/06/91 : n°4 -> n°1 (Joint experiment in Créteil)

EXCHANGE OF MATERIAL / METHODOLOGY :

n°1 -> n°3 and n°4 : Procedure for fibroblast growth evaluation within a matrix.
n°2 -> n°3 : exchange of fibroblasts cultures (03/10/91 and 02/03/92).
n°3 -> n°1 : mRNA extraction from fibroblasts embedded within a collagen matrix.

LIST OF PUBLICATIONS WITH TRANS-NATIONAL AUTHORSHIP :

-n°1 : BERTAUX B., HORNEBECK W., EISEN A.Z. and DUBERTRET L.: Growth stimulation of human keratinocytes by tissue inhibitor of metalloproteinase. J Invest Dermatol 97: 679-685, 1991.
-n°2 : DELVOYE P., WILIQUET P, LEVEQUE J.L., NUSGENS B. and LAPIERE Ch.: Measurement of mechanical forces generated by skin fibroblasts embedded in a three-dimensional collagen gel. J Invest Dermatol 97: 898-902, 1991.
-n°2 : COLIGE A., ROUJEAU J.C., DELAROCQUE F., NUSGENS B. and LAPIERE Ch.: Abnormal gene expression in skin fibroblasts from Hutchinson-Gilford patient. Laboratory Investigation 64: 799-806, 1991.
-n°2 : LAMBERT C., SOUDANT E.P., NUSGENS B. and LAPIERE Ch.: Regulation of extracellular matrix macromolecules and collagenase synthesis at a pretranslational level by mechanical forces in collagen lattices. Laboratory Investigation (in press).
-n°3 and n°4 : SHARFFETTER-KOCHANEK K., KLEIN C.E., HEINEN G., MAUCH C., SCHAEFER T., ADEMANN-GRILL B.C., GOERZ G., FUSENIG N.E., KRIEG Th. and PLEWIG G.: Migration of human keratinocyte cell line (HACAT) to interstitial collagen Type I is mediated by a2B1-integrin receptor. J Invest Dermatol 98: 3-11, 1992.

OTHER PUBLICATIONS :

-n°1 : DUBERTRET L., BRUNER-FERBER F., MISITI J., THOMAS K.A. and DUBERTRET M.L.: Activities of human acidic fibroblasts growth factor in an in vivo dermal equivalent model. J Invest Dermatol 97: 793-798, 1991.
-n°1 : SANQUER S., COULOMB B., LEBRETON C. and DUBERTRET L.: Effect of acitretin on epidermal growth in a human skin model containing fibroblasts and serum from psoriatic patients. (submitted).
-n°2 : COLIGE A., NUSGENS B. and LAPIERE Ch.: Altered response of progeria fibroblasts to epidermal growth factor. Journal of Cell Science 100: 649-655, 1991.
-n°2 : NOEL A., MUNAUT C., BOULVAIN A. CALBERG-BACQ C.M., NUSGENS B., LAPIERE Ch. and FOIDART J.M.: Modulation of collagen and fibronectin synthesis in fibroblasts by normal and malignant cells. Journal of Cellular Biochemistry 48: 150-161, 1992.
-n°2 : COLIGE A., LAMBERT C., NUSGENS B. and LAPIERE Ch.: Effect of cell-cell and cell-matrix interactions on the response of fibroblasts to EGF in vitro: expression of collagen type I, collagenase, TIMP and stromelysin genes. Biochemical Journal (in press).
-n°2 : NOEL A., MUNAUT C., NUSGENS B., FOIDART J.M. and LAPIERE Ch.: The stimulation of fibroblasts collagen synthesis by neoplastic cells is modulated by the extracellular matrix. Matrix (in press).
-n°4 : BREITKREUTZ D., BOUKAMP P., RYLE C.M., STARK H.J., ROOP D.R. and FUSENIG N.E.: Epidermal morphogenesis and keratin expression in c-Ha-ras-transfected tumorogenic clones of the human HaCaT cell line. Canc. Res. 51: 4402-4409, 1991.
-n°4 : MACKENZIE I., RITTMAN G., BOHNERT A., BREITKREUTZ D. and FUSENIG N.E.: Influence of connective tissues on the in vitro growth and differentiation of murine epidermis. Epithelial Cell Biology (in press).
-n°4 : SMOLA H., THIEKÖTER G. and FUSENIG N.E.: Human dermal microvascular endothelial cells (HDMEC) stimulate growth and differentiation of skin keratinocytes in vitro. (submitted).

TITLE: Establishment of immortal differentiated hepatocyte lines from transgenic mice and their use for studying viral and chemical agents.

CONTRACT NUMBER: BRIDGE BIOT CT90 0189.

OFFICIAL STARTING DATE: March 1, 1991.

PARTICIPANTS: Prof. Dieter Paul, Fraunhofer Inst. Hannover, FRG

Dr. C.Guguen-Guillouzo, INSERM 49, Rennes, France

OBJECTIVES SET FOR REPORTING PERIOD: To establish one adult hepatocyte line from transgenic SV-202 mice and initiate characterization of established hepatocyte line BGK-202. Transfection of cells using the CaPO4-DNA coprecipitation with the HBV genome and studies on the control of the cell cycle in these cells.

MAJOR PROBLEMS ENCOUNTERED:

- The need to obtain new tools more efficient than thymidine incorporation for defining the differences between normal and immortalized hepatocytes. They include identification of cdc2, CDK2 and cyclin A and B kinase expression in the cells.

- Transfection of liver cells using the CaPO4-DNA coprecipitation technique causes high toxicity to hepatocytes and the yield of tansfectants is therefore low. This is presumably due to alterations of intracellular Ca++ in hepatocytes, to which hepatocytes have been shown to extremely sensitive. Alternative techniques are being tested, including electrporation.

- Morphological criteria suggest that cell populations of the established line BGK-202 are heterogeneous.

- Fetal hepatocyte line FMH-202 is useful for assays to identify indirectly acting mutagens. However, highly active phase II enzyme activities appear to render genotoxic metabolites of aflatoxin B1 and cyclophosphamide inactive and can therfore not be assayed by employing chromosome aberrations as an endpoint. In contrast, induction of sister chromatid exchanges can readily be identified, presumably because distinct genotoxic metabolites initiate SCEs as compared with chromosome breaks (clastogenic actovity).

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 characterize the control of the cell cycle of adult line BGK-202, it was demonstrated that cells express the kinases cdc2, CDK2 and cyclins. Sequential phosphorylation processes were shown that indicated that cdc2 clearly participates in the control of G2/M but not in G1/S transition. Attempts are being made to obtain stably transfected cells using the complete HBV genome. Fetal transgenic mouse cell line FMH-202 was employed as target for assaying indirectly acting mutagens in attempts to set up a short-term toxicity assay for the identification of indirectly acting mutagens. It was shown that the fetal line lends itself for such tests, however further validation is needed for standardizing the assay systems for routine use.

1) Establishment of an adult hepatocyte line from transgenic SV-202 mice

An adult transgenic hepatocyte line was established from a 12 week old SV-202 mouse. As expected, the line is not clonal, since cells require neighbors, i.e. cell-cell contacts for growth and maintenance of differentiated hepatic functions. The cells grow slowly in MX-83 medium free of serum in response to EGF and insulin. Initially, transfers can only be conducted once every 12 - 16 days. After about 4 passages, the cultures apear homogeneous as judged by morphological criteria. Initial data suggest that the cells multiply every 48 hours, however transfer efficiency is, as expected on the basis of earlier experiments, quite low. Additional work has to be conducted to characterize the general properties of the cell line when it has adapted well to the culture conditions and can be passaged without great loss of cells.

2) Characterization of the fetal transgenic mouse hepatocyte line FMH-202 as a target cell in short-term test systems for the identification of for indirectly acting mutagens.

FMH-202 Cells were employed for experiments to assay for the genotoxic activity of indirectly acting mutagens including Dimethyl-benzanthracene (DMBA), Benzo(a)Anthracene (BaA), cyclophosphamide (CP) and aflatoxin B1 (AFB1). Few known cell lines are

endowed with metabolizing enzyme activities that are required for the activation of each one of these compounds to generate genotoxic metabolites which are responsible for the induction of mutations, and no cell line has been described that responds to these compounds by metabolizing them into genotoxiic intermediates. Endpoints chosen in our assays were both clastogenic activity as measured by chromosome aberations and the induction of sister chromatid exchanges (SCEs).

All four compounds used in this study clearly induced SCEs at low, non-cytotoxic concentrations in the culture medium. However, only DMBA and BaA, however not CP or AFB1, induced chromosome aberrations. Since the cells produce metabolites of these compounds and since the cells express phase II-enzymes (sulfatase, glucuronidase), the genotoxic intermediates of these compounds that were presumably generated are inactivated quickly and before they can interact with DNA to form adducts. Form these preliminary data it appears that cell line is appropriate for detecting indirectly acting mutagens of different classes without the need of their metabolic activation in vitro, e.g. by S9 liver extracts or by preincubation with freshly isolated hepatocytes in primary culture prior to be added to the target cell culture. Additional work is being conducted to define the suitability of this cell line for short-term in vitro toxicological test systems.

3) Characterization of hepatocyte line BGK-202 with respect to cell growth regulation to define alterations of the cell cycle controls that lead to progression towards immortalization.

For this purpose we have developed tools and experimental conditions for determining the kinase proteins which are known to control G1/S and G2/M transition, the two main limiting steps of cell cycle progression. Little is known this general field concerning eukaryotic cells 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. We have clearly shown hat cdc2 participates in the control of G2/M transition, like in many other cell types, but not to that of G1/S transition. Furthermore, we have shown that CDK2 could play a critical role in G1/S transition. Moreover, we have defined the sequential expression of various oncogenes, particularly those normally expressed during G1, plus p53 tumor suppressor gene which could be, together with the Rb protein, a substrate for the cdc2 kinase. Abnormal p53 expression has frequently been reported for human hepatocarcinoma. A manuscript is in preparation on this topic. Experiments are in progress for anlayzing expression and activity of these kinases in the BGK-202 hepatocyte line.

4) Use of immortalized hepatocyte line BGK-202 to analyze mechanisms by which HBV favours development of malignant cells.

Transfection of the BGK-202 cell line using the CaPO4-DNA coprecipitation procedure has met with limited success and/or reproducibility. This has led us to analyze the efficiency of electropo-

ration to increase efficiency in obtaining transfectants. Transfections of the complete HBV DNA genome into a new human hepatocyte hepatoma line HBG, which fails to grow in soft agar and to develop tumors in nude mice, have been performed. The experiments show an increase in c-myc expression in transfected cells and not in non-transfected cells. The same induction was also observed when HBX gene was transfected, suggesting that c-myc could in part be involved in the mechanism by which HBV induces hepatocellular carcinoma.

HIGHLIGHTS/ MILESTONES: Establishment of a new adult transgenic hepatocyte line:

WIDER CONSIDERATIONS: Novel differentiated liver cell lines were established for immediate use in biotechnology, virology, biochemistry and toxicology. The cells appear to be useful for short-term toxicological test systems in vitro.

COOPERATIVE ACTIVITIES: No meetings were organized during the initial year of the contract. During the second and third years at least three meetings including exchanges of pe rsonnel are being prepared.

PUBLICATIONS:

SALASSIDIS, K., KULKA, U., PAUL, D., & BAUCHINGER, M.. Induction of chromosome aberrations and sister chromatid exchange by indirectly acting mutagens in immortal mouse and rat hepatocyte lines. Mutagenesis 6:59-63 (1991)

O'SULLIVAN, J.F., SCHMIDT; G.H., & PAUL, D. Spontaneous mutation rate in the mouse. Nature 352:200-201 (1991)

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., & Guillouzo, A.Eds. Les Editions INSERM, Paris.John Libey Eurotext, London, 1992. Vol. 216, pp. 277-285.

TÖNJES, R.R., XANTHOPOULOS,K.G., DARNELL, J.E. & PAUL, D. Transcriptional control in hepatocytes of normal and c14CoS albino deletion mutant mice. EMBO J. 11:127-133 (1992).

DIOT, C., GRIPON, P., RISSEL, M., & GUGUEN-GUILLOUZO. Replication of hepatitis B virus in differentiated adult rat hepatocytes transfected with cloned viral DNA, J. Med. Virol. (in press).

DEVELOPMENT OF AN IMMORTALIZED HUMAN ARTICULAR CARTILAGE CELL LINE AND ITS USE IN PHYSIOLOGICAL, PHARMACOLOGICAL AND TOXICOLOGICAL IN VITRO INVESTIGATIONS

CONTRACT NUMBER: BIOT 0196
OFFICIAL STARTING DATE: 01-03-1991
COORDINATOR: EM.VEYS, Ghent University Hospital, B-9000 GENT.

PARTICIPANTS:
EM VEYS, G VERBRUGGEN, Ghent University Hospital, B-9000 GENT.
M ADOLPHE, Ecole pratique des hautes études, F-75230 PARIS.
L DE RIDDER, University of Ghent, B-9000 GENT.
GS PANAYI, Guy's and St.-Thomas Medical School, LONDON, UK.
A CLOAREC, Laboratoires UPSA, F-92506 RUEIL-MALMAISON.

OBJECTIVES SET FOR THE REPORTING PERIOD:
The aim of this EEC-BRIGDE project was to study the possible use of in vitro cultured phenotypically stable human cartilage cells in pathophysiological, pharmacological and toxicological investigations.
Functional characterization of this cell line involved the study of synthesis, secretion and turn-over of proteoglycan and collagen in an artificial extracellular matrix. To this end, the metabolic functions of the human chondrocyte maintained in suspension culture had to be characterized.
The validity of this system for the study of the effects of disease mediators (cytokines, O^{-}_{2}-radicals, co-cultured macrophages) and of pharmacological substances on mediated chondrocyte metabolism had to be determined.
In order to obtain full control of the environment and complete standardization of this system, it had to be established in how far single or combined growth factors could replace serum in the incubation medium.
Cartilage cells were obtained from human subjects with spontaneously occurring or from animals with experimentally induced degenerative and inflammatory joint diseases. It had to be determined whether an in vivo observed abnormal function of these cells persisted in vitro.
Since such cells are difficult to obtain, it was decided to construct immortalized human chondrocyte cell lines. To our knowledge, human articular chondrocyte immortalization has never been done. Because of a variety of control mechanisms, such as an efficient repair potential, the genetic apparatus of the human cell is relatively stable compared to that of most other animal species. It was necessary to study the problems inherent in the immortalization and propagation of the human chondrocyte in particular and of human cells in general. To this end, several clones of immortalized human cartilage cells had to be developed. It had to be defined in how far different clones of immortalized and transformed human chondrocytes differed from the original non-immortalized cell.
The preservation of the original (in vivo) functions with respect to the homeostasis of extracellular matrix macromolecules in an in vitro model (culture in different gelified matrices) had to be studied.
Fully characterized immortalized chondrocyte cell lines may be

an interesting tool for researchers in university or industrial laboratories. Consequently, the possibility to preserve these cells at -180°C in a chondrocyte bank had to be evaluated.

MAJOR PROBLEMS ENCOUNTERED:
One of the major difficulties encountered was the fact that after the initial transformation the cells remained in a 6 to 9 months long latency or 'crisis' period. It took almost a year to obtain the first clones of immortalized normal human articular cartilage cells.
Attempts to immortalize human and animal chondrocytes from diseased cartilage (human osteoarthritic cartilage, adjuvant arthritis rabbit cartilage) had to be postponed.
Since chondrocytes in articular cartilage of patients with rheumatic conditions may be involved in the pathogenesis of autoimmune diseases (antigen presentation), it was decided to focus on chondrocytes obtained from rheumatoid arthritis cartilage. However, so far it has been impossible to isolate sufficient numbers of these cartilage cells.

RESULTS
Several batches of human articular chondrocytes were isolated at autopsy from articular cartilage of human femoral condyles. The investigations performed in 1991 resulted in :

1- DEVELOPMENT OF A PROTOCOL FOR BIOLOGICAL FREEZING AND PRESERVATION OF VIABLE CHONDROCYTES AT -180°C.
A collection of 'viable chondrocytes' is a useful tool when human articular cells of different origin (normal and various inflammatory, metabolic or inherited diseases) are studied. Having access to 'the bank' obviates the researcher's dependence on the availability - at a particular moment - of a certain type of cell. A biological freezing program for human lymphocytes was slightly adapted to the needs and used to test the effects of dimethylsulfoxide (DMSO) as a protective agent on human articular chondrocytes suspended in a semisynthetic culture medium. Five percent of DMSO were found to be an optimal concentration. Approx. 80% of viable cells were recovered at this concentration. ^{35}S-incorporation in newly synthesized proteoglycans was studied in cells recovered from -180°C and in the control cultures. The cryopreservation procedure did not affect proteoglycan synthesis rates or the proportions of the different proteoglycan subtypes (Table I).

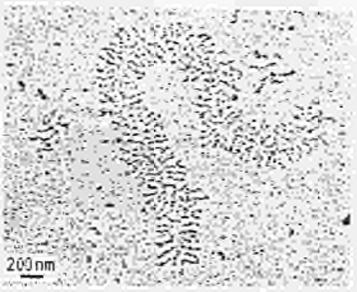
TABLE I: PROTEOGYCAN SYNTHESIS (picog SO^4incorp/10^6cells/hour) AND PROTEOGLYCAN SUBTYPES (%).

	total PG	%-aggr	%-mono	%-debr
controls	2628 (14.6)	42.6 (9.7)	36.5 (8.5)	20.9 (6.9)
after-180°C	2526 (9.6)	44.5 (6.5)	35.8 (7.8)	19.8 (3.4)

aggr: PG-aggregates; mono: PG-monomers; debr: turn-over products and low mol weight PG; coefficients of variation between ( ).

2- CHARACTERIZATION OF THE METABOLIC FUNCTIONS OF THE HUMAN CHONDROCYTE CULTURED IN SUSPENSION CULTURE IN AGAROSE

Human chondrocytes were shown not to divide in 1.5 % of gelified agarose. They continued to synthesize proteoglycans (PG) at constant synthesis rates for at least 8 weeks.
Three subpopulations of PG were recovered from the agarose gels and from the incubation media: PG aggregates, monomeric PG and low molecular weight material (turn-over products, minor PGs); PG aggregates consisted of monomeric PG linked on a hyaluronan backbone, as was confirmed by electron microscopic analysis of the PGs (fig 1).



1 2

Fig 1: electron microscope picture of native de novo synthesized PG. Fig 2: chondrocyte cultured in agarose with Alcian blue fixed PG surrounding the cell.

Over 90% of the PG-aggregates were immobilised in the gel and remained in the immediate vicinity of the chondrocyte (fig 2). Approx. 50% of monomeric PG and the bulk of the turn-over material were lost in the incubation medium.
The biochemistry of the synthesized extracellular matrix macromolecules shows that these cells remained in their differentiated state in the ex vivo condition. The immobilization of the newly synthesized PGs in the artificial surrounding matrix was comparable with that of in tissue cultured cartilage.
It is an interesting finding that the total ^{35}S-incorporation rates correlated with the age of the donor. Others have found that PG synthesis rates decline with age in human and in rabbit cartilage tissue culture. The results of these experiments allowed to conclude that articular cartilage cells keep their in vivo ^{35}S-PG-synthesis rates in this culture condition.
The total PG-synthesis rate of the chondrocytes was comparable with values observed in vivo in humans.

3- STANDARDIZATION OF THE CULTURE CONDITIONS - COMPLETELY DEFINED CULTURE MEDIA.

Routinely, cells or tissues are grown in vitro in semisynthetic nutrient media. In order to supplement growth and differentiation factors, fetal calf serum is added. Fetal serum contains these factors in variable and unknown concentrations.

In a series of experiments fetal calf serum (FCS) was replaced by a single growth factor or a combination of these biological mediators. In the absence of FCS, PG synthesis rates dropped to about 10-15% of the values obtained in the presence of serum. 0.1% of bovine serum albumin did not improve metabolic activity.

Human recombinant insulin, at concentrations ranging between 10.0 and 100.0 nG per ml, increased PG synthesis rates to about 60 % of the values obtained with FCS. Higher concentrations did not result in further improvement. Both insulin-like growth factor I and II were unable to enhance the effect of insulin. This was not surprising, considering that these three factors bind to the same receptors.

Transforming growth factor beta (TGF-b), at concentrations of 10 nG per ml, caused an increase in PG synthesis rates to about 50 % of the values obtained with FCS. In combination with 100 nG/ml of insulin, 10 nG of TGF-b completely restored the original PG synthesis rates.

4- STUDY OF THE INFLUENCES OF VARIOUS BIOLOGICAL MEDIATORS AND SOME PHARMACOLOGICAL AGENTS ON CHONDROCYTES.

Chondrocyte metabolism is known to be affected by various cytokines and drugs (i.e. NSAIDs).

Interleukin(IL)-1 stimulates PG catabolism and decreases PG synthesis of chondrocytes cultured in agarose in a dose-dependent manner. IL-1 dramatically enhances the release of IL-6 by these chondrocytes. More than 100,000 pg of IL-6 per ml of incubation medium are found when the chondrocytes are stimulated with 1 U of IL-1/ ml. IL-6 has no effect on chondrocyte PG synthesis.

Tumor necrosis factor-a also stimulates PG catabolism and decreases proteoglycan synthesis of chondrocytes cultured in agarose in a dose-dependent manner. The effects are less pronounced than those of IL-1.

Human recombinant interferon-g (IFN-g) inhibits PG-synthesis. PG-catabolism is not affected.

NSAIDs were tested in this culture model. The inhibitory effects of salicylate (SAL), acetylsalicylic acid (AC-SAL) and niflumic acid (NA) on PG-synthesis were reproduced. Piroxicam (PIR) did not seem to depress PG synthesis by these cells.

5- DEFINITION OF THE NONIMMORTALIZED CARTILAGE CELL.

In order to analyse any changes in cell behaviour, or loss of specific functions after immortalization the 'normal' chondrocyte has to be defined. In table II the functions of the cartilage cell are listed. These functions will be tested in immortalized chondrocytes.

TABLE II: METABOLIC FUNCTIONS OF NORMAL HUMAN CHONDROCYTES

EXPORT PRODUCTS	PROTEOGLYCAN				COLLAGEN TYPES		
	CS	KS	DS	AGGR	I	II	IX
	++	++	--	++	-	+	nd

INFLUENCES	IL-1	TNF	IFN	IL-6	SAL	A-SAL	NA	PIR	MNC
PG-SYNTHESIS :	--	--	--	=	--	--	--	=	--
PG-CATABOLISM :	++	++	=	=	=	=	=	=	++
PGE_2-RELEASE :	++	nd	nd	nd	--	--	--	--	nd
IL-6-RELEASE :	++	++	nd	---	---	---	---	---	++
PROLIFERATION :	--* ++**	nd	--	nd	---	---	---	---	---

CS: chondroitinsulfate; KS: keratansulfate; DS: dermatansulfate; AGGR: aggregates. =: no effect; nd: not done; ---: irrelevant; * 'young' monolayer cultures; ** 'old' monolayer cultures

6- STUDIES OF THE SUITABILITY OF THIS CELL FOR RESEARCH ON CELL-CELL (MACROPHAGE-CONNECTIVE TISSUE CELL) INTERACTIONS IN PATHOLOGICAL CONDITIONS.

For co-culture, 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 peripheral blood monocytes (MNC) are grown. The chondrocytes (in agarose) are thus maintained on top of the MNC (fig 5). Bacterial lipopolysaccharides (LPS) and IFN-g are used to activate these cells. Activated MNC release IL-1, TNF-a and other biologically active compounds.

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 nutrient medium, their PG-metabolism is greatly disturbed. They stop synthesizing PG and release metalloproteinases which destroy the formerly synthesized PG.

7- DEVELOPMENT OF IMMORTALIZED CHONDROCYTE CLONES ORIGINATING FROM DIFFERENT HUMAN SUBJECTS.

Transfections were performed on human chondrocytes originating from 7 different donors. Cells were named AMA, PAPY, HUM, DEK, DAC, PIF and PAF. The calcium phosphate mediated DNA transfection procedure with plasmid encoding for various oncogens was used. Cells were co-transfected with a plasmid carrying the Neo gene to allow the selection of successfully transfected cells in the presence of G418. G418 treatment was continued on for at least 3 weeks.

One of the major difficulties was the fact that after the initial transformation, the cells remained in a 6 to 9 month latency or 'crisis' period.

Three batches of cells (AMA, PAPY, HUM) were transfected with a plasmid carrying the temperature sensitive SV40 large T antigen.

AMA cells: After a 9-month latency period, 11 clones emerged from the transfected cells (transfection efficiency $2x10^{-6}$). 10 of these clones have been isolated but have not adhered to the culture dishes as yet.
PAPY cells: After a 6-month latency period, 21 clones emerged from the transfected cells (transfection efficiency $3x10^{-6}$). 15 of these clones have been isolated and expanded.
HUM cells: After a 6-month latency period, 30 clones emerged from the transfected cells (transfection efficiency $5x10^{-6}$). 27 of these clones have been isolated and 14 of them expanded.
Two batches of cells (DEK, DAC) were transfected with a plasmid encoding the SV40 large T antigen. After transfection these cells failed to adhere to the plastic culture dishes. Immortalized clones have not been detected as yet. PIF and PAF cells were transfected with a plasmid encoding the c-myc and the Polyoma large T antigen respectively. Immortalized clones have not been detected as yet.
The characterization of the clones isolated from PAPY and HUM cells was started recently. SV40 large T antigen was detected by immunofluorescence. For some clones, 100% of the cells were intensively labeled. For others, labeling intensity varied according to the observed fields.
Indirect immunofluorescence staining for type II and I collagens showed a wide variety of phenotypes (table III). These results will be confirmed by the study of the RNA transscripts. Biochemical characterization of the collagen types and of the PG synthesized by the chondrocytes is in progress.

TABLE III :	COLLAGEN TYPE		T-ANTIGEN
	COLL II	COLL I	
CLONES (5th or 6th passage)			
HUM-1,-15; PAPY-12,-14,-15	+	-	++
HUM-9 ; PAPY-6	+	-+	-+
HUM-18,-23,-24; PAPY-4,-10	-+	-/-+	++/+
PAPY-7	-+	-+	-+
HUM-2,-3,-16,-25;			
PAPY-1,-2,-3,-5,-11	-	-	++/+
HUM-19, PAPY-8,-9,-19	-	-	-+

The metabolic properties (degree of transformation) of these cell lines are currently investigated.

HIGHLIGHTS/MILESTONES
To our best knowledge, these are the first human articular cartilage cell (chondrocyte) lines. The cells are cultured as phenotypically stable articular cartilage chondrocytes in a suspension culture system. They export extracellular matrix products typical of articular cartilage.

WIDER CONSIDERATIONS
Immortalized human articular cartilage cells (chondrocytes) can be used in in vitro assay systems for the study of physio(patho)logical processes in human diseases e.g. the destruction of joints in rheumatic disorders. Both beneficial and deleterious effects (toxicology) of pharmacological substances on cartilage cells can be investigated in such a model. The need of using experimental animal models could be partly obviated.

Title:

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.

Contract No:

BIOT-CT91-0266

Official Starting Date: 1 March, 1991

Coordinator: Dr Peter Bach, Polytechnic of East London, London, UK.

Participants:

Dr. Jacque Diezi, University of Lausanne, Lausanne, Switzerland.

Dr. W Lentzen, Dr. Madaus AG, Koln, Germany.

Dr. Jean-Paul Morin, University of Rouen, France.

Dr. Walter Pfaller, University of Innsbruck, Austria, (Austrian Research Council P-7968 MED).

Dr. Michael P. Ryan, University College Dublin, Dublin.

Dr. Mechthild Soose, University of Giessen, Germany

Dr. Hilmar Stolte, Medical School Hannover, Germany

Objectives: The optimization of culture conditions, functional characterization of cell systems, response of cells to biotechnology products and validation of new cell systems, with a major emphasis on the design of novel human and animal renal cell lines. Current objectives include tailoring new cells, fully characterising them and developing sensitive methods to document cell modulation by potentially toxic molecules.

Major Problems Encountered: None

Results:

Use of cell lines: Groups in Dublin, Rouen and Lausanne have concentrated on LLC-PK_1 (of proximal tubular origin) and MDCK (of distal tubule and collecting duct origin). Cells are being characterized in terms of enzymatic profiles, growth patterns from labelled precursor incorporation into DNA, mRNA and protein synthesis under control conditions and following exposure to nephrotoxins. Receptor characterization has confirmed the presence of angiotensin II receptors and cell responses have been monitored by flow cytometry. Work is in progress on effects of atrial natriuretic peptides and novel inhibitors of the renin-angiotensin-aldosterone system.

Electrophysiological investigations: Techniques for growing cells on microporous membranes in Dublin, Innbruck, Rouen and Lausanne have been successfully employed and instrumentation developed to assess immediate alterations in ion transport properties for both low- or high-resistance transepithelial membranes under control and test conditions. A dose-response relationships to effects of gentamicin and metal ions has been established and confirmed by enhanced fluxes of ^{14}C-mannitol. This could be prevented by treatment with magnesium-L-aspartate hydrochloride.

Weakness of proximal tubular cell lines: The LLC-PK_1 and OK cell lines of proximal tubule origin have been biochemically and functionally characterized and the LLC-RK_1 cell line is under study the LLC-PK_1 cell line lacks of gluconeogenic function, has high glycolytic function and an inappropriate pattern of adenylate cyclase response to hormonal stimulation. OK cell line lacks expression several brushborder marker enzyme and the lack of methyl-*O*-D-glucopyranoside transport. The Rouen group has thus concentrated on primary culture of rabbit proximal tubule cells developed under strictly controlled conditions because LLC-PK_1 or OK cells may only be partially suitable for mechanistic studies and extrapolation to the *in vivo* situation.

Primary rabbit cell lines: Major biochemical and functional phenotypic improvements have been gained on primary cultures of rabbit kidney proximal tubule cells, through the modulation of culture medium composition and the adaptation of the cell culture environment. The use of hormonally defined conditions, using permanent rocking monolayers on uncoated plastic petri dishes in a 99.5% air 0.5% CO_2 atmosphere has been optimized in Rouen.

Cell fusion experiments: An OK-cell subline with new properties in culture media where glucose is replaced by pyruvate to stabilise expression of gluconeogenesis is being cytogenetically and biochemically characterized in Innbruck. More recent collaboration between the Dublin, Rouen and Innbruck groups has addressed the goal of tailoring new immortal renal epithelial hybrid lines which express the sum of the properties of their *in vivo* ancestor cells.

Fusion was performed between gluconeogenic LLC-PK_1 $FBPase^+$ with OK cells using specific procedures to select the fusion hybridomas, which is the most crucial step. We have transfected a gene imparting resistant against neomycin toxicity. Cells that lack this gene will die in the presence of high concentrations of the antibiotic. The other selection criterion is a metabolic property in the non-neomycin resistant fusion partner which is absent the neomycin resistant cells. We have succeeded in establishing hybridoma lines between neomycin resistant renal cell lines with those lines expressing the property of gluconeogenesis, i.e. being able to grow and proliferate in the absence of glucose from the culture medium. Trials to fuse neomycin resistant immortal cell lines with cells freshly isolated from rat, rabbit and man (which can be kept transiently in the absence of glucose) are under investigation.

The production of hybridoma cell lines between rabbit primary culture cells LLC-PK_1 wild type OK wild type and LLC-RK_1 wild type has just started. To characterize the response pattern of our model to gentamicin, we have examined the activities of two plasma membrane marker enzymes, two lysosomal marker enzymes, a mitochondrial marker enzyme, two enzymes involved in the detoxication metabolic system, the protein and DNA synthesis and methylglucose transport. The biochemical, metabolic and transport properties of the newly tailored cell lines are being characterised and their usefulness for testing the effects of cephalosporins and recombinant Streptase is being investigated.

Surrogates for glomerular and medullary cells: The London group has used 3T3 fibroblasts as surrogates for glomerular epithelial and medullary epithelial and interstitial cells (active peroxidase system). 3T3-cells are sensitive to low concentrations of chlorpromazine, the

mechanism of which is thought to be due to inhibition of Na^+ ,K^+ -ATPase-mediated active transport, membrane stabilization, or metabolic activation of chlorpromazine to reactive species. Cytotoxicity was assessed for chlorpromazine, several metabolites and analogues and defined in terms of the concentration required to inhibit mitochondrial reduction by 50% (IC_{50}). The metabolite 7,8-dihydroxychlorpromazine and the analogue nor-1-chlorpromazine had greater cytotoxic (IC_{50} of 2uM and 5uM respectively) than chlorpromazine, 6,9-dihydroxychlorpromazine and 7-hydroxychlorpromazine HCl (all about 15uM). The remaining compounds were less cytotoxic (IC_{50} 100-1000uM or > 1000uM). The structural feature that are essential for cytotoxicity include the N,N-dimethylpropanamine moiety and either an unoxidised 6,7,8,9-ring, or 6,9-dihydroxy groups. Other data which suggest that oxidative metabolism is the key to chlorpromazine's cytotoxicity are the protective effects of vitamin E, L-ascorbic acid and glutathione, and to a lesser extent ethoxyquin and catalase.

Human mesangial cells: Workers in Giessen, Hannover and Koln have concentrated on human glomerular mesangial cells. These provide a useful *in vitro* model to study the metabolism by which fibronectin is released from cells and incorporated into the extracellular matrix, and then degraded as indicated by fibronectin fragments in the extracellular culture compartments. Exposure of cultured human glomerular mesangial cells to adriamycin resulted in an accumulation of newly synthesized fibronectin in both the culture medium and the extracellular matrix. This is assumed to be due to an inhibition of fibronectin degrading proteinases rather than an increased fibronectin synthesis. This is corroborated by the diminished fibronectin fragments in the culture medium and by the unchanged fibronectin-mRNA level of adriamycin-treated human glomerular mesangial cells. This suggests that the extracellular environment modulates fibronectin expression *via* direct cell-matrix interactions.

Similarly work from Hannover on isolated hagfish glomeruli indicate that adriamycin reduced the aminoacid uptake into glomerular cells, due to adriamycin effects on membrane components, but increased aminoacid incorporation into glomerular proteins was increased. This was possibly due to metabolic disturbances in protein synthesis and/or protein degradation, with no concomitant increase in RNA-synthesis. These *in vitro* findings from human glomerular mesangial cells and isolated fish glomeruli confirm *in vivo* data on adriamycin-treated rats where a loss of urinary fibronectin-degradation products are of renal orign are an early marker for degenerative functional changes.

Higher order systems: In Lausanne isolated S_2 segments of rabbit proximal tubules are being incubated *in vitro* and the effects of chemicals on the basolateral uptake of standard organic anions and cations investigated. The technique is being validated with a selection of nephrotoxins and peptide effects will be evaluated.

Specialised techniques: In Dublin flow cytometer (using fluorescein diacetate and propidium iodide) has been used to assess cell viability after exposure to nephrotoxins. The technique is objective, accurate and provides instant statistical analysis of cell responses. Other probes such as Semi-Naptho-Rhoda-Fluor (SNARF-1) fluorochrome shows that intracellular pH is reduced in both LLC-PK1 and MDCK cells exposed to gentamicin and Indo-IAM indicates an increase in $[Ca]_i$. Flow cytometry has the potential to be used as an extremely sensitive

tool in monitoring initial interactions between biotechnology products and renal cells. Flow cytometry with appropriate antibodies will also be extremely useful in sorting cells from different nephron segments and also in monitoring and separating cell fusion products during development of new lines. The London group is developing similar probe based techniques to quantitate intracellaular ion changes by confocal laser microscopy. The role of shifts of intracellular calcium ions, measured simultaneously with membrane conductance in the same epithelial cell lines is going to be investigated by the Lausanne, Innbruck, Dublin and UK groups.

Highlights and Milestones: The first tailored cells and cells with much improved phenotypic expression are being characterised biochemically and morphologically. The combined use of electrophysiology and fluorescent probes, and specialised biochemical and molecular biological techniques are giving new insight into the effects of chemicals and biotechnology products on cells.

Wider Considerations: Several novel techniques and cell culture systems have already been developed which should allow for design of safer biotechnology-derived products and earlier detection of nephrotoxicity leading to more effective treatment.

Co-operative Activities: Contractors met on 7/12/1991 to discuss exchange of work and personnel between laboratories. The techniques of primary cell culture have been transferred from Dublin to Innsbruck to Dublin, and cell fusion from Innsbruck to develop fused primary animal and human cell lines. Characterization of new cell lines will be carried out in both laboratories with Innsbruck concentrating on biochemical and enzymatic characterization and Dublin concentrating on pharmacological and receptor characterization. Similar exchanges are planned with Dr. Morin, France. All BRIDGE groups will meet 24/4/1992 in London, at the *First European Workshop on Biotechnology Applications for Microinjection, Microscopic Imaging and Fluorescence.*

Publications

McGlynn, H. et al., (1991). Electrophysiological and flow cytometric studies on mechanism of gentamicin nephrotoxicity in cultured renal cells. Arch. Toxicol. Suppl. 15, Medical Toxicology, 140-143. Dublin group

McGlynn, H., et al., (1991). Effects of gentamicin on glucose transport, transepithelial resistances and cell viability in established renal cell lines. Br. J. Pharmacol. Proc. 104, 77P. Dublin group

Soose, M., et al., (1991) Altered renal fibronectin excretion in early Adriamycin nephrosis of Rats. J. Pharmacol. Exp. Ther., 257:493-499. Hannover group

Kastner, S., et al., (1991) The effects of Adriamycin and cis-platin on glomerular protein metabolism. Studies on the Atlantic hagfish Myxine glutinosa. Bull. Mt. Des. Isl. Biol. Lab., 30:120-122. Hannover group

Radermacher, J., (1991) The effect of arginine depletion on glomerular and tubular kidney function: Studies in the isolated perfused rat kidney. Amer. J. Physiol., 261: F779-786. Hannover group

Kastner, S., (in press) Mechanistic studies on the influence of Adriamycin on aminoacid uptake and aminoacid incorporation into isolated glomeruli of the Atlantic hagfish Myxine glutinosa. Bull. Mt. Des. Isl. Biol. Lab. 31, in press. Hannover group

Kastner, S., et al., (1991) Metabolic studies on isolated rat glomeruli: A valuable tool to investigate glomeruli damage. In: Nephrotoxicity: Mechanisms, Early Diagnosis and Therapeutic Management, (Edit. Bach, P.H., Gregg, N.J., Wilks, M.W. and Delacruz, L.), pp. 467-474. Marcel Dekker, New York. Hannover and London groups

Bach, P.H. (1991) Better diagnosis and mechanistic understandings of nephrotoxicity using histochemistry and cytochemistry. *Progress in Histochemistry and Cytochemistry*, **23**, 178-186.

Thorndike, J.M., Bach, P.H. and Moore, M.N. (1992) Chlorpromazine Cytotoxicity in 3T3-Cells. *Marine Environmental Research*, , -. In Press London group

Thorndike, J.M., et al., (1992) Lectin binding sites in the digestive gland of mussel. *Marine Environmental Research*, , -. In Press London group

Wilks, M.F., et al., (1991) Effects of heavy metals on metabolic functions in isolated glomeruli and proximal tubule fragments. In: Nephrotoxicity: Mechanisms, Early Diagnosis and Therapeutic Management, (Edit. Bach, P.H., Gregg, N.J., Wilks, M.W. and Delacruz, L.), pp. 363-366. Marcel Dekker, New York. London group

Wilks, M.L. and Bach, P.H. (1992) Renal enzymes as the basis for alternative methods of safety screening in vitro and understanding the mechanism of nephrotoxicity. In: Urinary Enzymes in Clinical and Experimental Medicine, (Edits. Jung, K., Burchardt, U., and Mattenheimer, H.), pp. - . Springer-Verlag, Heidelberg. London group

Bach, P.H. and Wilks, M.L. (1992) *In vitro* techniques for screening for nephrotoxicity, mechanism of injury and new discovery. In: *In Vitro* Toxicology: An Alternative to Animal Testing?, **(Edits. Jolles, G and Cordier, A.), pp. - . Academic Press, London.** London group

Communications

McGuinness, S., et al., (1991). Comparison of renal tubules and slices for in vitro nephrotoxicity studies. Presentation at 'Newer Aspects of Renal Cell Injury'. A Forefronts in Nephrology Symposium. International Society of Nephrology, Cape Cod, Massachusetts, U.S.A. Oct. 6-9, 1991. Dublin group

McGlynn, H., et al., (1991). Flow cytometric and electrophysiological studies of gentamicin nephrotoxicity in renal cell lines. Presentation at 'Newer Aspects of Renal Cell Injury". A forefront in Nephrology Symposium. International Society of Nephrology, Cape Cod, Massachusetts, U.S.A. Oct. 6-9, 1991. Dublin group

McGlynn, H. & Ryan, M.P. (1992) Models and mechanisms of gentamicin nephrotoxicity in cultured primary rat renal cells. Presentation at 'First National Scientific Medical Meeting", Dublin, Ireland. March 13-14. 1992. Dublin group

McGlynn, H., et al., (1992). Flow Cytometric assessment of gentamicin nephrotoxicity in established renal cell lines. Presentation at 'First European Workshop on Biotechnology Applications of Microinjection, Microscopic Imaging and Fluorescence (BAMMIF). Polytechnic of East London, London, April 22-24. Dublin group

TITLE: Analysis of gene transfer between microorganisms and plants

CONTRACT NUMBER: BIO-CT91-0282 (SSMA)

OFFICIAL STARTING DATE: 1 October, 1991

COORDINATOR: Charles Thompson, Institut Pasteur (IP), Paris, France

PARTICIPANTS:

R. Guerrero, University of Barcelona (UBA)	**SPAIN**
A. Puehler, University of Bielefeld (UBI),	**GERMANY**
P. Simonet, University Claude Bernard (UCB)	**FRANCE**
A. Tsiftsoglou, University of Thessaloniki (UT)	**GREECE**
M. Lebrun, Rhône Poulenc Agrochimie (RPA)	**FRANCE**

OBJECTIVES SET FOR THE REPORTING PERIOD:

Not formalized.

MAJOR PROBLEMS ENCOUNTERED:

Work on the projects outlined in our proposal has been initiated in several laboratories but it should be noted that delays in receipts of funding has meant that most participants have not been able to start work promptly.

RESULTS:

We are interested in the exploring the possibility that genetic information can transfer horizontally between distantly related organisms. A better understanding of this question is necessary in order to establish rules for for biological containment of genetically manipulated organisms which are consistent with potential hazards to man and the environment. We would like to establish both genetic and physiochemical factors which affect gene transfer both under well controlled conditions in the laboratory and in the natural environment. Our studies are designed to understand more about possible mechanisms which allow DNA transfer including conjugation, transformation, or unknown processes which might allow gene transfer between plants and microbes. In order to monitor gene transfer, the following genes were engineered to allow selection in bacteria and/or plant cells:

- *aacC1*, the gentamicin acetyltransferase-3-I gene, mediates gentamicin (Gm) resistance in a broad range of Gram-positive and Gram-negative bacteria
- *pat*, the phosphinothricin N-acetyltransferase gene, confers resistance to phosphinothricin itself (Pt) or phosphinothricin incorporated into a tripeptide (Ptt, bialaphos). *pat* has been modified to contain prokaryotic or eukaryotic transcriptional and translational sequences to allow expression in plants as well as bacteria (UBI)
- *nptII*, the aminoglycoside phosphotransferase, confers resistance to neomycin, kanamycin, G418

I. Effects of environmental conditions on conjugative gene transfer (UBA)

Various physio/chemical factors such as water content, pH, temperature, as well as nutritional conditions may effect the efficiency of conjugation. Effects of these parameters on genetic exchange between *Rhizobium meliloti* and *Escherichia coli* in a soil environment are ongoing. Studies carried out under an previous BAP project had shown that soil water content influenced gene transfer frequency probably as a result of limited bacterial mobility in non-fluid conditions.

In an extension of this work, we are now measuring the effects of pH and the nutritional conditions on rates of dispersion of *E. coli* and *R. meliloti* in soil. Dispersion

rates of *R. meliloti* were were altered only at highly acidic or alkaline conditions over a pH range between 3 and 9 whereas *E. coli* strains showed a more stenotopic response. Both species displayed maximal frequencies of gene transfer at neutral pH.

Adverse environmental condition probably exert their effects on gene transfer, at least partially, by altering the the metabolism of the bacteria. Polyhydroxyalkanoate (PHA), an energy and carbon polymeric reserve compound in most bacteria, has proven to be to be a good indicator of physiological state of microbial communities under natural conditions. The level of cellular PHA content is directly related to growth in soil ecosystems. In addition, there is some evidence that membrane-bound PHA could play a role in entry of DNA molecules into the cell. Our preliminary results show that *R. meliloti* cells having higher PHA levels show slightly higher gene transfer rates in intraspecific conjugation experiments. These results must be confirmed and tested in a soil microcosm.

II. Bacterial conjugative mechanisms (IP, UT)

Soil rhizospheres are largely populated by Actinomyces, Rhizobium, and Agrobacterium. We are now studying mechanisms by which these bacteria might transfer genetic material among themselves (intraspecifically and interspecifically) or with the plant roots.

Previous studies (BAP, IP) demonstrated that Gram-negative bacteria (*E. coli*) can transfer genetic material to Gram-positive bacteria (*Streptomyces* and *Mycobacteria*). These RP4 and RSF1010 conjugative processes rely on some specific transfer functions supplied in trans as well as an origin of transfer carried by the conjugative plasmid. 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 which may allow mixing of the two cytoplasms or even genetic recombination between the two organisms. However, these points of contact between Gram-positive actinomycetes and Gram-negative *E. coli* are not dependent on plasmid-specific transfer functions. Genetic evidence (Institut Pasteur) has indicated that similar fusion events can take place between Streptomycetes strains. Apparently, these fusion events, in conjunction with plasmid fertility genes, allow chromosomal recombination. We are now pursuing studies of streptomycete plasmid genes (pIJ101) which either induce recombination directly or possibly in conjunction with chromosomal genes.

We have developed methods for analyzing possible gene transfer between agriculturally important organisms. Previous studies carried out under the previous BAP project (UBA, UT) have indicated that the *aacC1* gentamicin acetyltransferase gene can be transferred by conjugation from genetically engineered *Rhizobium leguminosarum* $VF3_{a\text{-}c}$ into plasmidless *Agrobacterium tumefaciens* via an RP4 mobilizing element. Attempts to transfer the gene from this recipient into *R. leguminosarum* and *Pseudomonas syringae* in the absence of the RP4 mobilizing system have revealed that no efficient gene exchange occurs.

Presently, we (UT) are pursuing these studies using as recipients our own soil isolates of *A. tumefaciens*. We have characterized these isolates both genetically (antibiotic resistance spectrum) and genetically by DNA analyses. In addition, we have begun to to define experimental conditions for conjugation experiments which will be carried out under both laboratory and field conditions. Transfer of the *aacC1* gene will be detected both by selecting for gentamicin resistance and by hybridization using the *aacC1* gene as a probe.

III. Plant-bacteria transfer - Investigation of possible gene transfer from *Niconiana tabacum* to *Agrobacterium tumefaciens* (UBI, RPA)

Interkingdom gene transfer is a potential risk factor to be considered when genetically manipulated plants are released into the environment. The *A. tumefaciens*-induced callus is a natural environment where the close contact between bacteria and plant cells, creates conditions which might favour potential horizontal transfer of genetic

material. The transfer of genes from *Nicotinana tabacum* to the *A. tumefaciens* cells within the callus is currently under investigation.

Leaf-discs of the wild type *N. tabacum* were infected with *A. tumefaciens* containing the plasmid pIV16.41-G. This plasmid contains *aacC1*, *pat*, and *nptII* genes. The regenerated shoots were placed on rooting medium (supplemented with Pt) to induce root formation. The stems of the plants were infected with *A. tumefaciens* (gentamicin sensitive) to induce calli formation. Nine calli were excised aseptically and plated on medium supplemented with gentamicin to select for potentially recombinant bacteria which might have received the *aacC1* gene from the tobacco cells in the callus. In all more than 10^8 *A. tumefaciens* cells were recovered from the callus and a total of 429 were gentamicin resistant. When these colonies were screened using *aacC1* as a hybridization probe, 26 gave positive signals These results need to be confirmed by more precise Southern blot hybridization experiments.

Similar experiments are being planned for field trials in France. We are in the process of submitting a request to the French "Commission du Génie Biomoléculaire" in order to get authorization for field release of these transgenic plants in 1992. Field trials are planned for 1992 at RPA's experimental farm facility in Bergerac region of France.

IV. Characterization of DNA/soil interactions and and its influence on transformation of plants and bacteria in the soil (UCB).

There is very little information available related to what happens to DNA released from lysed organisms in the soil. The stability of this DNA as well as its efficiency in processes of natural transformation of other organisms must be a function of soil chemistry.

We will investigate both physical interactions and reactivity of DNA with different soil components. Investigations will be focused on the relationships between DNA molecules and soil particles such as clay minerals and humic acids. Preliminary studies indicate that efficiency of DNA adsorption onto soil particles is related to to surface characteristics such as charge, hydrophobicity, and microaggregate formation. Adsorption of DNA on clay minerals, a major component of soil, will be investigated in the future using an adsorption isotherm technique. Parameters such as DNA concentration and chemical composition of the environment (presence of cations) will be studied. In order to investigate the natural fate of clay-adsorbed DNA in the soil, we will investigate the role of adsorption in protecting DNA against nucleolytic activities that would naturally exist in the soil. We will also determined whether adsorbed DNA remains active for transformation of microorganisms. This will be done using an artificial model composed of chromosomal or plasmid DNA, montmorillonite, DNaseI and competent *E. coli* cells. We expect that studies outlined above will allow us to optimize conditions for recovery of DNA from the soil by chemical techniques or by transformation of a microbial host.

HIGHLIGHTS/MILESTONES:

Although the results need to be confirmed by more precise hybridization experiments, it is very interesting to note at this preliminary stage that the UBI team has recovered *Agrobacterium tumefaciens* strains from tobacco calli which may may have received an antibiotic resistance gene as a result of horizontal interkingdom DNA transfer from plant to bacteria.

WIDER CONSIDERATIONS:

Nothing to report.

COOPERATIVE ACTIVITIES:

Nothing to report.

LIST OF PUBLICATIONS/PATENTS WITH TRANS-NATIONAL AUTHORSHIP:

Nothing to report.

TITLE : Fate of GEM's and GEDS in some environmental hot spots : polluted soils, river sediments, decaying and diseased plants, role of broad host range plasmids and development of containment systems.

CONTRACT NUMBER : BIOT - CT91 - 0284 (SSMA).

OFFICIAL STARTING DATE : 1/10/1991.

COÖRDINATOR : Dr. Max Mergeay, V.I.T.O., 2400 Mol, Belgium.

PARTICIPANTS : Dr. John Fry, University of Wales, Cardiff, UK.
Dr. José Marques, I.B.E.T., Oeiras, Portugal.
Dr. Willy Verstraete, University of Gent, Gent, Belgium.

OBJECTIVES SET FOR THE REPORTED PERIOD :

a) Monitoring of the fate of GEM's and GEDS in potential environmental hot spots with special emphasis on the role of broad host range (BHR) plasmids.
   - Construction of GEM's and GEDS.
   - Monitoring of their fate in the microcosms.
b) Study of the genetic transfer potential in the environmental hot spots using "exogenous plasmid isolation".
c) Development of a rapid method for total extraction of DNA from soils.
d) Development of bacterial containment systems.

MAJOR PROBLEMS ENCOUNTERED : No significant scientific problems to be reported.

RESULTS :

a) Construction of GEM's and GEDS.

Different GEM's and GEDS were constructed which will be used in the different microcosms to study gene escape. The GEM's contain marker genes based on heavy metal resistance and organic xenobiotioc degradation. The genes have been cloned and will be cloned in different replicons (Tra^-Mob^+ vector, Tra^-Mob^+ vector, chromosome) in the GEM's which include E. coli and P. putida, as models for accidental and deliberate release respectively.

The czc (encoding resistance to cadmium, zinc and cobalt) and cnr (encoding resistance to cobalt and nickel) operons derived from Alcaligenes eutrophus CH34 were cloned in 1) a pBR322 based vector (ColE1,Tra^-,Mob^-) 2) RSF1010 (IncQ) and 3) RP4 (IncP1) based vectors (Tra^-,Mob^+) by the group of Mol and have been introduced in E. coli and P. putida (Table1). These genes are not expressed in E. coli and P. putida but are expressed if they are to be transferred into A. eutrophus. As such, the heavy metal resistance genes can be used as useful marker genes to study gene transfer from P. putida and E. coli to A. eutrophus in microcosms as they allow the use of very selective media against donor and the natural microbial population (also through a combination of selective metals).

On the other hand, in order to study the escape of genes from an introduced micro-organism to the indigenous micropopulation, marker

genes are needed which are expressed in a broad range of bacteria. Genes encoding resistance to mercury (mer) and encoding the meta-cleavage of 2,3-dihydroxybiphenyl (bphC) or catechol (tdnC) into a easily detectable yellow cleavage product were found to be expressed in a wide range of bacteria at least in the β- and γ-subgroup of the Proteobacteria (Cardiff, Mol).

The biphenyl degradative genes of A. eutrophus A5 were found to be borne on a large transposable element, Tn4371. The bphC gene of the pathway was cloned in pRK415 (IncP1, Tra^-, Mob^+, Tc^r) by the group of Mol and subcloning is going on. The tdnC gene of P. putida and mer gene were cloned together on a Tn5 transposon by the group of Cardiff and were used to mark broad host range (BHR) plasmids.

b) Study of gene transfer in the microcosms.

The transfer from E. coli to A. eutrophus of Mob^-Tra^+ and Mob^-Tra^- plasmids carrying the czc genes encoding resistance to cobalt, cadmium and zinc as marker genes (see above) was studied in unpolluted and heavy metal polluted soil microcosms by the group of Gent as a model to study the risks associated with accidental release of GEM's. Special emphasis was put on the presence of BHR plasmids in the microcosms.

Direct transfer of the non-conjugative plasmid pDN705 (pRK290::czc;IncP1; Tra^-Mob^+;Tc^r) from an E. coli strain harbouring the TRA-genes of RP4 in the chromosome to A. eutrophus was observed in polluted and non-polluted soils in sterile as well as in non-sterile conditions . Whereas in sterile conditions the presence of heavy metals in the soil did stimulate the formation of transconjugants, this was not the case in natural conditions. Moreover, only when nutrients were added to the soil, transconjugants were detected under natural conditions.

Furthermore, in sterile conditions, it was shown that the IncP1 plasmid, RP4, was able to mobilize plasmid pMOL155 (pKT210::czc; IncQ; Tra^-Mob^+; Cm^r) by direct transfer, in triparental matings and by retromobilization from E. coli to A. eutrophus, as well in unpolluted as heavy metal polluted soils. Moreover, the presence of a selective pressure i.e. the presence of heavy metals in the polluted soils again stimulated the formation of transconjugants.

In addition, it was shown that the czc heavy metal resistance marker cloned in pBR322 (=pMOL149:pBR322::czc;ColE1;Tra^-Mob^-;Tc^r;Ap^r) can be picked up by plasmid RP4::Mu3A in E. coli and transferred to A. eutrophus in triparental matings and by retromobilization in sterile soil microcosms. Again a positive effect was observed in heavy metal polluted soils. These results emphasize the role of transposons and that even genes on a Mob^- plasmid can be transferred towards other strains in soil conditions.

Several bacterial systems were evaluated for use as donor-recipient pairs by the group of Oeiras. A pair of E. coli K12 strains (XL-2 and RB85) was chosen for further "accidental release" studies. Strain XL2 bears an F' plasmid conferring tetracyclin resistance and strain RB85 is streptomycin resistant. The strains were used to study the colonizing patterns on the roots of maize and the level of transconjugant formation. Results suggest differences in spatial and temporal colonization patterns of the two strains. This may explain the low

frequency of transfer over roots detected with this system. In future studies, the E. coli and P. putida GEMS and gene escape system constructed by Mol will be assessed in the plant-soil microcosms.

c) Study of the genetic transfer potential in the environmental hot spots using "exogenous plasmid isolation".

Experiments were done to isolate BHR plasmids from some of the proposed environmental hot spots i.e. River Taff epilithon (Cardiff) and organic polluted soils and sludges (Gent) by exogenous plasmid isolation. Exogenous plasmid isolation is a technique that isolates plasmids from mixed bacterial communities by transfer into a known recipient during mating on filters.

Plasmids were isolated from the river epilithon by the group of Cardiff using 9 recipient strains : 5 strains of P. putida, 2 strains of P. aeruginosa , 1 strain of E. coli and 1 strains of A. calcoaceticus. Mercury resistance was used as a selective marker. The recipients received plasmids from the epilithic bacteria although with various transfer frequencies. Most of the plasmids were very large (50-300 kb) and a few very small (<10 kb), but none were between 10 kb and 30 kb. 15 (24%) of the isolated plasmids in E. coli were capable to transfer to E. coli and P. putida.

To test the host range, transfers of two of the isolated plasmids (pQM705,52 kb and pQM706, 55 kb) were carried out (Table2) from P. putida into a variety of recipient bacteria selected on their position in the 16S rRNA evolutionary tree. Two other BHR plasmids were used for comparison : another natural epilithic plasmid (pQKH6) and a marked RP1 derivative designated as pQM899 (= RP1::Tn5::mer::tdnC). pQM899 transferred most widely and was found to transfer even into the distantly related Cythophaga heparina. pQM706 and pQKH6 transferred into almost all the proteobacteria but not to Cytophaga heparina. None transferred into the Gram + bacteria tested. Natural BHR plasmids are thus present in river epilithon.

Exogenous isolation of plasmids from polluted soils and activated sludge was done by the group of Gent. They used a modification of the procedure used by Cardiff. The plasmids were isolated by means of triparental matings in which the indigenous microbial population delivers mobilizing plasmids to mobilize pMOL155 (see above) from E. coli to a Rif^r A. eutrophus recipient strain. Using this procedure, several mobilizing plasmids were isolated from organic polluted soils and their characterization is going on. Moreover, several plasmids were isolated from activated sludge. They all had a size of around 60 kb, were able to mobilize pMOL155 and to self-transfer from A. eutrophus to E. coli and showed Tc, Ap and Kn resistance.

d) Development of a rapid method for total extraction of DNA from soils.

To study the transfer of genes from released bacteria into the natural population and in particular to none-culturable bacteria, total DNA extraction and the use of appropriate probes and PCR may be a suitable way. Therefore, a rapid method was developed to purify DNA from soils by the group of Mol. The procedure takes no longer than 3 hours and the purified DNA can be used for DNA-DNA hybridization. The detection limit

for a single chromosomal target gene-carrying bacterium is 100 000 CFU/0.5 gram of soil. Experiments are going on to see if the extracted DNA is applicable for PCR to lower the detection limit.

e) Development of bacterial containment systems.

The presence of natural broad host range plasmids in the environment may represent an enhanced risk considering the escape of genes from introduced GEM's as they may be able to invade the GEM and to pick up genes from it e.g. by means of retrotransfer. Therefore, the use of bacterial strains deficiënt in conjugation exchange (unable to be invaded by BHR plasmids) might be interesting to decrease the risks.

A mutant of E. coli ED8739, defective in conjugation with a P-type donor ($ConP^-$) was isolated by the group of Mol. The mutant was shown to be 50 to 100 times less efficiënt as recipient in conjugations with an IncP1 trans- fer system but was not affected in transformation efficiëncy nor in the capacity to maintain plasmids. Moreover, the Con P^- mutant was conjugation-proficient for IncW plasmids and was not affected in its donor capacities for IncP1 plasmids.

A mutant of P. aeruginosa impaired in survival (sss) was isolated by the group of Gent. This mutant shows promisses for a possible containment system.

HIGHLIGHTS/MILESTONES :

1. Retrotransfer of a Mob^-Tra^- pBR- plasmid into soilbacteria by means of a IncP1 BHR plasmid in soil microcosms (RP4::Mu3A).
2. Isolation of new BHR plasmids from river epilithon which can play a role in gene transfer in the river (RP1::Tn5::mer::tdnC).
3. Transfer of the IncP1 BHR plasmid RP1 into Cytophaga.
4. Isolation of a mutant of E. coli defective in conjugation with a P-type donor (towards a possible containmant system)

WIDER CONSIDERATIONS : The present of BHR plasmids (especially those provided with efficient transposons) in a soil microflora do influence the dissemination of genes cloned in pBR-vectors. This puts a limit on the safety of such vectors.

COOPERATIVE ACTIONS : A first meeting was hold in Mol (19/3/1992) attended by all partners to discuss practical details and time table the progress of the work, exchanges of people, organisms and methodologies etc.

LIST OF JOINT PUBLICATIONS/PATENTS WITH TRANS-NATIONAL AUTHORSHIP :
Work in progress.

OTHER PUBLICATIONS/PATENTS :
Isolation of a mutant defective in conjugation with a P-type donor ($ConP^-$) that is lacking the major outer membrane protein OmpF. D. van der Lelie, E. Top, G. Nuyts and M. Mergeay; submitted to Microbial Releases.

Characterization of the retrotransfer phenomenon using mechanistic mathematical modeling. E. Top, P. Vanrolleghem, M. Mergeay and W. Verstraete; submitted to Journal of Bacteriology.

Characterization of the retromobilization phenomenon by use of a mutant defective in conjugation with a P1-type donor (ConP1$^-$). E. Top, D. van der Lelie, W. Vertstraete and M. Mergeay; submitted to Microbial Releases.

Identification of a transposable element, Tn4371, encoding biphenyl and 4-chlorobiphenyl degradation in A. eutrophus A5. D. Springael, S. Kreps and M. Mergeay; submitted to Journal of Bacteriology.

Table 1 : GEM's and GEDS for accidental release based on heavy metal resistance genes (Mol).

Fragment genotype	Cloned in	Inc group	Resulting plasmid	Vector markers	E. coli Host	Application
czc	pBR325	ColE1	pMOL149	Tc, Ap	HB101	Mob$^-$,Tra$^-$
czc	pRK290	P	pDN705	Tc	S17/1	Mob$^-$,Tra$^+$ direct transfer
czc	pKT210	Q	pMOL155	Cm	S17/1	Mob$^-$,Tra$^+$ direct transfer
czc	pKT210	Q	pMOL155	Cm	BHB2600	Mob$^-$,Tra$^+$ trip.mating retro-transfer
cnr	pRK415	P	pMOL154	Tc	S17/1	direct transfer
cnr	pKT210	Q	pMOL164	Cm	DH5	retro-transfer

Table 2 : Host range of some BHR plasmids from epilithic bacteria

Transfers were from P. putida UWC3 on membrane filters with Plate Count Agar into rifampicin resistant recipients at 30° C for 24 h; nd = not yet done.

		Mean transfer frequency per recipient (n=2) for			
Recipient	Proteo-bacteria group	pQKH6	pQM705	pQM706	pQM899
Comamonas acidovorans	β-1	1.7×10^{-2}	nd	1.2×10^{-1}	9.4×10^{-5}
Pseudomonas putida	γ-3	7.0×10^{-1}	3.0×10^{-1}	3.7×10^{-6}	3.7×10^{-1}
Pseudomonas cepacea	β-2	2.5×10^{-3}	nd	1.2×10^{-5}	5.5×10^{-1}
Pseudomonas maltophilia	γ-3	3.6×10^{-2}	nd	1.7×10^{-6}	4.5×10^{-1}
Acinetobacter Calcoaceticus	γ-3	1.6×10^{-2}	3.2×10^{-4}	2.4×10^{-1}	4.2×10^{-5}
Alcaligenes eutrophus	β-2	1.8×10^{-2}	nd	2.1×10^{-4}	3.8×10^{-4}
Cytophaga heparina	FLAVO	$<2.2\times10^{-9}$	nd	$<1.1\times10^{-9}$	1.6×10^{-2}
Escherichia coli	γ-3	1.4×10^{-3}	4.0×10^{-2}	3.1×10^{-2}	1.5×10^{-2}
Rhizobium leguminosarum	α-2	5.4×10^{-4}	nd	nd	1.3×10^{-5}

Title **The effects of selection on gene stability and transfer in populations of bacteria in soil.**

Contract number BIOT 910285

Official starting date: 1/10/91

Coordinator:
Dr E. M. Wellington, Biol. Sci., Univ. Warwick, Coventry. CV4 7AL U.K.

Participants: Dr. A. Karagouni, Inst. Gen. Bot., Univ. of Athens, 15784 Athens. GR ,
Dr. A. Akkermans, Dept. Microbiol., Agricultural Univ., Wageningen, N L

Objectives set for the period of reporting

The primary aim for the first 6 months was to obtain and distribute amoungst the participants, genetically marked Streptomycetes. Genes for antibiotic resistance to thiostrepton and neomycin/kanamycin were sought as these antibiotics had been selected for subsequent soil enrichments. The second objective was the development of a soil microcosm system which required the selection and characterisation of soil at specified sites by each participant, and use of batch, fed-batch and continuous microcosms with methods for the extraction of bacteria and antibiotics from the soil. The characterisation of selected soils, to be used throughout the two year study period, involved physical, chemical and biological study. The latter required assessment of indigenous resistance levels to thiostrepton and neomycin in streptomycete populations.

The antibiotic extraction techniques were required for detection and identification of antibiotics produced in soil and added to soil. As the former was known to be very low, a high level of sensitivity was required.

Cell extractions were required for monitoring both populations and DNA in soil. The aim here was to enable monitoring of cell replication during sporulation. Thus methods allowing differentiation of spores and vegetative cells were needed to determine if the fed-batch and continuous microcosms were allowing rounds of replication. Molecular methods were intended as a secondary source of information concerning the fate of introduced genes. In this case differential extraction of DNA from spores and vegetative cells was needed together with exploitation of polymerase chain reaction (PCR) to improve sensitivity for detection of genetic markers.

Division of labour: Each participant was responsible for development of a microcosm system and the workload for construction of strains was divided between Warwick and Wageningen, the former was responsible for development of chromosomal and plasmid markers and the latter for transposons (introduction of transposons on the chromosome and in plasmids). Warwick was responsible for developing methods for the extraction of

thiostrepton and athens and Wageningen for extraction of neomycin from soil.

Major problems encountered

A present no major problems have been encounted, except that transposons were found to be extremely unstable and an alternative strategy for chromosomal marking was developed. This involved the use of the plasmid series pGM (Muth *et al.* 1989) which allow insertion of markers into the streptomycete chromosome as these plasmids act suicide vectors, unable to replicate at 35°C. At present plasmids are being constructed with chromosomal fragments to allow integration at specific sites.

Results

1) *Selection of marked strains* : The plasmid pIJ673 was stabily inherited in strains selected from the different sites and was selected for all studies. It carries both *tsr* and *aph*II genes for resistance to the target antibiotics thiostrepton and neomycin. Greek isolates were transformed with plasmid DNA and selection of transformants is still in progress.

Attempts were made to obtain transposon marked strains but all were found to be too unstable for use. Because of this problem an alternative strategy has been devised. This involved the use of integrating plasmids, specifically pGM4 and pGM9 (Muth *et al.* 1989). These plasmids are stably inherited at temperatures below 34°C, but are lost at incubation temperatures above this, allowing insertion of markers into the streptomycete chromosome. The effect of addition of antibiotics to soil can also be used to assess the retardation effect on loss of these plasmids.

At Warwick a strain of *S.lividans* (TK64 KT) (P. Kaiser, pers. comm) which contains a chromosomal amplification containing both the *tsr* and *aph*II genes has been chosen for study.

2) *Characterisation of soil*: Work has begun to assess local soil streptomycete populations from Warwick, Wageningen and Athens with respect to antibiotic resistances. Resistance to thiostrepton in the indigenous population has not been found in Wageningen, but in Warwick soil low levels were observed (resistance to 0.5 μg/ml thiostrepton). Up to 10% of Wageningen indigenous soil streptomycetes expressed resistance to neomycin (1-10 μg/ml) and at Warwick a small population of streptomycetes were kanamycin resistance up to 100 μg/ml.

Resistant streptomycetes have were isolated and these will be probed using the *aph*I, *aph*II and *aph*V genes in Wageningen, the *tsr* and *aph*II genes in Athens and the *dag*, *tsr* and *aph*II genes at Warwick. This probing should allow the determination of the origin of resistance phenotypes in these streptomycetes.
In addition to analysis of the indigenous streptomycete population, soil from each laboratory has been subject to physicochemical analysis, wetting and drying curves and water holding capacity studies.

3) *Development of fed-batch and continuous microcosms*: In order for selection to be effected by an antibiotic it is necessary for a streptomycete to be in the active mycelial state. It is of great importance to determine the morphological state of this organism *in situ*. Many workers have shown that in soil *Streptomyces* species are almost entirely present as spores, which are by their nature resistant to antibiotics being investigated in this study. In addition, when streptomycete spores are added to natural soil, only a brief period of limited germination occurs, followed by an almost indefinate period of residence as spores. To overcome this problem, fed-batch microcosm systems were developed that resulted in repetitious rounds of germination induced after the initial period of activity. This involves feeding the system with nutrients as well as adding more soil in order to provide a habitat for the inoculated streptomycetes to colonise.

In both Warwick and Wageningen fed-batch systems are being investigated. In Wageningen nutrient amendment, without soil replacement, was studied, whereas in Warwick and Athens between 10-20% of soil from microcosms was continually replaced at set intervals over a time period of several weeks, while ensuring that percentage water in the system remains constant. In Warwick, to date, this work has meant that continuous rounds of germination occurred, a phenomenon not observed in previous fed-batch systems (Fig. 1). Continuous rounds of germination were possible and antibiotic induced selection could be implemented during these cycles of activity.

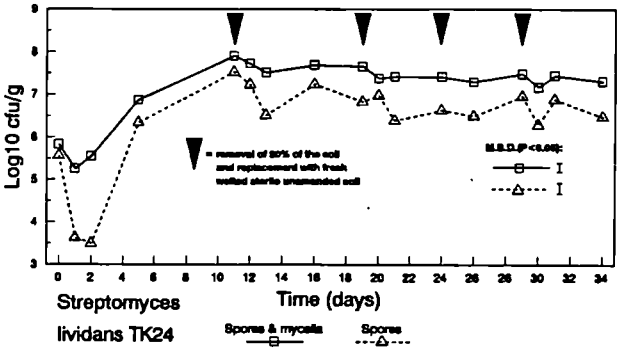


Fig 1. Iterative germination and sporulation in the dynamic fed-batch soil system.

In addition to fed-batch systems both Athens and Warwick are developing continuous microcosms, in which it is hoped that liquid nutrients (containing antibiotics if appropriate) can be added continuously to a soil body allowing, in addition to nutrient replacement, the leaching of spores for counting and possibly the continuous availability of micro-habitats for colonisation by the streptomycetes.

4) *Extraction and analysis of biomass and antibiotics from soil*: All three laboratories are developing methods to monitor antibiotic concentration and stability in soil. While Wageningen has only recently begun to study the fate of neomycin in soil, Warwick has spent several months developing existing methods for thiostrepton detection and quantification. This method involved recovery of the antibiotic from soil using a solvent, a

number of which are presently under study, including chloroform, acetic acid and ethyl acetate, and subsequent separation by TLC, HPTLC and HPLC (Fig.2).

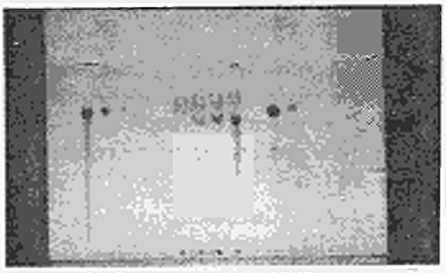


Fig 2. TLC plate showing extracted thiostrepton from soil. Lanes 1-3 25, 2.5 and 0.25 μg thiostrepton $CHCl_3$, lanes 4-7 soil extract 0, 50, 100,500 μg added thiostrepton, 8-10 25, 2.5 and 0.25 μg thiostrepton, CH_3COOH.

To confirm the antimicrobial activity of thiostrepton the TLC plate was subjected to bioautography and placed onto an agar plate seeded with *Staphylococcus aureus*. Detection levels of thiostrepton by TLC was as low as 0.25 to 0.05 μg, although the detection level from soil is yet to be determined. The detection of extracted thiostrepton from soil was analysed by addition of a thiostrepton-containing soil extract to filter discs on seeded agar plates. Detection levels for this method were around 0.5 ng thiostrepton.

All three laboratories investigated improved methods for extraction and analysis of biomass. Such methods include the use of Chelex which allows the specific extraction of spores and bead beating which extracts DNA from both spores and mycelia within the soil. Both methods are being continually assessed and updated. In addition to the isolation of spores and mycelia, methods for extraction and monitoring of DNA were investigated. Once DNA has been extracted from soil, probes can be used to monitor the presence of specific genes within the gene pool. This has previously been achieved using ^{32}p labelled probes, which allow the detection of as few as 0.174 mg dry weight mycelia / g soil. Attempts by Warwick have recently been made to use an alternative method of probing, in this case the non-radioactive digoxygenin labelling kit. Initial experiments using this system have allowed the detection of 10 mg dry weight mycelia / g soil, although it is likely that this value can be considerably improved. ^{32}p labelled probes will also be further investigated.

When DNA of sufficient purity has been obtained it is hoped that PCR can be used to detect low levels of a marked bacterial population, theoretically down to as little as a single genome. Primers for use in PCR were developed, including those for the *tsr*, *dag* and *aph*II genes. At present both *tsr* and *dag* have been amplified from purified plasmid controls using these primers, although no detection of specific genes isolated from soil DNA has yet been acheived. One possible reason for this is the inhibition of the PCR reaction by

impurities in soil extracted DNA. Methods of "cleaning" the DNA to remove impurities such as humic acid from the soil are still under investigation.

Highlights/Milestone

Development of a new soil model microcosm,the fed-batch system, allowing continuous rounds of germination, previously unavailable. This will provide a more accurate and realistic assessment of antibiotic resistance gene transfer and stability in soil.
Detection of antibiotics in soil, new methods for sensitive extraction and identification of antibiotics produced in, or added to soil.

Wider considerations

The role of natural or artificial selection in soil must be measured to accurately assess the fate of genes in the natural environment and evaluate any risks associated with new gene introductions. Enabling technology is being developed to study the process of selection in the soil within a realistic time-scale.

Cooperative activities

Over the past six months there has been one plenary meeting at Wageningen 8-14th Dec 1991, all participants were involved and plasmids and strains exchanged.
Further meeting and exchange between Warwick and Athens from 9th-11th March 1992.
Vist made to University of Kaiserslautern, 7th-9th Feb. 1992 to obtain plasmids from Prof. John Cullum, previous BAP partner and obtain technical expertise.

List of joint publications with trans-national authership

Survival and detection of a bald, auxotrophic *Streptomyces lividans* strain in soil.
Toth I. K., E. M. H. Wellington, A. Vionis, A. Karagouni, J. Cullum, B. Kochte-Clemens and D. McDowell. In preparation.

Other publications

Factors affecting the mobility of antibiotic resistance genes within populations of streptomycetes in the soil. E. M. H. Wellington, I. K. Toth, N. Cresswell, P. Marsh and M. Schilhabel.
Submitted for publication in Proceedings ISME6, Barcelona, Spain 1992

References

Muth G, B. Nußbaumer, W. Wohlleben and A. Puhler. 1989. A vector system with temperature-sensitive replication for gene disruption and mutational cloning in streptomyces. Mol. Gen. Genet. 219:341-348.

SAFETY ASSESSMENT OF THE DELIBERATE RELEASE OF TWO MODEL TRANSGENIC CROP PLANTS, OILSEED RAPE AND SUGARBEET.

CONTRACT NUMBER:	BIOT-CT91-0298
OFFICIAL STARTING DATE:	01/10/92
COORDINATOR:	P. Rüdelsheim, Plant Genetic Systems, Gent, Belgium
PARTICIPANTS:	M. Renard, INRA, Le Rheu, France W. Stiekema, CPO, Wageningen, The Netherlands P. Dale, IPSR, Cambridge, United Kingdom A. Buchter-Larsen, Danisco, Copenhagen, Denmark P. Gouyon, Université Paris Sud, Paris, France S. Waters, Monsanto Europe, Brussels, Belgium J. Streibig, RVAU, Frederiksberg, Denmark S. Jensen, NFNA, Horsholm, Denmark
OBJECTIVES SET FOR THE REPORTING PERIOD:	Nothing to report
MAJOR PROBLEMS ENCOUNTERED:	Nothing to report
RESULTS:	Nothing to report. Much of the work is season dependent and the first results are expected to become available after the summer of 1992.
HIGHLIGHTS/MILESTONES:	Nothing to report

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 not now 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 there 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.

TITLE: STABILITY, GENETIC TRANSFER AND ECOLOGY OF FUNGI USED AS BIOCONTROL AGENTS

CONTRACT NUMBER: BIOT - CT91 - 0290 (SSMA)

OFFICIAL STARTING DATE: 01/10/1991

COORDINATOR: RIBA GUY, INRA, Lutte biologique, GUYANCOURT, F.

PARTICIPANTS:

BRYGOO Y., UNIV PARIS XI, Lab. Cryptogamie, ORSAY, F.
RHODES J., ICI, AGROCHEMICALS, Microbiology, BRACKNELL, UK
GULLINO L., UNIV STUDI TORINO, DIVAPRA,TORINO, I.
PELSENEER-COREMANS J., ULB, Parasitologie, BRUXELLES, B.
TURNER G., UNIV. SHEFFIELD, Mol. Biol. Biotec., SHEFFIELD, UK

OBJECTIVES SET FOR THE PERIOD REPORTING PERIOD:

* Genetic stability of heterologous DNA in *Beauveria*
* Stability of homologous recombinants of*Beauveria*
* Natural genetic transfer in *Fusarium* and *Beauveria*
* Methodology for ecological studies

MAJOR PROBLEMS ENCOUNTERED: Major problems encountered consist in :

* the selection of the most informative and reproductible molecular technics to fungal strain characterization
* the karyotyping of *Fusarium spp.*
* the adaptation of molecular technologies for soil microbiology
* the adaptation of molecular technologies to study fungal populations.

RESULTS:

1 Genetic stability of heterologous DNA (INRA and Univ. PARIS XI): The strain *Beauveria bassiana* 147 has been transformed by cotransformation procedure using the glucuronidase gene from *E. coli* and the nitrate reductase gene nia D from *Aspergillus nidulans*. Several transformants are stable and their virulence is identical to that of the parental strain. Now, the transformants will be submitted to several cultural and environmental conditions defined in microcosms **(INRA, ICI)**.

2 Stability of homologous recombinants (SHEFFIELD Univ, PARIS XI, INRA, ICI): A lambda bank of *Beauveria* is under construction and several selenate resistant mutants of this species have been selected. From this material it is aimed to isolate the ATP sulphurylase gene of *Beauveria bassiana*. Then frequency of homologous recombination during mitotic growth and plasmid stability in laboratory conditions will be estimated.

3 Natural genetic transfer (INRA, Univ. TORINO, ULB):

A Genetic characterization First we need to characterized the strains in order to follow their genetic stability within a mixture of several strains. Also it is necessary to estimate the genetic distances through the phylogenetic studies of strains, species and generous comparing rRNA sequences. Informative DNA probes are researched in *Beauveria bassiana, B. brongniartii* and *Fusarium spp.* **(INRA, PARIS XI)**. After selection, 1.5 to 2 kb probes are amplified by PCR and used for

RFLP analysis. The molecular karyotyping of *Fusarium* was obtained by pulsed field gel electrophoresis (CHEF). Nine chromosomal bands which ranged in size from 1.6 to 6.3 Mb have been found (**Univ. TORINO**). Then characterized strains will be mixted in polyclonal artificial populations and submitted to controlled or natural conditions in order to estimate competitivity of strains, selectivity of environmental factors and genetic transfer.

B Parasexuality under natural conditions: Artificial mutants (benomyl resistant or orange colored) have been used to study parasexual exhanges between antagonistic or pathogenic strains of *Fusarium* (**Univ TORINO**).
Hypervirulent interspecific diploids have been obtained by protoplast fusion of one *B. bassiana* and one *B. sulfurescens* strain. Now it is engaged to estimate the probability for those two strains to have genetic exhange within an insect host or in the soil. Also the genetic stability and the competitivity of those diploids is calculated in microcosm and natural conditions (**INRA, ULB**).

C Transposition (Univ. PARIS XI, INRA): Some strains of *Fusarium* have a natural genetic instability for some characters. Following the genetic instability of nitrate reductase gene from such strains, 3 new transposons have been observed. They belong to the bacterian types, however the homology between those 3 transposons is very low. More, studying repeated dispersed sequences a retro-transposon has been isolated. We are using the same strategy to detect transposons within *Beauveria bassiana*.

4 Methodology for ecological studies (INRA, Univ. PARIS XI): Tagging of fungal strains required specific and susceptible technics. Investigations are engaged in researching specific probes, or heterologous non coding sequences which could be amplified and used for detection in hosts or in the soils.

HIGHLIGHTS/MILESTONES: Three major original achievements must be underlined:
* Discovery of new type of transposons in fungi
* Isolation of stable interspecific hypervirulent diploids within *Beauveria spp.*
* Conception of molecular technics for fungal strain characterization

WIDER CONSIDERATIONS: The discovery of transposons and interspecific parasexual phenomenum in fungal organisms under laboratory conditions, underlines the absolute necessity to develop researches to estimate the risk assessment linked to the agronomic use of fungi under natural conditions.

COOPERATIVE ACTIVITIES:
* **INRA** and **Univ. PARIS XI** have daily exhanges and collaborative activities
* 20 march 92: Meeting between **INRA** and **ULB** staff to define protocols for ecological studies.
* April 92: Exhange of strains from **ULB** to **INRA**
* June 92: A one day meeting between **INRA, ICI** and **Univ. SHEFFIELD.** Coordination of researches and exhange of material.
* March 93 to August 93: One researcher from **INRA** will stay in **Univ. SHEFFIELD** for a cooperative program on homologous recombination in *Beauveria bassiana.*

LIST OF JOINT PUBLICATIONS: Nothing to report yet

OTHER PUBLICATIONS:

DABOUSSI M-J, LANGIN T., BRYGOO Y.- 1992. Fot 1, a new family of fungal transposable elements. Mol. Gen. Genet. (under press).

RIBA G. FORTINI D.-1992. Hypervirulent interspecific diploids between *Beauveria bassiana* and B. *brongniartii*. 25th Internat. Meeting Soc. Invertbr. Pathol., Heidelberg, Germany

TITLE: Genetic tools for constructing GEMs with high predictability in performance and behavior in ecological microcosms, soils, rhizospheres and river sediments.

CONTRACT NUMBER: BIOT-CT91-0293(SSMA)

OFFICIAL STARTING DATE: 01/10/91

COORDINATOR: Juan L. Ramos, C.S.I.C., Granada, Spain

PARTICIPANTS: Soren Molin, TUD, Lyngby, Denmark
Kenneth N. Timmis, GBF-I, Braunschweig, Germany
Victor de Lorenzo, CSIC, Madrid, Spain
Daryl Dwyer, GBF-II, Braunschweig, Germany
F. O'Gara / D. Dowling, UC, Cork, Ireland

OBJETIVES SET FOR THE REPORTING PERIOD:

I. Specialized vectors to obtain gene expression under environmental conditions and stable maintenance of engineered traits.

II. Barriers to gene transfer.

III. Conditional suicide systems. Analysis and performance of killing functions and adaptation of conditional suicide systems to suicide circuits. Improvement of TOL regulatory elements.

IV. Construction of PCB-degraders.

V. Validation tests under laboratory conditions and in microcosms.

RESULTS

To achieve the proposed goals, laboratory work was organized in three teams, so that a series of recombinant bacteria with and without a biological conditional suicide system were constructed. The work of the three teams should convergence in the near future into a large series of validation tests under laboratory growth conditions and in soil microcosms, including rhizospheres. 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, while the University of Cork group and group II at GBF were in charge of objective IV. The latter two teams also performed preliminary soil and rhizosphere microcosm tests.

For objective I the following two actions have been undertaken:

1. Construction of transposon probes to detect promoters responsive to growth phase. We plan to use promoters active under starvation conditions as expression systems for heterologous genes under environmental conditions. We chose to develop a series of genetic probes for Pseudomonas putida as a straighforward means of identifying this type of promoter. We based some of our work on the transposons previously developed to identify sporulation genes in Bacillus (1). First, we constructed three lacZ-tet cassettes which contain, as a NotI restriction fragment, a promoterless bicistronic reporter system able to distinguish the type of promoter under study (lacZ: ß-galactosidase; tet: resistance to tetracycline). One of the cassettes was tailored to produce translational/transcriptional fusions, whereas the other two were used for generating exclusively transcriptional fusions. These last two differed in the nature of the lacZ reporter, which was either an authentic intact lacZ gene or a trp-lacZ fusion. To examine whether the reporter system could be used as desired, we cloned the cassette with the lacZ-tet genes in front of the Pu promoter of the TOL plasmid (which is under strict growth phase regulation) and made a mini-Tn5 transposon (2) containing the whole unit, for insertion into the chromosome of Pseudomonas putida. The colonies containing this insertion showed intense luminescence on plates containing the Pu inducer p-chloro-benzylalcohol after they were sprayed with MUG, a fluorescent subtrate of ß-galactosidase, but they were unable to grow on plates with tetracycline, thus confirming the expected phenotype. We then constructed three mini-Tn5 transposons containing each of the lac-tet cassettes, in such a way that upon

random insertion in the chromosome of a target strain, a defined phenotype could be followed to identify insertions downstream from genes subjected to growth-phase regulation. Development of a simple procedure to utilize the cognate promoters for heterologous expression is under way.

2. Study of two new nonantibiotic selection markers: Resistance to silver salts and to the herbicide glyphosate. One of the concerns in the use of GEMs for environmental release is the undue spread of resistance genes to antibiotics of clinical importance. An alternative is the use of resistance to heavy metals and herbicides. We have obtained strains of *E. coli* and *Pseudomonas stutzeri* resistant to silver salts, and the determinant genes will be used as selection markers for vector construction. We have used PCR to clone the gene *aroA* of *E. coli* (5-enolpyruvyl-3-phosphoshikimate synthetase), a variant of which provides resistance to the herbicide glyphosate, which is also used as a nonantibiotic marker.

For objective II the following strategy has been used:

1. The Colicin E production/immunity/lysis system was used as a source of killing functions for biological containment. Inhibition of the horizontal transfer of DNA by GEMs requires the construction of genetic circuits to avoid entry of recombinant genes by bacteria present in the same ecological niche, be it through conjugation, transformation or transduction. The specific approach that we are attempting should avoid all types of transfer, by associating at least two killing functions cloned in the chromosome. Acquisition of the recombinant material along with the killing function but without immunity should then result in the death of the recipient cells. The assembly of this type of circuit requires, first of all, the availability of universal killing functions and their corresponding immunity genes. Among possible candidates we have picked the colicin E3 system. The production and immunity genes have been independently cloned and expressed, and their combination with adequate reporter genes is in progress to test the above mentioned concept.

The experimental work devoted to objetive III has been aimed at designing a suitable biological containment system to be used in concert with the degradation of xenobiotics. The strategy chosen has been to couple a killing gene to the regulatory loop of degradative pathways, such that expression of the killing function is repressed during the period of metabolization of the

the xenobiotic; after the compound has been removed, the killing function should be derepressed. The model system under study is a modified TOL meta-cleavage pathway for the metabolism of alkylbenzoates (3) combined with the lac operon and the suicide gene, gef.

1. The host killing function gef. The E. coli gef gene has been shown to express a very toxic cell killing polypeptide of approximately 50 amino acids. The lac gene was inserted downstream of the strong, synthetic lac promoter in pVHE24-3 which has a very low basal level (uninduced), and a very high expression level after induction. Transcription is controlled by the lacI repressor. Induction of Gef protein results in killing of E. coli.

2. Killing of P. putida bacteria by Gef protein. The killing cassette (P_{lac}::gef; lacI) was subsequently inserted in a mini-Tn5 transposon (3), and transferred via bacterial conjugation into the chromosome of P. putida 2440. It was found that induction of Gef protein resulted in cell killing. In particular, killing of P. putida 2440 was as efficient as that of E. coli. We therefore concluded that a single copy of the gef gene is sufficient to kill this organism.

3. Coupling the killing cassette with the TOL degradative pathway. Previously, a fusion between a promoterless lacI gene and the Pm promoter of the meta-pathway of the TOL genes was constructed; in E. coli the simultaneous presence of a P_{lac}::gef fusion resulted in cell death in the absence of benzoate acting as an inducer of the repressor (3). After insertion of the P_{lac}::gef fusion on the chromosome of P. putida bearing the fusion Pm::lacI on plasmid pCC102, similar results were obtained, indicating that benzoate-conditioned killing of the cells can be transferred to P. putida. However, due to the high dose of the lacI gene, the system was not sufficiently effective, and the next important step is therefore to move all components into the chromosome of P. putida.

4. Survival of P. putida bearing gef gene in soil microcosms. The wild type Pseudomonas putida 2440 bearing recombinant TOL plasmid pWWO-EB62 and pCC102 (Pm::lacI), and the same strain but also carrying a chromosomal insertion of the killing cassette P_{lac}::gef, were introduced at low doses (about 10^4 CFU per g $soil^{-1}$) in two different soils from the Granada area (a cambisol soil [poor in organic matter] and a fluvisol soil [rich in organic matter]) unamended or amended with 0.1% 3-methylbenzoate (3MB) or 0.1% glucose. In the cambisol soil unamended or amended

with glucose, the bacteria without the killing function became established at about 10^3 CFU g $soil^{-1}$, while survival of the bacteria bearing the killing cassette was notably reduced. The number of CFU decreased with time, to less than 20 CFU per g $soil^{-1}$ after 3 weeks. In contrast, the number of CFU per g $soil^{-1}$ was greater than 10^3 in soils amended with 3MB. In the fluvisol soil, in samples amended with glucose or without amendement, the number of CFU remained low with time (about 10^4 CFU g $soil^{-1}$), while in the soils amended with 3MB, the number of bacteria increased to about 10^7 CFU g $soil^{-1}$.
Our current results show that gef prevents multiplication in soils of P. putida bearing rDNA. However, the high dose of lacI makes the system less efficient than desired. A series of constructions bearing all components in the chromosome of P. putida 2440 is being prepared. New control systems including improved promoters of the TOL plasmid upper pathway are under development.

The third team of laboratories has directed their efforts to the construction of PCB-degrading strains of Pseudomonas fluorescens, which are good colonizers of the sugar beet rhizosphere, and P. putida KT 2442. The engineered strains were characterized to see if colonization efficiency had been altered by the addition of the new genes under selective and nonselective conditions in the rhizosphere microcosms. Specific actions involved:
1. Introduction of PCB genes into rhizosphere strains. Three rhizosphere strains, Pseudomonas F114 and M113, and P. putida KT2442, were chosen as recipients for the genes encoding biodegradation of PCBs, which were confined within a PCB cassette. Potential PCB degrading strains were constructed by mating recipients with a permissive E. coli donor carrying the PCB cassette on a suicide plasmid pDDPCB. Pseudomonas transconjugants bearing the transposon on the chromosome were selected as able to grow on biphenyl (BP) as a sole carbon source. Transconjugants were recovered at low frequency (10^{-6} per recipient) and were confirmed by spraying with 2,3-dihydroxybiphenyl, (2,3OHBP) an intermediate in the breakdown pathway of PCBs, which results in a yellow color if the product of the bphC gene is expressed.
2. Genetic stability in vitro. The stable maintenance of the PCB genes was assessed by growth in nonselective media followed by testing for the retention of the ability to grow on BP. Both F113PCB and KT2442PCB grew stably on minimal medium with BP after

40 generations in nonselective media.

3. **Growth rates of F113 and F113PCB.** Transconjugant F113PCB was chosen for further study. Growth rates in different media were compared with those of its parent strain, F113Km. No differences were found in growth rates, suggesting that the insertion of the transposon did not cause changes in the GEM.

4. **Behavior in natural settings: The sugar beet rhizosphere.** The GEM F113PCB was applied to sugar beet seeds prior to sowing, and colonization and maintenance of the PCB degrading trait were followed over a period of 4 weeks. Strains containing the PCB cassette could be identified by their ability to use biphenyl (BP) as a sole carbon source, and by the production of a yellow product when sprayed with 2,3-OHBP. Inoculated seeds were sown in nonsterile soil with and without 100 ppm 4-CBP in a growth cabinet. Bacteria were isolated from the root system at different times and plated on selective medium. Total CFU were estimated, and those retaining the PCB genes were found by spraying with 2,3-OHBP and by growth on BP as the sole C-source. Colonization efficiency of the GEM was found to be the same as that of the wild type in both amended and unamended soil.

5. **Stability in soil.** The cassette was found to be stable, with 100% of recovered F113PCB still retaining the BphC$^+$ phenotype after 4 weeks in the rhizosphere.

REFERENCIAS

1. Youngman, P., Zuber, P., Perking, J.B., Sandman, K., Igo, M. and Losick, R. (1985). Science 228: 285-291.

2. Herrero, M., de Lorenzo, V., Timmis, K.N. (1990). J. Bacteriol. 172: 6557-6567.

3. Contreras, A., Molin, S., and Ramos, J.L. (1991) Appl. Environm. Microbiol. 57: 1504-1508.

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, soil microcosms and rhizospheres. Genes (including suicide killing functions) were expressed as expected and appeared to be stable. This proves that our strategy is an efficient approach to the engineering of strains for various environmental purposes.

MILESTONES

1. A number of useful tools have been designed to obtain an efficient expression system under environmental starvation conditions.
2. New nonantibiotic markers for tracking herbicide and heavy metal resistance in GEMs are being developed.
3. The TOL meta-cleavage pathway regulatory elements, the Pm promoter and the xylS regulator have been coupled to the gef gene, whose gene products kill cells when overexpressed.
4. Pseudomonas putida bacteria bearing a recombinant TOL plasmid and the suicide function under Pm/XylS control have been shown to function both in the laboratory and in soils.
5. The GEM F113PCB was sucessfully constructed using a suicide delivery system (pDDPCB).
6. The PCB phenotype was stably maintained both in vitro and after growth in a nonsterile soil during 4 weeks.
7. Colonization of sugar beet roots by the GEM was monitored, and was found to be similar to colonization of the parent in both BP amended and nonamended soil.

COOPERATIVE ACTIVITIES

Lars B. Jensen from the Technical University of Denmark visited J.L. Ramos's laboratory for three months to carry out joint work.
J.L. Ramos visited Dr. Soren Molin to further define collaborative work.
Dr. V. de Lorenzo provided partners with new suicide transposons.
The PCB genetic cassette used in the University of Cork was constructed in K.N. Timmis's laboratories.
Dr. D. Dwyer visited the University of Cork to further define details of collaboration.
K.N. Timmis and J.L. Ramos exchanged strains.
A plenary meeting will be held in June in Granada.

TITLE: Experimental and modelling studies on the fate in soil of introduced biologically-contained bacteria

CONTRACT NUMBER: BIOT-CT91-0288

OFFICIAL STARTING DATE: 01/10/91

COORDINATOR:
Dr. J.D. van Elsas, Inst. for Soil Fertil. Res., Wageningen, NL

PARTICIPANTS:
Dr. J.D. van Elsas, Inst. for Soil Fertil. Res., Wageningen, NL
Dr. N. van der Hoeven, IMW-TNO, Delft, NL
Dr. S. Molin, Techn. U. of Denmark, Lyngby, DK
Dr. S. Kjelleberg, U. of Goteborg, Goteborg, SW

OBJECTIVES SET FOR THE REPORTING PERIOD:
The objectives set for the working period are apparent from the work planning chart which makes part of the original workplan (annexed). It includes the insertion and further development of inducible killing genes into soil isolates, microcosm testing of non-biologically-contained (parent) bacteria, development of detection methodology from soil, selection of crucial parameters for a model, and studies on responses of bacteria to nutrient and other stresses.

MAJOR PROBLEMS ENCOUNTERED:
There are so far no major obstacles to the success of the project. As already indicated in the workplan, a crucial point in the project will be the availability of suitably working "active" killing genes for the soil bacteria in use. This hurdle is expected to fall between month 6 and 12 of the project. An alternate strategy based on modification of starvation survival genes (e.g. *dnaK*) is being worked out should the development or functioning of active principles prove unexpectedly difficult.

RESULTS:

The involvement of the 4 groups in the project can be found in the work plan. Here, progress of each of the 4 groups will be briefly treated.

The aim of the ISFR GEM project, of which this endeavour makes part, is the biocontrol of dipteran insects which damage gramineous plants. It is focussed on the insertion of the 3'-truncated fragment of the δ-endotoxin gene from *Bacillus thuringiensis* var *morrisoni* (*cry*IVB) in soil bacteria adapted to the rhizosphere of graminea. For this purpose, two *Pseudomonas* strains, *P.fluorescens* R2f and *P. cepacia* P2, were isolated. *P. fluorescens* R2f was first selected as a carrier for *cry*IVB, since it is most amenable to genetic modification. Soil studies with marked derivatives revealed a decline of introduced organisms, and a stimulation in the rhizosphere of young wheat plants. Further work focused on competition between GEMMO and parent strains in soil. Two derivatives of a rifampicin-resistant R2f mutant, Ar-1 (chr::*npt*II) and Art-3 (chr::*npt*II-*cry*IVB) were tested versus the parent in loamy sand microcosms planted to wheat. During the 15-day experimental period, the introduced *P. fluorescens* populations decreased slightly in the root free soil, while there was an increase of the *P. fluorescens* cfu counts in the rhizosphere. Roots were also colonized. The proportion of both GEMMOs in the introduced populations (Ar-1/R2f and Art-3/R2f mixes) decreased in bulk and rhizosphere soil and in the rhizoplane. There was no such trend in the mixture Ar-1/Art-3. The similarity in behaviour between strains Ar-1 and Ar-3 led to the conclusion that they were equally impaired in their fitness in soil as compared with the parent strain, either due to an effect of the *npt*II gene product on cellular physiology or to an impairment of functioning of genes flanking the chromosomal insertion site.

Effects of antibiotic pressure on GEMMO performance was a further focus, with the aim to study possible selective advantage in soil and rhizosphere of GEMMOs carrying antibiotic resistance markers. Transposon Tn5 carrying *P. fluorescens* R2f which expresses resistance to neomycin (kanamycin) and streptomycin, was used. Soil was supplemented with 100 or 1000 µg of either neomycin or streptomycin per ml soil moisture (equivalent to 20 and 200 µg per g of dry soil). Neomycin did not affect inoculant numbers, whereas streptomycin at 1000 µg/ml stimulated the inoculant in bulk soil and in the wheat rhizosphere. These results indicated a slight competitive

advantage for introduced strains carrying streptomycin resistance genes in soil microsites where streptomycin is present at high concentrations.

For field release of the *cry*IVB-loaded strains, it is necessary to reduce putative hazard using biological containment. One strategy could be based on killing outside the rhizosphere. For this, promoter activity specifically responding to signals in root-free or rhizosphere soil was screened for in the *Pseudomonas* strains. To select promoters responding to signals from soil, conditions likely to occur in soil, e.g. carbon starvation, low temperature (10°C) and low water activity were taken as factors. Estimations of the amount of organic carbon dissolved in soil water revealed concentrations of about 100 ppm (equivalent to 250 ppm glucose) in the soil used. Organic carbon released by wheat roots was estimated to amount to 300 - 3000 ppm per ml soil water (equivalent to 800 - 8000 ppm glucose) per cm root. Therefore, mineral medium (M9) supplemented with glucose at concentrations of 100, 250, 1000 and 4000 ppm was selected as a medium. Glucose was shown to be growth-limiting at 100 ppm for P2 and at 100 and 250 ppm for R2f. Carbon starvation promoters were looked for using 100 and 250 ppm for the "on" response and 2000 and 4000 ppm for the "off" response. Temperatures in Dutch soils may differ between below-zero and 25-30°C. Both R2f and P2 were shown to grow on M9 agar plates supplemented with glucose at temperatures ranging from 10 - 27°C.

To obtain differentially induced promoters, we used the promoter probe system of Simon *et al.*, which is based on Tn5 containing a promoter-less *lacZ* gene. Tn5::*lacZ*, located on suicide plasmid pSUP102, was delivered to rifampicin-resistant mutants of R2f and P2 via conjugation, using kanamycin and rifampicin selection, and promoter activity upstream of the Tn5::*lacZ* element was screened by assaying ß-galactosidase activity.

Two R2f mutants were obtained, which gave a 20 - 26 fold higher ß-galactosidase activity in M9 medium containing 100 ppm glucose compared to 4000 ppm glucose. One, RA92, revealed differential ß-galactosidase activity in both the late exponentional and stationary phase, whereas the second, RA74, only gave a differential response in the stationary phase. Further, 7 mutants of P2 responded to carbon starvation; expression studies with these mutants will be performed. Furhter, a P2 mutant (PD19) was isolated which responded to 10°C as opposed to 27°C, whereas no cold-induced R2f mutant was found.

The experimental work at the Techn. U. of Denmark has been aimed at designing a suitable biological containment system to be employed in concert with the design of the *Pseudomonas* biopesticides. The strategy chosen has been to couple a killing gene to a stochastically induced expression system. The purpose of this design is to limit the long-term survival of the bacteria as a consequence of a constant rate of elimination of cells from the population. Several model systems are being designed and tested. Isolates of *Pseudomonas*, capable of colonizing plant roots, including strains R2f and P2, are the model organisms. The killing gene is either the *E. coli gef* gene or nuclease genes isolated from different microorganisms. The stochastic induction will be based on either homologous recombination or site-specific recombination during which process a negative expression control element is deleted.

The *E.coli gef* gene expresses a very toxic cell-killing polypeptide of roughly 50 amino acids. The gene was inserted downstream of the strong, regulatable promoter in plasmid pUHE24-2 (H.Bujard, personal communication). The promoter is a synthetic *lac* promoter which has a low basal level (uninduced) and a very high expression level after induction. Transcription is controlled by the *lacI* repressor. Induction of Gef protein resulted in killing of *E. coli*. We have recently also designed killing systems based on highly active nucleases. The major idea is to kill and remove contaminating DNA at the same time. Two different extracellular nucleases have been redesigned such that they no longer are excreted (removal of signal peptide sequences). Induction of these enzymes results in severe growth inhibition, but optimization is still required for efficient killing to occur.

The killing cassette thus obtained was subsequently inserted in the mini-Tn5 transposon system developed by de Lorenzo *et al.* (1990), and transfer of the derived transposons via bacterial conjugation resulted in chromosomal insertions in a number of gram-negative bacterial species. In all but one case it was found that induction of Gef protein resulted in cell killing. In particular, killing of *P.putida* strain 2440 was as efficient as that observed in *E. coli*. We therefore conclude that a single copy of the *gef* gene is sufficient for killing of this organism.

A stochastic induction system based on homologous recombination has been designed in which two direct repeats of a 1000 bp sequence flank a marker gene (Km-resistance) and a *lacI* gene. On a separate replicon
the above P*lac-gef* fusion was inserted, and it was shown that this design had the expected phenotype of inhibiting growth in slow-growing cultures in contrast to fast-growing cultures. The system appears

useful for multicopy plasmids, whereas the recombination frequencies with chromosomal inserts are probably too small.

An alternative strategy was started based on the plasmid resolution system of the broad host range plasmid RP4. In this system two repeats of ca. 100 bp will recombine very efficiently through the action of a site-specific resolvase. The advantages of this system are: the high rate of recombination, the requirement for only short sequences and the presumed broad host range of the recombination system. The first result using reporter genes looks very promising.

Research of the U. of Goteborg group has focussed on stress responses and starvation survival of marine bacteria, i.e. *Vibrio proteolyticus* S14 (CCUG 15956), and, for comparison purposes, *V. anguillarum*, *V. vulnificus*, *V.* DW1. The findings reported here will serve as a basis for comparison of stress responses in soil pseudomonads, as well as for alternate containment strategies. The role of C-starvation as the determinant for the differentiation into starvation- and stress-resistant cells has been established. It was shown that N- and P-starvation are not sufficient triggers for induction of a successful adaptation programme. C-starvation, however, offered complete protection against a variety of inducers and allows for long-term survival. Similarly, experiments in which overall macromolecular synthesis rates and the signal molecule ppGpp were determined, identified C-starvation as the determinant for total or multiple starvation.

Furthermore, starvation-induced differentiation appeared to include resistance against predation. In collaboration with Dr. Ingve Børsheim at the University of Trondheim, it was shown that growing cells of *Vibrio* are taken up and digested by a microflagellate, while starved cells are not. Biological containment that prevents the development of predation resistance may facilitate removal of released GEMMOs by predators. The control regulons that are part of this response, such as the stringent control regulon, are likely targets for containment constructs.

MiniMu (*lac*) insertion fusions, in which the reporter gene responds to starvation promoters, induced at different times during the initial period after growth arrest, have been generated. These mutants show little loss of viability as compared to the wild-type. The genes involved have not yet been identified. However, killing genes may be inserted in these sites to allow for a programmed die-off. These fusions are further essential for the identification of regulatory genes of the carbon starvation stimulon, with predicted loss of viability during C-starvation.

In collaboration with the U. of Denmark, pUT and pLOF transposon constructs have been evaluated and reconstructed. In particular, a mini-Tn 10 construct was very efficient in yielding high frequencies of transconjugants with single inserts in the chromosomes of several strains. This construct will be used for identifying late starvation promoters, for deletion or inactivation as well as for insertion of killing genes. Furthermore, this construct will be used to identify genes specifically involved in the upshift response: a series of *mat* (maturation) genes are induced sequentially and transiently - prior to induction of growth genes - during recovery by nutrients of long-term starved cells. Inactivation of these genes, or insertion of killing genes behind *mat* promoters may prevent the cells from exiting starvation and proliferating.

Four genes have been identified as major regulators of the starvation-induced differentiation response. Three of these, *rel*A, *dna*K and a Tn generated regulatory mutant, were explored while the fourth gene, *kat*F, so far has only been shown to exist in *Vibrio* by hybridization with an *Escherichia coli* probe. *Kat*F is a newly discovered stationary phase or starvation sigma factor in *E. coli*; it regulates at least 20 stationary phase or starvation genes, including morphogenes that allow for conversion of the rod to the coccus shape, *cst* (carbon starvation) and *pex* (post exponential) genes in *E. coli*. A *kat*F mutant rapidly lost viability after growth. The *rel*A gene product (stringent factor) controls formation of ppGpp in the stringent control response. The *rel*A gene was found by insertion of V. S14 DNA into a *rel*A⁻ *E. coli* and screening for restoration of wild-type *E. coli* stringent physiology on aminotriazol plates (prevents growth of *rel*A-strains). *Dna*K encodes a Hsp10 heat shock protein, a major chaperone in prokaryotes. A *dna*K gene was cloned by PCR.

The role of the stringent control as a regulator of the initial phase of starvation-induced differentiationy is well established. Evidence for DnaK as a major regulator has also accumulated. DnaK was shown to be synthesized at increased rate following starvation of 2 *Vibrio* spp. Two C-starvation induced types of Hsp70 were found. It is suggested they are key regulators during starvation. *Dna*K may also be induced by the low water content of starved cells.

A mutant in a regulatory gene of the C-starvation stimulon may prove to be lethal during starvation (possibly for *kat*F-, *rel*A- and *dna*K-). By inserting a second transposon (mini-Tn 10) in a strain carrying a mini-Mu (*lac*) insertion in a gene which was induced exclusively during C-starvation, a clone no longer induced by C-starvation was obtained. Viability was reduced 100-fold after 4 days of

starvation. This regulatory mutant is presently being compared with its parent using 2D gel electrophoresis of pulse-labelled cells. Proteins of the C-starvation stimulon will thus be identified.

Several genes of potential interest for the design of disabled strains have thus been identified. Their inactivation, deletion or utilization for insertion of a lethal gene will allow us to evaluate the behaviour of organisms carrying them in microcosm experiments.

The work of the IMW-TNO focused on models for the fate of bacteria in soil. This report examines the structure of models to be developed for the prediction of the fate of GEMMOs in soils, including biologically-contained ones. The model will be structured using factors which affect the fate of these bacteria in soil. These are: 1) cell properties (growth rate, survival, activity of killing genes, mobility) as affected by environmental conditions, 2) soil structure, 3) the localization of the bacteria in soil, i.e. in micropores of different size classes or in the rhizosphere, 4) the presence of competing bacteria or predators, and 5) the availability of nutrients. The distribution of nutrients throughout soil compartments determines the growth potential in these compartments; replacement of used nutrients is also relevant. Transport will be modelled assuming mobility of bacteria in soil takes place with water flow, soil animals or mechanically with soil particles. The fate of the introduced genes depends on, besides on host fate: 1) the probability of transfer to other bacteria and 2) the persistence of DNA in soil following cell lysis.

Factors important for modelling the fate of *P. fluorescens* in soil are obtained from experiments at ISFR. However, since the influence of several factors is unknown, some simplifying assumptions have to be made. Important points for the model are:

- The structural diversity of the soil: sites (pores) with higher or lower growth capacity (nutrient availability), more or less predation, different bacteria already present,
- the first come, first served principle: resident bacteria are assumed to have an advantage over intruders, not implying invasion is impossible,
- transport by water fluxes: a simple model would only consider an overall transport probability from one point to another, neglecting the fine distribution of water,
- The effect of a host killing gene: the mortality rate will be asuumed to be enhanced by the host killing gene,
- Gene transfer: gene transfer will be assumed to occur only between bacteria in one patch; transfer is proportional to donor and recipient density.

A tentative model description is as follows:

Let there be K patches (index k (or l), from 1 to K), and J bacterial species (strains) (index j (or i), from 1 to J). The volume of patch k is V_k, in that patch are N_{jk} bacteria of species j, so the bacterial density in that patch is $X_{jk} = N_{jk}/V_k$. The growth minus mortality rate of species j in patch k depends on the density of all other bacteria in that patch, and can be described by the function:

$R_{jk}\,[1-f_{jk}(X_k)]$, where $f_{jk}(0,0,...,X_{jk},..,0,0) \leq f_{ik}(0,0,...,X_{jk},..,0,0)$ for each i. This rule ensures that an occupied patch cannot be invaded by intruding bacteria. The predation rate in a patch, P_{jk}, may depend on the patch and may differ between bacterial species. Transport between patches goes through a kind of 'transport patch', where no growth occurs. It will first be assumed that only one 'transport patch' exist, i.e., from each patch bacteria will go with a certain probability to the transport patch, and from that patch each normal patch can receive bacteria. Transport is a random process, occurring in discrete units. The effect of the transfer of bacteria from a 'normal' patch to the 'transfer' patch on the bacterial density in that 'normal' patch will be considered negligible. If more than one transport patch is assumed, rules for transport between such patches have to be formulated, and each 'normal' patch has to be linked to a specific 'transport patch'. A take-over of a patch by another species will generally only be possible if more than one bacterium enters that patch within a short timespan. Densities which correspond to less than one bacterium are considered to be 0. Most patches will only contain one bacterial species. For such a model, the function f_{jk} has to be chosen and its parameters have to be estimated, just as the parameters R_{jk} and P_{jk}. Furthermore, estimates have to be made about the transfer rate and the volumes of each patch. Simple assumptions about these functions and parameters are: $f_{jk}(X_k)$ is a linear function in X_k and only a limited number of different patches (i.e. patches with different parameter values and volumes) exist, for instance: 1) pore patches: no predation, low invasion rate, small volume, 2) rhizosphere patches: predation and a high growth rate, intermediate invasion rate, 3) standard patches: predation and a low growth rate, high invasion rate. This model will lead to a long coexistence of many bacterial species (those which are initially occupying a patch). In the long run, successful invasions in other patches will lead to a reduction in the number of species. The time span of this process, however, will make this species reduction not very relevant. When the number of patches becomes very high, it may be appropriate to start working

in densities of patches with a certain bacterial species, instead of in bacterial densities. This method has to be worked out further.

Basic assumptions of this model are: 1) Spatial inhomogeneity of soil is a crucial aspect controlling soil microbiology; simplification of this leads to a "poor" model, 2) The effect of nutrients can be simplified to a competition model with direct competition between species instead of competition through depletion of nutrient, 3) Each patch can be considered to be homogeneous, 4) The transport mechanism chosen in this description is relevant for soil, 5) The mutual exclusive principle as a result of interaction is correct.

Other modelling options have been considered and will receive attention in the following period in parallel to the model proposed.

HIGHLIGHTS/MILESTONES:
The project is in a too early stage to have major achievements. It is foreseeable that a suitable biological containment system for use in soil will become available in the following period.

WIDER CONSIDERATIONS:
A biological system is being developed for the efficient control of bacterial populations released into soil for biocontrol purposes. The system is based on genes naturally present in bacteria.

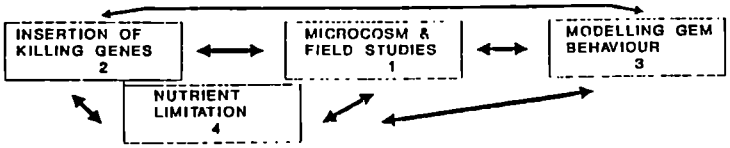
COOPERATIVE ACTIVITIES:
The main contact between all participating groups has been a group meeting organized march 26 and 27, 1992 in Wageningen. Progress and strategies for the next period were discussed. Further, a collaborator of the ISFR group and collaborators of the U. of Goteborg visited the Techn. U. of Denmark laboratories for discussions and some cooperative work (dec 1991). The IMW-TNO collaborator has been in frequent contact with the ISFR for detailed discussions about factors for a model describing fate of inoculants in soil. Further contacts have been via normal communication channels.

JOINT PUBLICATIONS:
Time has not permitted any joint publications. The first output may be expected in the next experimental period.

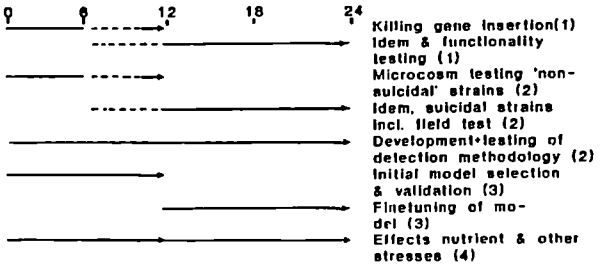
OTHER PUBLICATIONS:
See under joint publications

WORK PLANNING CHART

I. Interactions and flow of information:



II. Time (bar) chart:



TITLE: An Experimental Approach To Investigate Horizontal Gene Transfer Between Organisms.
CONTRACT NUMBER: BIOT-CT91-0287 (SSMA).
OFFICIAL STARTING DATE: 1 January 1991.
COORDINATOR: Dr Richard Peter Oliver, UEA., Norwich, G.B.
PARTICIPANTS: Dr. R.P.Oliver
Dr. M.A. Grandbastien (Contractor INRA Versailles).
OBJECTIVES SET FOR THE REPORTING PERIOD:

1. The creation and testing in *C.fulvum* and Tomato of chimeric marker genes.
2. The creation of marked copies of both transposons and transformation into *C.fulvum.* and Tomato.
3. Collection of world-wide *Solanaceae* strains.
4. Screening of the plants by hybridisation and PCR and sequencing.
5. Collection of world-wide *C.fulvum* strains.
6. Screening of the *C.fulvum* strains by hybridisation and PCR and sequencing.

MAJOR PROBLEMS ENCOUNTERED: The research work has not been in progress in Norwich for the duration of the reporting period. The post-doctoral position was not filled in Norwich until 17 February 1992 and the technician position was not filled until two weeks later.
RESULTS:1 Chimeric marker genes have been created in both laboratories. In Norwich chimeric genes for either hygromycin phosphotransferase or ß-glucuronidase (GUS) using the gpd promoter have been created, whilst in Versaille these marker genes have been constructed using the CaMV 35S promoter.These chimeric marker genes have been exchanged between the participating laboratories and are currently being tested in *C.fulvum* and tomato.

2.The creation of marked copies of both transposons is under way in both laboratories. Difficulty was encountered in the creation of marked copies of *Cf*T-1 since no unique site within a non coding region of the cloned retrotransposon was available for the insertion of a marker gene. These problems have largely been overcome and the construction of a marked copy of the *Cf*T-1 retrotransposon is nearing completion.

3. The French group have initiated the world-wide collection of *Solanaceae* strains.

4. In Versaille a Tnt1 LTR-GUS construct has been shown to be specifically activated by cell wall hydrolases.

5. To date 31 *C.fulvum* strains have been collected world-wide. The possibility of obtaining further strains from South America is in progress.

6. These 31 *C fulvum* strains have been screened by hybridisation. A probe P5 has hybridised to all 31 *C.fulvum* strains so far collected and although most fragments of the genome containing this element are the same between strains there are some which show polymorphisms. The probe P5 encodes part of the Reverse Transcriptase gene of *Cf*T-1 and shows homology to other Reverse Transcriptase genes and was used as a probe since it appears to be the most highly conserved region of the element.

HIGHLIGHTS/MILESTONES: Nothing to report.
WIDER CONSIDERATIONS: An assessment of the risks involved in the release of genetically engineered organisms must take into account the frequency of horizontal gene transfer. The frequency of horizontal gene transfer places an upper limit on the safety of the deliberate release of engineered organisms because it will indicate the likelihood of gene-escape to unrelated species in the environment. The observation that an element related to the *C.fulvum* retrotransposon *Cf*T1 is present in tomato may indicate recent interspecies genetic exchange. The objective of this research is to experimentally measure the frequency of horizontal gene transfer between eukaryotic organisms.
COOPERATIVE ACTIVITIES: The participating members met on the 31 March 1992 at INRA Versaille and discussed major scientific aspects of the project. Chimeric marker gene were exchanged together with tomato seeds. Freeze dried fungal exudates have been sent to Versaille to determine if they will specifically activate the Tnt1 retrotransposon.
LIST OF JOINT PUBLICATIONS/PATENTS WITH TRANS-NATIONAL AUTHORSHIP: Nothing to report.
OTHER PUBLICATIONS/PATENTS: Nothing to report.
GENERAL REMARKS/SUGGESTIONS/COMPLAINTS: Nothing to report.

TITLE: Safety of genetically engineered retroviruses used for gene transfer

CONTRACT: BIOT - CT91 - 0286

OFFICIAL STARTING DATE: 1/10/1991

COORDINATOR: Finn Skou Pedersen, Aarhus University, Aarhus, DK (UA)

PARTICIPANTS: Walter H. Günzburg, GSF-München, Neuherberg, DE (GSF)

John Guardiola, IIGB, Napoli, IL (IIGB)

Gerard Verdier, Université Claude Bernard, Lyon, FR (UCB)

Claire-Michelle Calberg-Bacq, Université de Liege BE (UL)

OBJECTIVES SET FOR THE REPORTING PERIOD:

Task 3 (ii): Effect of steroid hormones on expression of integrated proviruses.

Task 5 (i): Screening of infected animals for the presence of virus.

Task 5 (iii): Production of amphotropic and ecotropic producer cells.

MAJOR PROBLEMS ENCOUNTERED:

Delay in transfer of money from EC-administration to the coordinator caused a delay in initiation of experimental work.

RESULTS:

UA: Personnel has been employed as foreseen in the contract. Work is progressing with respect to transfer, expression and genetic stability of murine leukemia virus derived vectors in cultured murine cells. Oligodeoxynucleotide primers for PCR amplification and sequence analysis have been designed and synthesized, and nucleotide sequence information of integrated vector proviruses has been obtained. Cell clones with integrated proviruses selected for higher expression have been characterized by Southern hybridization and PCR sequence analysis. Experiments directed towards the analysis of long term stability of expression of integrated proviruses have been initiated and differences among individual proviruses have been observed. Tools for analysis of recombination between infecting vector proviruses and endogenous sequences are being generated. Discussions and scientific visits between the project leaders of the UA and GSF groups have led to new strategies based upon these materials and resulting in mutual exchange of co-workers. Studies analysing the relative influence of promoter-enhancer elements of the vector and of the host chromosomal integration site are continuing. As part of this, studies of the inducibility of individual integrated vector proviruses by the steroid hormone dexamethasone have been completed and a manuscript is in preparation. Discussions between the groups of UA and GSF have led to further collaborative efforts concerning retroviral vector infection of embryonal cells.

GSF: Personnel has been employed as foreseen in the contract. Initial experiments aimed at determining the elements involved in the tissue specific expression of genes in the mammary gland have been completed and a manuscript has been submitted for publication. Based upon these initial findings, strategies have been set for the construction of tissue specific retroviral vectors targeted towards the mammary gland. PCR primers required for the construction of such retroviral vectors have been synthesized. In approaches aimed at the targeted integration of retroviral vectors at specific genomic loci, three classes of retroviral vector have been constructed carrying sequences of a impaired test gene, the Herpes Simplex Virus thymidine kinase gene in different locations within the vector. A number of cell lines have also been established carrying single copies of the test target gene (thymidine kinase). Using positive and negative selection strategies, the number of homologous recombination events between the retroviral vector carrying the test gene and the target gene in the cell can be determined. Amphotropic and ecotropic packaging cell lines containing the vector have been established and experiments carried out to increase the titre of infectious virus by "ping-pong" infection. Such virus has been used to infect the target cell lines and preliminary results as to the recombination efficiency have been obtained. In collaboration with the IIGB group, retroviral vectors have been introduced into animals to determine the risk of infection of the germ line. Progeny animals have been tested for acquisition of retroviral vector sequences. New rapid protocols for the screening of large numbers of progeny animals have been established. Discussions between the GSF and UA participants have led to new sub-projects related to retroviral infection and expression in embryonal cells.

IIGB: MHC class II genes are expressed in mature cells of the lymphoerythropoietic system. A tissue-specific MHC class II promoter may thus constitute an ideal tool for targeting expression to different cell types of the lymphoid and the erythroid cell compartment. A promoter sequence from the human major histocompatibility class II gene HLA-DQA1 has been fused to the CAT reporter gene, for use in transient transfection assays, or the hph (hygromycin resistance) gene for permanent transfection studies. The HLA-DQA1/CAT and the HLA-DQA1/hph constructs have been introduced into retroviral vectors as well as conventional expression vectors. In order to establish the minimal promoter sequences required for tissue specific expression of MHC class II genes in different cell lineages, different fragments from the HLA-DQA1 promoter have been assayed in various human cells types in vitro. The ability of the minimal promoter thus identified to direct correct expression was verified upon stable transfection of HLA-DQA1/hph constructs in the same cell types. We found that the minimal promoter element was faithfully regulated also when integrated at different chromosomal locations; the permanent transfectants obtained are now being studied in greater details. The HLA-DQA1/hph fusion was subsequently cloned into a retroviral vector, pLJ, in either orientation and transfected into helper cells. The retroviral particles obtained have been used to infect helper free cell lines. We have found that the presence of retroviral sequences interferes with regulated expression of the HLA-DQA1 promoter used. We are now in the process of characterizing the clones obtained after infection in order to understand the mechanisms by which this interference is brought about. In collaboration with the group at the GSF we have continued our effort in the study of the ability of retroviral vectors to infect stem cell of the male germline in mice. Preliminary experiments have been performed in order to test the infectivity of retroviral particles in vivo after injection into the testes. Further, a methodology has been developed to transplant retrovirus producing cells into the testis of the animals, without affecting their ability to fertilize female mice and produce offspring.

UCB: The main focus of the UCB participant has been to develop avian leukosis virus based vector systems with an emphasis on reducing the risk of generation of helper viruses and on targeting vector infection and expression to specific tissues. To derive safe helper cells, packaging-mutants from ALVs have been crippled by separating gag-pol and env into two transcription units and by deleting noncoding sequences required for a complete virus life cycle. Shut-off of expression of the proviral genes in the packaging cells has been a major problem in past work. Such problems have been overcome by insertion of genes for selectable markers (hygro, phleo) into the transcriptional units of viral genes. These new packaging cells stably produce the packaging functions provided they are cultivated under selective pressure (hygromycin and phleomycin). The packaging cells generated from quail cells have been checked for absence of production of replication-competent viruses and for stable production of retroviral vectors in high titres. In contrast, the use of a chicken cell line as a helper that bears and expresses avian leukosis virus related sequences resulted in the generation of replication-competent viruses after various times. Preliminary analyses demonstrate a risk of transmission of some of the endogenous RNAs to the target cells causing either recombination or modification of the vectors and experiments are in progress to characterize such recombination mechanisms. With the overall aim of targeting vector expression to specific tissues, double expression vectors have been constructed. Two selectable marker genes are expressed from the retroviral promoter and from an SV40 promoter in an internal transcriptional unit, respectively. Interference from the strong avian leukosis virus promoter may cause problems with respect to expression from an internal promoter. To reduce this problem the retroviral integration signals have been manipulated to allow integration of the provirus in a disorganized form. Infected cells of the expected characteristics have been obtained and the detailed structures of inserted vector genes are presently under investigation.

UL: The UL participant has mainly been concerned with tasks dealing with the stability, infectivity, and penetration of retroviral particles into cells, all properties dependent upon the viral glycoproteins and on the integrity of the viral envelope. Current work uses three retroviral constructions, all derived from Moloney murine leukemia virus. Ecotropic and amphotropic producer cell lines have been prepared and the relative infectivity of the vector viruses produced are being characterized for mouse cells of different origin, made available in part through collaboration with the GSF-participant. In parallel experiments the infection risk of corresponding cell lines of human origin is being evaluated. Work relating to infection of cells in animals has been initiated. Experimental protocols have been set up to follow the fate of virus vectors after different routes of infection. Strategies aiming at sensitive and quantitative detection of retroviral vector infection and expression at the single cell level in animal tissues have been discussed among members of the participating laboratories.

HIGHLIGHTS/MILESTONES:

We have been able to reach all the milestones originally planned for this project period:

Task 3 (ii) Effect of steroid hormones upon expression of integrated proviruses. This part of the task has been completed and a manuscript reporting these results is in preparation by UA.

Task 5 (i) Screening of infected animals for the presence of virus. To establish the basis for such screening experiments by UL, a large number of progeny animals from parents

infected with retroviral vectors has been tested for presence of retroviral vectors and a new fast screening protocol has been developed in collaboration with GSF.

Task 5 (iii) Production of amphotropic and ecotropic producer cells. A number of such cell lines for murine viruses have been established and characterised (UL and GSF).

In accordance with the time chart, a review meeting with members of all research groups has taken place. The meeting was organized by the IIGB participant on Capri, from March 22 to March 24. A total of 18 members of the five groups participated in a schedule of presentations and discussions of results, research strategies and ideas for collaborative efforts.

WIDER CONSIDERATIONS:

Recent developments in the field of retroviral vector mediated gene therapy in humans have demonstrated that the areas covered by this EEC-BRIDGE project are of even greater importance than originally anticipated during the planning of this collaborative effort in the spring of 1990.

COOPERATIVE ACTIVITIES:

The GSF and UA groups will undertake the construction of replication competent retrovirus vectors carrying different envelope genes as part of a collaboration with the UL group. These vectors will then be employed by the UL group to study the spread of retroviruses in animals as well as the influence of the env variants upon infection spectrum. Replication competent MMTVs will be useful for the study of the effects of superantigens on the establishment of viral infections.

Currently there is little information about the ability of retroviruses endemic to one species (e.g. mouse) being able to package vector constructs based retroviruses endemic to another species (eg. chicken). We propose to take advantage of the expertise available among the participating labs of the EC Bridge project to address this in a collaboration between the UCB and the GSF. During the meeting on Capri it became apparent that these two groups have a common interest in the regulation of retrovrial integration using the microinjection of retrovrial cores as a test system. We propose to determine the ability of MLV cores to integrate into avian cells and ALV cores to integrate into murine cells, which would involve the UCB and GSF groups.

Recombination between retroviral sequences is a major interest of the UA and GSF groups. The stratagies employed by both groups have been streamlined so that they are complementary rather than competitive and it is envisaged that an exchange of constructs and expertise through exchange visits will occur. The efects of mutating the primer binding site (currently studied by the UA group) as well as the study of gag mutants may eventually lead to safer, more efficient retroviral vectors and the GSF and UA groups intend to form closer links with respect to these projects.

The UCB and UA groups will collaborate on studies aimed at determining the stability of expression of retroviral constructs in avian and murine systems. Both groups have preliminary data from their respective systems and they intend to determine how generally applicable their respective data are for retroviral vectors based on different

systems. Finally, the UA group will provide some of its currently available vectors to the IIGB group in an attempt to overcome the interference of viral sequences with the HLA-DQA1 promoter regulated expression that the IIGB group has observed.

During the last 6 months Finn Pedersen has visited the GSF on two occassions and Walter H. Günzburg spent four days (11-14/11/91) at the UA where he discussed possibilities for further collaborations and gave a seminar entitled "Negative regulatory elements of mammary genes:- a common mechanism of tissue specific expression?". Currently we are compiling a list of materials (such as vectors) and techniques that are available in each lab as well as an oligonucleotide bank within the framework of the EULEP-OLIGO bank.

PUBLICATIONS:

Sorensen, M., Duch, M.S., Paludan, K., Jorgensen, P. and Pedersen, F.S. (1992) Measurement of hygromycin B phosphotransferase activity in crude mammalian cell extracts by a simple dot-blot assay. Gene 112, 257-259.

Günzburg, W.H. and Salmons, B. (1992) Factors controlling the expression of mouse mammary tumour virus. The Biochemical Journal 283, 625-632.

TITLE: Assessment of environmental impact from the use of live recombinant virus vaccines.

CONTRACT NUMBER: BIOT - CT91 - 0289

OFFICIAL STARTING DATE: 1 October 1991

COORDINATOR:

Dr.D.N.Black,AFRC.,I.A.H.,Pirbright Lab,Woking,GU24 0NF,GB.

PARTICIPANTS:

Dr. A.L.J.Gielkens,,CDI.,P.O.Box 365,NL-8200 AJ,Lelystad,NL
Dr.M.A.Skinner,AFRC,I.A.H.,Houghton Lab.,Houghton,PE17 2DA,GB
Prof.P.P.Pastoret,Univ. de Liege,FMV.IVI.,BE-1070,Bruxelles,BE
Dr.R.M.Gaskell,Univ. of Liverpool, DVPMM, Neston, L64 7TE, GB

OBJECTIVES SET FOR THE REPORTING PERIOD:

i) Initial stages of the construction of the different recombinant viruses based on capripox, Aujeszky's disease and fowl pox virus.
ii) Construction of vectors to assess recombination between fowl pox virus and vaccinia virus.
iii) Recombination in vivo; Wild type ADV and modified ADV. Cowpox and vaccinia virus.
iv) Ecological survey; Sera and tissue collection and screening.
v) Pathogenesis of cowpox in mammals.

MAJOR PROBLEMS ENCOUNTERED: Nothing to report.

RESULTS:

Coordinator.

Recombinant plasmids containing a selectable gene (*E.coli* gpt), under the control of the vaccinia virus promoter p7.5, and either the rinderpest virus F gene or H gene, under the control of the vaccinia promoter p11, have been constructed. Several recombinant plasmids have been constructed each targeting the F (or H) gene to a different non-essential region of the capripoxvirus genome. Expression of the F gene and the H gene, with these plasmids, was demonstrated using transient expression in capripoxvirus infected lamb testis cells. Preliminary transfection studies have indicated it has been possible to generate a recombinant capripox F gene recombinant virus.

Plasmids containing the rinderpest F (or H) gene have been constructed for supplying to CDI for the construction of the recombinant ADV-rinderpest F (or H) recombinants.

CDI-Lelystad.

A recombinant ADV (NIA-3 gX$^-$,E1$^+$) containing the envelope glycoprotein E1 of classical swine fever (HCV) within the non-essential gX gene has been constructed. A recombinant plasmid (pMZ67) containing the HCV E1 gene fused in frame with the first 58bp signal sequence of the ADV gX gene was constructed. *Eco*RV-NCOI digestion generated a plasmid fragment containing the E1 gene flanked by NIA-3 sequences. Swine kidney cells were co-transfected with this fragment and virus NIA-3 DNA and the virus harvests screened for E1 expression using immunoscreening with E1 specific MAB. The recombinant virus was plaque purified and integration of the E1 gene verified by endonuclease analysis and blot hybridization.

The virulence of the "worst case" recombinant virus, NIA-3 gX$^-$ E1$^+$ was compared with that of the virulent parent virus NIA-3 gX$^-$. Five of the three-week-old SPF piglets were infected intra-nasally with 10^5 pfu of the parent virus NIA-3 gX$^-$ and the recombinant virus NIA-3 gX$^-$ E1$^+$. Preliminary findings indicate that both of the viruses cause sever and typical Aujeszky's disease and death. Three out of five died after inoculation with the recombinant and five out of five died after inoculation with the parent virus. Thus it appears that the "worst case" recombinant virus (a virus with the E1 gene of HCV located in the gX locus of a virulent field ADV) will not result in increased virulence of the virus in the field.

The development of a discriminatory PCR assay for discrimination between field viruses and any possible recombinants which may evolve in the field is in progress.

The gII plasmid and serum specific to GII have been prepared for transfer to the coordinator (IAH, Pirbright).

IAH. Houghton Laboratory.

i)Recombination between fowlpoxvirus

Recombinant poxviruses containing different marker genes inserted into different non-essential genes in the fowlpoxvirus genome have been constructed.

The first contains the *E.coli* gpt gene under the control of the vaccinia virus promoter p7.5 inserted into a non-essential genes within the terminal repeat of fowlpoxvirus DNA.

The second recombinant fowlpoxviruses contains the *E.coli* lacZ gene under the control of the vaccinia virus promoter p7.5 inserted in the D8 and D9 genes of fowlpoxvirus. The marker in the former recombinant will be used for the positive selection of possible recombinants.

ii)Recombination between heterologous viruses carrying a region of homology.

A plasmid containing the spike gene from infectious bronchitis virus has been prepared (pGSS1). Following removal of the vaccinia

virus promoter p7.5 from this plasmid a p7.5-*E.coli* gpt cassette was inserted into the spike gene sequences to produce the plasmid PGSS1/p7.5/Δ p7.5 gpt. This will be used to construct a recombinant plasmid, with the spike gene/p7.5 gpt cassette embedded in the terminal repeats of fowlpoxvirus genome.

A recombinant vaccinia virus containing the IBV spike gene has been constructed and characterised.

University of Liege.

Sera samples have been collected from wild mammals for analysis for the presence of orthopoxvirus antibodies. These will be screened both at the University of Liege and at the University of Liverpool.

Sera samples have also been collected from domestic animals in Continental Europe. These will be tested by Pirbright Laboratory for the presence of antibodies to capripox or capripox-related viruses.

University of Liverpool.

Samples from human and domestic animals with suspected cowpox have been screened. A further 14 strains of cowpox virus have been isolated, thus identifying geographical regions in which wild rodents will be sampled in an attempt to identify the reservoir host of the virus. Samples of these new cowpox virus strains have been sent to Prof. P.P. Pastoret (participant 04) for studies on the pathogenesis in foxes.

WIDER CONSIDERATIONS:

Preliminary assessment of recombinant Aujeszk's disease virus have indicated that no alteration, as compared to the parent virus,in their pathogenecity has occurred.

COOPERATIVE ACTIVITIES:

At this early stage of the programme only limited interchange has taken place. The Veterinary School,University of Liverpool has delivered samples of the recently isolated field strains of cowpox virus to the University of Liege. However, several recombinant plasmids containing specific virus genes have been prepared for distibution to the different participants.

LIST OF JOINT PUBLICATIONS/PATENTS
WITH TRANS-NATIONAL AUTHORSHIP: Nothing to report

OTHER PUBLICATIONS/PATENTS: Nothing to report

I TITLE

Identification of genes involved in latency and reactivation of pseudorabies virus, use in biological containmant study of viral genomes in pigs.

II CONTRACT NUMBER

BIOT CT 91-0297 (SSM)

III OFFICIAL STARTING DATE

October 1 - 1991

IV COORDINATOR

. A. JESTIN
. CNEVA
. 22440 PLOUFRAGAN
. FRANCE

V PARTICIPANTS

1 - A. JESTIN - CNEVA PLOUFRAGAN - FRANCE
2 - H.J. RZIHA - FRCVDA - TUBINGEN - GERMANY
3 - I. ALMEIDA - NVRL LISBON - PORTUGAL
4 - P. SHELDRICK - IRSC - VILLEJUIF - FRANCE
5 - M. RIVIERE - RHONE-MERIEUX - LYON - FRANCE

VI OBJECTIVE SET FOR THE REPORTING PERIOD

The goal of the project is the identification of viral genes influencing establishment of and reactivation from latency of Pseudorabies virus (PRV). The major objective will be a detailed analysis on the biosafety of PRV mutants specifically manipulated in latency-associated (LA) functions. LA transcripts (LATs) will be identified (cDNA bank, in situ cytohybridization), characterized, and the molecular organization of this genomic region will be elucitated.

Neural cell culture will be produced and in vitro assays on these porcine neural cells reflecting a natural terget cell of PRV, with strain and mutants can be studed as in vivo on pigs to analyse their latency characteristics.

VII MAJOR PROBLEMS ENCOUNTERED :

This common project was planned for three years in the first version and all partners were well linked : cDNA bank, neural cell cultures, in vitro and in vivo study. When the CEC reduced the programme to 2 years all partners reduced the objectives. Nevertheless the difficulties persist . Ex : neural cell culture, cDNA Bank.

VIII RESULTS :

PARTNER 1 : JESTIN CNEVA PLOUFRAGAN

Characterisation of strains in vivo

Characteristics of the mutants at the molecular level will be examined in acutely infected, latent and reactivated animals emphasising a comparison of these mutants to the wild type. Studies have been done on various organs and on various cells : white blood cells, bone marrow cells. Immediate early, early, and late transcripts will be looked for by in situ cytohybridization to determine any modifications of organ and cell tropism and latency reactivation properties.

PARTNER 2 : RZIHA FRCVDA TUBINGEN

Analysis of : (1) LAT ('latency-associated transcrip') region of PRV,
(2) Viral gene expression of lymphocytes from latently infected pigs

(1) One genomic region of PRV specifically transcribed during the latent state of infection could be determined by *in situ* cytohybridization (to detect viral RNA), Northern blot analysis and protection assays. It was found that LAT(s) start near the right end of 'unique long' part and pass into the internal repeat region of the PRV-genome comprising in total ca. 13 kbp. LAT is over-lapping and orientated antisense to the immediate early mRNA. As reported recently by Cheung, at least the large LAT is spliced with a 4.6 kbp intron. We accomplished a transcriptional map and the DNA sequence of the region around LAT. To this end, the ca. 8.6 kbp KpnI-fragment I was subcloned. Strand-specific probes (cRNAs) are used in Northen blot analysis of total and poly(A)-RNA prepared from infected cells. Up to now, a contiguous stretch of ca. 7 kbp of this viral DNA-fragment has been sequenced. The data obtained so far can be summarized as follows :
(a) Three open reading frames (ORF) and corresponding mRNAs have been identified in lytically infected cells, which are homologous to "UL3", "UL4" and "UL5" of herpes simplex virus (HSV).
(b) One additional gene specifiying a 1.1 kb mRNA has been detected which exhibits no homology to other known herpesviral genes.
(c) Upstream of the LAT start site an intergenic region could be identified in the PRV-genome, which is non-essential for virus replication.
(d) Overlapping and antisense to the LAT(s) a new early gene of PRV (EPO) was found with significant homology to an immediate early gene of HSV.
(e) Cloning of different parts of the PRV-LAT promotor region has been initiated to provide suitable plasmids for testing LAT promotor activity in different cells.

(2) A large number of organ tissues and lymphocytes of peripheral blood (PBL) and bone marrow (BM) derived from latently infected pigs was investigated for the presence of PRV DNA and virus-specific transcripts. Except of the well established neuronal latency of this alphaherpersvirus, additionally we found the regular persistence of PRV DNA in PBL and BM cells of latently infected animals. Using polymerase chain reaction (PCR), *in situ* hybridization and immunohistochemistry the following conclusions can be made :
(a) During latency the viral genome is harboured in lymphocytes of PBL and BM in the same or even a higher percentage of animals exhibiting PRV DNA in neural tissues. The number of PRV DNA-positive lymphocytes, abtained from different times p.i., remained constant over the tested time periode (1 to 7 months p.i.). This indicates that

usually no possible spontaneous reactivation of latent PRV in those cells does occur.
(b) During acute phase of infection and after reactivation of latency, a subset of PBL and BM cells was found productively infected. Comparison to other organ tissues of the same pigs indicated that reactivation of latent PRV is independent of virus production in e. g. neural tissues.
(c) More detailed analysis of *in vivo* latently infected lymphocytes demonstrated the expression of **both LAT** as well as **early and early-late** PRV genes in c.a. 0.1 % of these cells.

PARTNER 3 : I. ALMEIDA N.V.R.L. LISBON PORTUGAL

In Vitro Study of Aujeszky Virus Interactions with cultured Cells from the Nervous System

Several in vitro systems have been developed to assay the neuropathogenic properties of Pseudorabies virus strains (PRV) (Aujeszky).

The cellular tropism, viral cythopathic characteristics and replication were investigated in various cellular substrates.

Cell lines (human neuroblastoma, IMR 32 and mouse neuroblastoma, Neuro2A) and dissociated embryonic cells (obtained from rat dorsal root ganglia, DRG and the cerebral cortex) were assayed for their susceptibility to PRV virus infection. These cells were infected either with the Kojnok wild-type, or the Bartha vaccine strain. Infection of cells were evaluated by both virus titration and virus-specific fluorescence staining whereas the type of cells obtained from dissociated cultures was evaluated by GFA staining for glia and neurofilament or tetanus toxin markers for neurons.

A) Infection of Neuroblastoma cells

SUSCEPTIBILITY

Both cell lines are susceptible to the wild-type and vaccine strains. However, infection of Neuro2A cells is far more efficient (with a MOI in the range of 0.0001 pfu/cell for Kojnok and 0.001 pfu/cell for Bartha, whereas both strains infect the human neuroblastoma cells in the range of 0.1 pfu/cell).

CYTOPATHOLOGY

Kojnok-infected mouse neuroblastoma cells exhibit extensive cytopathology affecting all the cells, with PVR antigen-positive multinucleated cells whereas human neurons predominantly exhibit viral antigens in cytoplasm, only in foci of syncytial cells.

Bartha infections result in less number of infected Neuro2A cells than Kojnok with a nuclear localization of viral antigens and rather little evidence of alteration on cellular morphology. Surprisingly, IMR 32 infected cells exhibit similar pattern of infection as the Kojnok strain.

VIRAL YIELDS

The viral yields obtained from Kojnok strain-infected Neuro2A cells is in the range of a 10 fold increase compared to that obtained from IMR 32 infected cells, confirming the observations from susceptibility and cytopathic criteria.

Titration of Bartha viral yields is under way. It is presumed that viral titers would be lower.

B) Dissociated Embryonic Cultures of RATS

Preliminary results show that DRG derived cells are susceptible to Kojnok strain infection with an early and predominant involvement of glia. Some neurons are infected only later. Cytopathic effects as multinucleated cells affecting the glia and swelling of neurons are also observed. Forty-eight hours after infection, most neuritic extensions have collapsed.

C) Dissociated embryonic cultures of piglets

An attempt to culture dissociated cells from the porcine nervous system succeed in obtaining viable cells in preliminary trial. cells from DRG are collected on 48 days old fetus as cortex cells.

Conclusion

The data show clearly the high susceptibility of Neuro 2 A cells to Kojnok strain, and the restriction to infection of IMR-32 cells, mainly to Bartha strains. IMR-32 cells are apparently capable to down-regulate the expression of the PVR genome. This may be of interest for establishment of a persistently infected cell line. The fact that glia may be more susceptible to PRV infection than neurons in DRG cells may need further confirmation using glia cell lines.

Remark

The above experiments have been performed using the facilities and routine rat dissociated cultures from neural tissues at the Rabies Unit, Pasteur Institute, Paris (H. Tsiang).

PARTNER 4 : P. SHELDRICK Th FOULON IRSC CNRS VILLEJUIF FRANCE

Characterization of mRNA's expressed in tissues of latently infected pigs. Construction of a cDNA bank.

Using two herpesviral model systems, pseudorabies virus (PRV) and a cottontail rabbit herpesvirus (CTHV), the latter of which has in the past been extensively studied in our laboratory, we have examined cDNA banks produced from productively infected cells in tissue culture as a prelude to subsequent studies on latently infected animals. In particular, we are concerned with the reproducibility of various methods for the production of cDNA (enzymes, reaction conditions, etc) and the representativity (whether all mRNA's are detected) of the cDNA banks generated.

Our more complete results with CTHV indicate, for example, that a substantial fraction (ca. 20%) of the genome is refractory to cloning in several vectors and that the cDNA technique will not detect RNA from these regions. Our results since six months indicate that, in the regions of PVR examined thus far, lack of representativity is not a major problem, and we will soon proceed to an analysis of tissue samples (with initial emphasis on peripheral blood lymphocytes and bone marrow) supplied by the Tubingen laboratory (participant 2).

PARTNER 5 : M. RIVIERE RHONE MERIEUX LYON FRANCE

Not involved in this period.

IX HIGHLIGHTS MILESTONES :

The briefly described results of a latent reactivable infection of porcine lymphocytes in vivo has been not reported before. Thus, part of the studies of this project shall now focus on furthern more detailled characterization of lymphocytes supporting a latent and productive PRV infection. Because of the available tools experiments for testing LAT promotor activity and unequivocal proof of PRV lytic gene expression (e.g. by cDNA cloning) in those cells should be possible. The expected results will not only show whether regulation of latency in PBL/BM might be different from that in neuronal cells, but also might open new perspectives in testing and diagnosing latently infected pigs. Furthermore, this natural virus-host system will provide new insights in alphaherpesvirus latency, in general.

The preliminary results of primary neuronal cell culture has been not reported before. We can consider the protocole is well defined and stocks of neuronal cells cultured derived from dissociated embryonic DRG of piglets will be produced for in vitro characterization of mutants and molecular constructs.

X WIDER CONSIDERATIONS : NOTHING TO REPORT

XI COOPERATIVE ACTIVITIES :

- Meeting PARIS September 10th 1991 (all parts)
- Transfert of fetuses of piglets from PLOUFRAGAN to PASTEUR INSTITUT (ALMEIDA working in rabies lab, Dr TSIANG)
- Transfert of positive samples from TURBINGEN to VILLEJUIF to produce a cDNA bank)

TITLE: BIOSAFETY OF GENETICALLY MODIFIED BACULOVIRUSES FOR INSECT CONTROL

CONTRACT NUMBER: Biot-CT91-0291

OFFICIAL STARTING DATE: December 1, 1991

COORDINATOR: Dr. J.M. Vlak, Department of Virology, Agricultural University, Binnenhaven 11, 6709 PD Wageningen, The Netherlands.

PARTICIPANTS:

1. Department of Virology, Agricultural University, Binnenhaven 11, 6709 PD Wageningen, The Netherlands.
   Project leader: dr. J.M. Vlak
   Scientist(s) involved: dr. R. Mans

2. Institut für Biologischen Pflanzenschutz, Biologische Bundesanstalt für Land- und Forstwirtschaft, Heinrichstrasse 243, D-6100 Darmstadt, Germany
   Project leader: dr. J. Huber
   Scientist(s) involved: dr. K. Undorf

3. Institute of Virology and Environmental Microbiology, National Environmental Research Council, Mansfield Road, Oxford OX1 3SR, United Kingdom
   Project leader: dr. R.D. Possee
   Scientist(s) involved: dr. C. Hauxwell

OBJECTIVES SET FOR THE REPORTING PERIOD:

The objective of the project is the construction of baculoviruses with enhanced insecticidal activity, but reduced survival in the field. This will be achieved by introduction of a 'built-in' suicide mechanism or gene deletions into baculoviruses that would impair their survival, but not their short-term efficacy. After their construction these genetically modified, biologically contained viruses, will be tested in the laboratory (host-range) and

in a microcosm (persistence and spread). In this way information can be obtained about the biological behavior 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 start the construction of the various recombinant viruses (1 and 3), to determine their biological activity (2 and 3) and host range (3) and to preliminarily test those recombinants already available in a microcosm (1 and 2).

MAJOR PROBLEMS ENCOUNTERED:

Nothing to report.

RESULTS:

Construction of recombinants.

Experiments have been initiated to produce a "suicide" *Autographa californica* nuclear polyhedrosis virus (AcNPV) insecticide. The strategy involves the modification of the polyhedrin gene promoter to incorporate lacO (operator), together with a copy of the lacI under the control of the p10 promoter into a recombinant. Production of lacI in virus-infected insect cells in the absence of the substrate IPTG (binds lacI) should prevent transcription from the polyhedrin gene promoter and thus abolish polyhedra production. In the presence of IPTG the lacO is repressed and polyhedra should be synthesized normally.

Baculovirus transfer vectors have been constructed based on the polyhedrin gene region (containing the hybrid polyhedrin gene promoter lacO) and the p10 gene region (containing the lacI coding sequences). These have been characterized at the sequence level to confirm authenticity. The vectors are presently being used in co-transfection experiments to derive recombinant viruses; these will be characterized for polyhedra production in the presence (polyhedra-positive) and absence (polyhedra-negative) of IPTG.

The construction of baculovirus recombinants with deletions of genes that affect the virus persistence in the field has been focusing on three genes, which have been described not to be essential for viral replication, increase virulence and reduce persistence of the virus. These genes are p10, the polyhedron-envelope gene (pp34) and the ecdysteroid UDP-

glycosyl transferase (egt), respectively. 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 coloring of their plagues 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. The various transfer vectors are now being constructed.

The marker gene can be conveniently obtained from a previously designed transfer vector pAcDZ1 as a gene cassette in which the lacZ gene is under the control of a constitutive promoter (the *Drosophila* heat shock promoter, hsp70) and 3'-flanked by a SV40 transcription terminator sequence. This gene cassette avoids the need to clone the lacZ gene as a fusion with the gene of interest.

The availability of nearly all transfer vectors allows the introduction of the various single and double deletions and eventually the deletion of all three genes. Subsequently these recombinant viruses can be analyzed including the possible reduced capacity for survival in the field.

Behavior of recombinants in microcosms

The biological and behavioral properties of an AcNPV recombinant AS3 (p10-minus) and wild type AcNPV were analyzed in bio-assays and in a microcosm. The p10-minus recombinant AcNPV-AS3 has a deletion of the p10 gene and has a reporter gene (lacZ) cassette (see above). AcNPV-AS3 was serially passaged (four times) in insects by feeding third-instar larvae of *S. exigua* and *A. californica* through the diet. The DNA of progeny viruses was analyzed by DNA-gel-electrophoresis after each passage and found to be unaltered.

The biological activity of recombinant AcNPV-AS3 and wild type AcNPV expressed as LC_{50}-value (lethal concentration 50%) was compared in standard bioassays. Five concentrations were used against first instar larvae of *Spodoptera exigua* and *Autographa gamma* (Table 1). No statistically significant differences in infectivity was determined between wild type and recombinant AcNPV. Standard curves were made for interpolation of the results of soil sample bioassays.

A field release of the genetically manipulated AcNPV-AS3 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. Each of the two viruses (wild type AcNPV and recombinant AcNPV-AS3) was applied in a 0.4 m^2 compartment at a single dose of 4 x 10^7 polyhedral inclusion bodies (PIBs), equivalent to 10^{12} PIBs per hectare. After one larval generation (25 days), soil, plants and infiltration water were collected from the ecosystem and are being bio-assayed for virus quantitation.

Equal quantities of the wild type AcNPV and AcNPV-AS3 (p10-minus) applied to 200 larvae of *S. exigua* showed no difference in response to infection (82 and 81% mortality). Budgetary studies of NPV production and partitioning involved foliage and soil extractions are being carried out at present. This suggests that

Table 1: Bioassay of wild type AcNPV and (p10 minus) AcNPV-AS3 against first instar larvae of *S. exigua* and *A. gamma* after 6 and 12 days.

	S. exigua LC_{50} (PIBs/ml medium)	*A. gamma* LC_{50} (PIBs/ml medium)
AcNPV	7.12 x 10^4	2.22 x 10^5
AcNPV-AS3	7.87 x 10^4	3.03 x 10^5

WIDER CONSIDERATIONS:

Baculoviruses are insect pathogens which are successfully used as biological control agents of insect pests in agriculture and forestry. Notable successes have been in the control of forest, orchard and greenhouse pests. For instance, control of the pine sawfly, **Neodiprion sertifer**, with the respective nuclear polyhedrosis virus NsNPV, brassica pests by **Mamestra brassicae** NPV and the codling moth, **Cydia pomonella**, by its granulosis virus. 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 problem and may lead to the wider use of this ecorational and more environmentally sound control approach. Information on the ecological and environmental behavior of genetically modified baculoviruses is essential for the proper use in the future.

COOPERATIVE ACTIVITIES:

All participants exchanged ideas and materials (transfer vectors and recombinants). They also agreed to meet twice a year, if possible in conjunction with a major international meeting. In addition, site visits are envisaged to support the transfer of technology. A promotional pamphlet (Blue Brochure) is being prepared for public interest to explain the activities of the group.

LIST OF JOINT PUBLICATIONS / PATENTS WITH TRANSNATIONAL AUTHORSHIP:

Undorf, K., Huber, J. and J.M. Vlak. 1990. Freisetzung eines gentechnisch veränderten Kernpolyedervirus von *Autographa californica* (AcMNPV) in einem Model-Ökosystem. Mitteilungen der Biologischen Bundesanstalt. Berlin-Dahlem. 266: 322

Roelvink, P.W., Van Meer, M.M.M., De Kort, C.A.D., Possee, R.D., Hammock, B.D. and J.M. Vlak. 1992. Dissimilar expression of *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus polyhedrin and p10 genes. Journal of General Virology 73: 000-OOO.

OTHER PUBLICATIONS / PATENTS:

Hawtin, R.E., King, L.A. and R.D. Possee. 1992. Prospects for the development of a genetically engineered baculovirus insecticide. Pesticide Science 34: 9-15.

Martens, J.W.M., J.W.M. van Lent, G. Honée, D. Zuidema, L. Visser and J.M. Vlak. 1991. Insecticidal activity of a *Bacillus thuringiensis* toxin expressed by baculovirus recombinants. IOBC/WPRS Bulletin 14: 185-190.

Stewart, L.M.D., Hirst, M., Ferber, M.L., Merryweather, A.T., Caylay, P.J. and R.D. Possee. 1991. Construction of an improved baculovirus insecticide containing an insect-specific toxin gene. Nature 352: 85-88.

Undorf, K. and J. Huber. 1991. Quantitative assessment of MbMNPV in a model ecosystem. IOBC/WPRS Bulletin 14: 44-45.

Undorf, K. and J. Huber. 1991. Survival of genetically modified NPVs in a model ecosystem. IOBC/WPRS Bulletin 14: 171-174.

Vlak, J.M. 1991. Baculoviruses - Genetically engineered insecticides. In: "Plant Biotechnology: Combat of Pests". Butterworth en Heinemann Ltd., Oxford, U.K., p. 221-242.

TITLE: "Risk Evaluation for Genetically Modified Microbial Inoculants"

CONTRACT NUMBER: BIOT-CT91-0823

OFFICIAL STARTING DATE: 01.10.91

COORDINATOR: Marco P.Nuti, DBA - Univ. Padova (IT)

PARTECIPANTS: F.O'Gara, Univ. College - Cork (IE)
J.Beringer,Dept.Botany - Univ.Bristol(GB)
Marina Basaglia, HELIGENETICS, Gaiba (IT)

OBJECTIVES FOR THE FIRST YEAR OF THE PROJECT:

1. Development of vector and reporter systems
   1.1.Exploit the natural selection method to obtain various Rhizobium and Bradyrhizobium strains defective in thymidilate synthase
   1.2.Evaluate the performance of the thy vector in the rhizosphere under greenhouse and field conditions
   1.3.Construction of inducible reporter systems

2. Construction of a suicide system for R.meliloti
   2.1.Evaluate the performance of the R.meliloti thy in the rhizosphere under greenhouse and field conditions
   2.2.Genetic manipulation of the Lactococcus lactis thy cartridge in preparation for a conditional expression system

3. Evaluation of genetically modified inoculum strains
   3.1.During inoculant manufacturing process
   3.2.Evaluation at microcosm and greenhouse level

4. Evaluation of natural occurring populations
   4.1.To use pyrolysis mass spectrometry (PYMS) to look for distances between isolates of Bradyrhizobium japonicum isolated from soybean plants grown in Italy
   4.2.To study isolates from Italy using DNA hybridization
   4.3.To look at population of Rhizobium in soil of differing characteristics and from different geographic regions
   4.4.To look at the effect of factors, such as manipulating the water table, on survival

MAJOR PROBLEMS ENCOUNTERED:

Overall considered, the work programme is in progress as outlined. The following problems have been encountered to date:
- the PYMS equipment in Bristol university became unavailable to develop it as a research tool for this project and links are being established with Professor M. Goodfellow and G.Manfio at Newcastle University
- DNA hybrydization is too slow and the throughput of strains is

restricted: the use of PCR fingerprinting has started to overcame the above difficulties, but DNA hybridization still provides a very useful backup procedure for strain testing
- at the University of Bristol the team is waiting for a licence to import soil
- at UCC the experiments for the evaluation of GM rhizobia under greenhouse conditions are delayed because the soil intended for these experiments was found to contain a resident R.meliloti strain able to outcompeting the test strain
- at the University of Padova the team is awaiting for permission to carry small-scale field experiments with GM rhizobia; the notification and permit application were received in 1991 at the Ministry of Agriculture; the notification was found to comply with the rules set by the Directive 220/EEC; however the need to have the detailed regulatory framework approved through the Parliament has greatly affected the procedure for permit notification to the applicant

RESULTS:

1. Development of vector and reporter systems

1.1. The selection of spontaneous thy mutant strains in the presence of antifolate drugs has proven to be readily applicable for selection of R.meliloti thy mutant strains. Under greenhouse and field conditions, reversion to wild type has been observed. The R.meliloti thy gene has been cloned by complementation of a spontaneous R.meliloti thy mutant. The availability of this gene opens the way to a reverse genetic approach to obtain stable R.meliloti thy mutant strains.
The natural selection procedure has been proven to be unsuitable for the generation of B.japonicum thy mutant strains. These organisms displayed a high intrinsic resistance to the antifolate drug. We envisage cloning the B.japonicum thy gene by complementation of either R.meliloti or E.coli thy mutants. A reverse genetic approach will then adopted to obtain definite B.japonicum.

1.2. The thy vector system has been evaluated for R.meliloti under field conditions. It was found to perform exceptionally well. The R.meliloti thy mutant strain was found to revert back to wild type at a frequency of 85% . However, this figure may be distorted because the thy mutants are impaired in their capacity for survival. Even so, plasmid retention in the thy mutant complemented with an IncP plasmid was found to be virtually 100% . This is a significant improvement compared with 81% retention of the same plasmid in the wild type.
Analysis of symbiotic efficiency indicated a marginal reduction in acetylene reduction, but no effect on the shoot dry weight of the plants.

1.3. A synthetic promoter, which has proven to be of great efficiency in Rhizobium, has been linked to a promoterless lacZ gene; the lac operator, a ribosome binding site and a translation start codon have been conveniently placed. The creation of two additional restriction sites for the enzymes XbaI and BglII by site directed mutagenesis has allowed the addition of a functional $lacI^q$ gene which will provide a basis for the regulation of such reporter cassette. The mercury resistance gene is presently being cloned aside the lac unit and the

final product will be inserted in Rhizobium leguminosarum both chromosomally via homogenotization of a Rhizobium recA copy, and on a broad host range plasmid vector. In addition, the efficiency of lac+ and mercury-resistance as phenotypical markers in Rhizobium has been assessed. The data indicate 8 µg/ml HgCl2 as the tolerable limit for a strain containing the mercury-resistence determinant in TY medium, where the wild type strain will not grow above 1 µg/ml. As regards lactose utilization a strain bearing a lacZ gene showed a much better growth on minimal medium plus lactose.

2. Development of a suicide system for R.meliloti

2.1. The capability of the mutants to survive in the rhizosphere and to nodulate alfalfa was examined under greenhouse and field conditions. The interpretation of the field data was complicated by the fact that the thy mutant inoculum strain reverted to wild type and that it proved to be difficult to reisolate the mutants from the nodules. However, the fact that thy$^-$ bacteria could be reisolated from the nodules at the end of the growth season demonstrated that the mutants can survive the entire lenght of the experiment. It is possible that the reverted bacteria may be responsible for sustaining the thy mutants. Only one nodule out of 58 was found that was apparently entirely occupied by the thy mutant. Others were either 100% reverted or contained a mixture of thy$^+$ and thy$^-$ bacteria. Greenhouse experiments currently in progress are designed to evaluate if the host plant can sustain the thy mutant strain in hydroponic cultures.

2.2. The Lactococcus lactis thy gene is being cloned and manipulated in order to remove the expression signals and to facilitate the clonig of a suitable promoter to conditionally express the gene. The nature of the expression signals will be decided upon evaluation of the plant data currently being accumulated, concerning the survival and effectiveness of the thy mutant strains.

3. Evaluation of genetically modified inoculum strains

3.1. The conditions to process the vermiculite matrix to be used in the production of (genetically modified) inoculants were identified. From our experience and from data in literature, this carrier seems the most appropriate in ensuring both survival and performances of the inoculant. vermiculite samples packaged in polyethylene bags have been irradiated with 2.5, 5 and 7.5 Mrad.
The treatment with 5Mrad seems to provide the best sterile conditions vs. minimum radiation used. In order to carry out a serological identification test, a New Zealand rabbit has been immunized against B.japonicum 602/A. The immune serum will be used for rapid identification and confirmation tests in the experiments during the programme phases.

3.2. These experiments are expected to be terminated in the next phase.

4. Evaluation of naturally occuring populations

4.1. The PYMS analysis indicated that there was genetic drift, on the basis that some isolates differed slightly from the introduced strains. One isolate was very different and couid have been another strain used as an inoculant elsewhere in Italy. We are checking to see whether the strain had been released locally and are examining the isolates further.

4.2. Using a cloned insertion sequence as a probe for DNA hybridization studies, we were able to characterise strains in a very reproducible manner. However, this technique is restricted in throughput because it requires that good quality DNA be prepared from each strain to be tested.

4.3. It is possible to produce a fingerprint of an isolate by adding a small amount from a colony to an eppendorf tube, adding reagents for DNA synthesis, boiling briefly to lyse cells and denature DNA, and then synthesizing DNA using the polymerase chain reaction (PCR). The DNA is synthesized from "universal" primers which recognize similar sequences in the genome if conditions for DNA annealing are not too stringent. The patterns produced can be scanned and the data fed to a computer programme which will produce dendrograms showing relatedness. At least 50 samples a day can be handled with ease. This technique is being developed.

4.4. In order to assess the influence of water-table depth and soil pH on survival of B.japonicum the field is being prepared for the experiment. The water table will be controlled piezometrically and the instruments have been installed. Two sites have been identified, one at pH 6 and the other at pH 7.7. The physical-chemical traits have been characterized and the cropping history recorded. The main biological traits are being defined, including the number of resident B.japonicum by the MPN technique.

HIGHLIGHTS/MILESTONES:
The thy system proved highly effective in ensuring stable maintenance of the plasmid under field conditions. This was found to be the case even though the thy mutant strain was found to revert at a rather high frequency. This demonstrates that the presence of the thy gene on a self-replicating plasmid removes the pressure for the thy host strain to revert to wild-type.
The lac and lac/Hg^r cassettes provide a tool which is based on a catabolic inducible reporter system without antibiotic resistance markers. The expression levels, comparable or higher than those obtainable with tac promoter, allow unambiguous detection of the genetically modified microbe.

WIDER CONSIDERATIONS:
Stable vector system based on thy will bw available for the introduction of novel genetic traits in commercial inoculant strains. They will also give improved consistency to experiments designed to evaluate the effects of such traits on symbiotic efficiency of the host organism.

COOPERATIVE ACTIVITIES:
One progress meeting has been organized at Gaiba on december 17th, 1991. Representatives of all partecipating institutions were present.

LIST OF JOINT PUBLICATIONS: -

OTHER PUBLICATIONS/PATENTS:
Nuti, M.P., Squartini A., Guerra E., Ollero F.J., Corich V., and Giacomini A. (1992). Monitoring genetically modified soil microbial inoculants. In "Genetically modified organisms for the 1990's", G.Tzotzos et al. Eds. (in the press).

TITLE : METHODOLOGY FOR THE FAST DESIGN OF FUNGAL DNA PROBES AND PCR PRIMERS

CONTRACT NUMBER: BIOT - CT91- 0301
STARTING DATE: DECEMBER 1st, 1991

COORDINATOR OF THE CONSORTIUM:

W. MOENS, IHE - Biosafety rDNA & Biotechnologies, Brussels, BE

PARTICIPANTS:

J. FRISVAD, Danmark Teknische Højskole, Dpt of Biotechnology, Lingby, DK
F. GANNON, University College Galway, Dpt of Microbiology, Galway, EI
N. NOLARD, IHE- IHEM Fungal Culture Collection , Brussels, BE
O. RASMUSSEN, Biotechnological Institute, Lingby, DK
V. RUBIO, Inst. de Investigaciones Biomedicas, Lab. de Hongos Filamentosos, Madrid, ES

OBJECTIVES:

Main

Fungal strains representative of the major genera and species involved in several areas of biotechnology have been selected by the consortium and will be studied under two coordinated approaches:

-taxonomy and culture collection standardization,
-molecular typing (including validation and sampling) and computerization of results.

Potential genera-, species-, strain- specific PCR primers and derived DNA probes are designed and validated by the participants. The specificity of the primers or probes is defined and controled on basis of the ecological niches to which belongs the fungi of interest..
The aim is to use PCR primer design for fungal molecular typing and taxonomy, identification and ownership certification, environmental & health biosafety and description of fungal biodiversities.

Reporting period

1) Assessment of the methodological model: design of PCR primers for the taxon identification and diagnostic of human pathogenic fungi of general epidemiological interest .
-Standardization of a fast preparation method for PCR-quality fungal DNA and building a bank of certified DNA batches
-Standardization of PCR formats and validation by participants
2) 1st contact group meeting
-Taxonomy & culture collection management
-Coordination of PCR and sequencing strategies
-Management of Bibliographic review
-Definition of the specific contribution of each participant
-Negociations about formal consortium foundation
3) Next objectives
-Testing families of consensus primers
-Standardization of common PCR and PCR products sequencing protocols
-Computer networking and design of shared core of laboratory and results database

MAJOR PROBLEMS ENCOUNTERED:

The available fungal DNA sequences of the ribosomal 18S subunit only provide a low number of target for species-specific primers. A first alternative strategy aims to systematically sequence and align interspaced regions between the fungal 18S and 23S subunits. In the second alternative, the same approach is applied to the description of the DNA sequences upstream the 5' part of the 18S subunit. These regions of the ribosomal genes being under lower evolutionary pressure, it is expected to increase the number of species-specific PCR priming areas.

RESULTS

Coordinator's

The first 4 months of this concerted action include the results of a research undergone one year during the grant application of the project. They concerns:
-the set up of the standardized DNA preparation method and PCR format;
-the develoment of fungal genera- and family-specific PCR consensus primers
-the assessment of the whole methodology when applied to the seek of PCR primers for the taxonomical certification and the clinical diagnostic of fungi pathogenic or lethal to immunocompetent and immunocompromised humans.

From the later, highly specific primers for 4 very important fungal pathogens where designed and validated in the coordinator's laboratory. The biological background of the primers involves 32 fungal species representative of the most prominent species encountered in the human ecological niche. The DNA bank builded from certified strains of the IHEM collection -a culture collection specialized in fungi of environmental and health interest- allowed to systematically study the inter- and intraspecificities but also the intergenera specificities of several PCR primer candidates within 5 months. Till now, this work has involved about 90 strains and the results will be continued along the building of the consortium fungal DNA bank.

Apart from the usefullness of the found primers, this study has shown that the concepts underlying the methodology are at first glance correctly defined.

Participants

1)Along the first contact group meeting, the participants have agreed on the basic concepts underlying the methodology of the concerted action and on the quality insurance aspects.

Among the last, three major requirements were defined:
-Taxonomical certification by Prof. Frivad and Dr. Nolard and registration of all fungal strains in the IHEM collection before starting any DNA study;
-Harmonization of the DNA preparation, oligonucleotide synthesis, DNA sequencing and PCR protocols in Gannon's, Moens', Rassmussen's and Rubio's teams.
-Use, exchange of strains, DNA batches, PCR products, and DNA sequences in the frame of a common DNA bank and common raw data and sequence databases.

2) Fungal DNA batches, consensus primers and protocols have been distributed to the participants for control and improvement at start of the concerted action.

3) Each participant has selected a set of fungal strain of taxonomical and/or biotechnological interest for which species-specific PCR primers have to be designed.
This goals involve computer analysis of fungal ribosomal DNA sequences and further production of new DNA sequences, particularly in the intergenic DNA sequences of the

ribosomal operon (ITS region). The ITS region will be amplified by PCR in one or two parts using consensus primers for the 3' end of the 18S subunit, the 5' start of the 23S subunit and the middle of the 5S subunit. The two first consensus primers available in the literature failed to prime DNA amplification in several families of fungi. Therefore the consensus primers will be improved to match the broadest interfamily consensus (F. Gannon and W. Moens). Family consensus for the 5S subunit are presently designed and studied (W. Moens). Sequencing of PCR products is a key part of the study and requires a good interlaboratory standardization (O. Rassmussen). Automated sequencing facilities are available for large scale sequence production (V. Rubio and F. Gannon).

HIGHLIGHTS/MILESTONES

A set a species-specific PCR primers for fungi pathogenic to humans has been designed in a very short period of time along a background of 32 species involving a set of 90 strains.
Along the building of the consortium DNA bank and ribosomal DNA sequence database, the rate at which PCR primers can be designed for a given species in a given ecological niche should increase rapidly.

WIDER CONSIDERATIONS

The European consortium for fungal molecular typing aims to the fast design of PCR primers and DNA probes of clinical, agronomical, aquatic, food, environmental and taxonomical interest. Each participant may act has a representative of the consortium for contacts with third party.
When engaged by third parties, the consortium manages the research according to the specific requirements of the demander . Given the adaptative character of such system of laboratories without wall and his concerted data sharing, any collaboration with the consortium further increases his potentialities.

COOPERATIVE ACTIVITIES

Next contact group meeting: June 25-26th, Madrid
Exchange of scientist:
From coordinator's lab an V. Rubio's lab to O. Rassmussen's lab: 2 and 1 scientists for 1 week
Exchange of certified material: fungal strains and/or DNA batches
Shared material: PCR primers, PCR products, DNA sequences, sequence alignments

LAST NON JOINT PUBLICATIONS

T. Barry, G. Colleran, M. Glennon, L. Dunican and F. Gannon (1991)
The 16S/23S Ribosomal Spacer Region as the target for DNA probes to identify Eubacteria in "PCR methods and Applications" CSHLP ISSN 1054-9803/91 p51-56

O. Rassmussen and J.C. Reeves (1992)
DNA probes for the detection of plant pathogenic bacteria (Minireview)
Accepted in "Journal of Biotechnology"

O. Rassmussen et al. (1991)
Listeria monocytogenes Isolates can be classified into two major types according to the sequence of the Listeriolysin gene
Infection and Immunity 59:3945-3951

O. Rassmussen et al. (1991)
A rapid polymerase Chain Reaction (PCR) -based assay for the identification of Listeria monocytogenes in food samples
Intern. J. Food Microbiology

C. Dawson and B. Van Vaerenbergh, N. Nolard and W. Moens (1992)
A model for the fast design of taxon-specific PCR primers: application to the diagnostic of the four major human fungal pathogens, Aspergillus spp, C. albicans, C. Krusei and Cryptoccus neoformans
(in preparation)

BRIDGE

T-PROJECTS

T-PROJECT

SEQUENCING OF THE YEAST GENOME

TITLE	SEQUENCING OF THE YEAST GENOME
CONTRACT NO.	BIOT 0167
STARTING DATE	January 1st 1991
OBJECTIVES	Sequencing of the entire chromosomes II (840,000 bp) and XI (630,000 bp) and of 230,000 bp duplications for controls (15%) and 40,000 bp overlappings (700 bp for each of the 60 cosmids). A total of 1,800,000 bp is thus to be sequenced between January 1991 and December 1993.
ADMINISTRATIVE COORDINATOR	Françoise Foury, Université de Louvain, B
DNA COORDINATOR	Chromosome II, Horst Feldmann, Universität München, D (see page 355) Chromosome XI, Bernard Dujon, Institut Pasteur, Paris, F (see page 358)
INFORMATIC COORDINATORS	Werner Mewes, Martinsried Institut für Protein Sequences (MIPS), D (see page 366) John Sgouros, MIPS, chromosome II Martina Haasemann, MIPS, chromosome XI

SEQUENCING SUBCONTRACTORS

Michel Aigle/Marc Crouzet, Université de Bordeaux, F
Wilhelm Ansorge, European Molecular Biology Laboratory, Heidelberg, D
Claus Christiansen, Genetic Engineering Group, Lyngby, DK
Horst Domdey, Genzentrum, München, D
Bernard Dujon, Institut Pasteur, Paris, F
Karl-Dieter Entian, Universität Frankfurt, D
(with Martin Brendel, Goethe-Universität, Frankfurt, D and
Fred Zimmerman, Technische Hochschule Darmstadt, Darmstadt, D)
Horst Feldmann, Universität München, D
Walter Fiers, Rijksuniversiteit Gent, B
Françoise Foury, Université Catholique Louvain, B
(with Claude Jacq, Ecole Normale Supérieure, Paris, F)
Hiroshi Fukuhara, Institut Curie, Orsay, F
(with Monique Bolotin, Université de Paris-Sud, Orsay, F)
Nicolas Glansdorff, Research Institute CERIA-COOVI, Bruxelles, B
Les Grivell, Univesiteit van Amsterdam, NL
François Hilger/Daniel Portetelle, Faculté Sciences Agronomiques,
Gembloux, B
Corn Hollenberg, Institut für Mikrobiologie, Düsseldorf, D
Michel Jacquet, Université de Paris-Sud, Orsay, F
Jean-Claude Jauniaux, Université Libre de Bruxelles, Bruxelles, B
Antonio Jimenez, Universitad Autonoma Madrid, Madrid, E
David McConnel, Trinity College, Dublin, IRL
Steve Oliver, Manchester Biotechnology Center, Manchester, UK
Peter Philippsen, University of Giessen, Giessen, D
Rudi Planta, Vrije Universteit Amsterdam, Amsterdam, NL
Fritz Pohl, Universität Konstanz, Konstanz, D
Michael Rieger, Biotechnologische und Molekularbiologische Forschung,
Wilhelmsfeld, D
Claudia Rodrigues-Pousada, Instituto Gulbenkian de Ciencia, Oeiras, P
Piotr Slonimski, Centre de Génétique Moléculaire, Gif-sur-Yvette, F
Yde Steensma, Rijksuniversiteit Leiden, Leiden, NL
George Thireos, Institute of Molecular Biology and Biotechnology,
Heraklio, GR
Dieter von Wettstein, Carlsberg Laboratory, Copenhagen, DK

PREPARATION OF CHROMOSOMAL LIBRARIES FOR THE NEXT BIOTECH PROGRAMME

Chromosome VIII: Steve Oliver, Manchester Biotechnology Center, Manchester, UK (see page 363)
Chromosome X: Francis Gallibert, Bernard Dujon, Hôpital St. Louis and Institut Pasteur Paris, F (see page 364)
Chromosome XIV: Peter Philippsen, Universität Giessen, D (see page 365)

RESULTS

DNA COORDINATION

Thanks to the preparative work carried out in 1991 by Horst Feldmann and Bernard Dujon, all sequencing subcontractors have received their first cosmid in February or March 1991.

SEQUENCING

At the end of March 1992, the 28 principal subcontractors had submitted a total of 542,161 bp, including about 50,000 bp of preliminary data. This amounts to near 15,000 bp per year and per principal contractor. During the first 14 months of the BRIDGE Programme, only 11 subcontractors have submitted more than 25,000 bp, 7 others have submitted less than 10,000 bp and 4 have not submitted any data yet.

DATA ANALYSIS

Two listing of homologies of the open reading frames have been circulated to YIP members and the coordinators.
Each contractors have received a summary listing of the homologies of the open reading frames detected in their submitted sequences.

PREPARATION OF ORGANIZED COSMID CHROMOSOMAL LIBRARIES FOR THE NEXT BIOTECH PROGRAMME.

The preparation of cosmid libraries from chromosomes V, VIII, X, XIV, and XVI were considered. However chromosomes V and XVI were abandoned. The construction of the chromosome X library is terminated, that of chromosome XIV is expected to be finished in 1992.

ADMINISTRATIVE COORDINATION

All principal contractors have received an advance payment of 45,000 ecus within the two weeks after signature of their subcontract. A second payment of 45,000 ecus has been made within two weeks after reception by the administrative coordinator of the approval by the DNA coordinators of the quality statement (including the description of sequencing strategy) provided by the sequencing contractors.

COMMENTS

During the remaining 22 months of the BRIDGE programme about 1,300,0 bp of additional final sequence will have to be carried out. This correspor to a steady submission of slightly more than 26,000 bp per year and principal sequencing subcontractor.

The average rate of sequence submissions will have to be increased by 73% during the 22 remaining months compared to the submissions performed during the first 14 months. In particular, the problems encountered by the slow sequencers have to be sorted out

TITLE:

Sequencing of Yeast Chromosome II **BIOT - 0167**

OFFICIAL STARTING DATE:

January 1st, 1991

COORDINATOR:

Prof. Dr. Horst Feldmann, Universität München, D

PARTICIPANTS:

The following laboratories participated in sequencing

Prof. Dr. Michel Aigle, Université de Bordeaux, F
Dr. Claus Christiansen, Genetic Engineering Group, Lyngby, DK
Prof. Dr. Horst Domdey, Genzentrum, München, D
Prof. Dr. Karl-Dieter Entian, Universität Frankfurt, D
Prof. Dr. Horst Feldmann, Universität München, D
Prof. Dr. Walter Fiers, Rijksuniversiteit Gent, B
Dr. Francoise Foury, Université Catholique Louvain, B
(with Prof. Dr. Claude Jacq, Ecole Normale Supérieur, Paris, F)
Dr. Nicolas Glansdorff, Res. Inst. CERIA-COOVI, Bruxelles, B
Prof. Dr. Les Grivell, Universiteit van Amsterdam, NL
Prof. Dr. Michel Jacquet, Université de Paris-Sud, Orsay, F
Prof. Dr.David McConnell, University of Dublin, EI
Prof. Dr. Fritz Pohl, Universität Konstanz, D
Prof. Dr. Pjotr Slonimski, CNRS, Gif-sur-Yvette, F
Prof. Dr. H. Yde Steensma, University of Leiden, NL

OBJECTIVES SET FOR THE REPORTING PERIOD:

Each of the above contractors should receive one appropriate cosmid clone of chromosome II for mapping and sequencing, whereby the sequencing strategy was left to his discretion. The data (final contigs >5kb) should be entered into the dataset administered by Martinsried Institute of Protein Sequences (MIPS) under the supervision of the DNA coordinator. The data should be analysed for genetic entities and other criteria 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. Some of the sequencing data were transmitted to MIPS rather late. A few contractors have delivered no sequencing data as yet.

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 used to assemble a physical map and to correlate it with the genetic map of chromosome II known at that time. It was found that both of these maps were largely co-linear. On this basis, a cosmid library of strain αS288 was established and clones for chromosome II (840 kb) were selected and mapped. A schematic overview is presented in Figure 1.
In a first round, each contractor received a (non-overlapping) αS288 cosmid clone containing 30-40 kb of yeast DNA insert to undertake restriction fine mapping and sequencing. Extending cosmids have been supplied, whenever the contractor had submitted >25 kb of final data from his first cosmid.

The status of data obtained from the contractors (as of the end of February 1992) is documented in Table 1.

Analysis of the sequence data was carried out by MIPS in collaboration with the DNA coordinator. The results are listed in Table 2. Altogether, 130 open reading frames were encountered including 6 genes for tRNAs, several singular delta, sigma and tau elements, and a complete Ty2 element. 27 of the protein encoding entities can be attributed to known functions or already gentically mapped loci. For 35 ORFs, significant FASTA scores were obtained, from which putative functions can be predicted.

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. 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. As it appears, the leftmost cosmid clone contains telomeric sequences.
Among the new proteins on chromosome II are several that reveal significant similarity with interesting regulatory factors from other species, particularly higher eukaryotes.

WIDER CONSIDERATIONS:

The complete sequences of yeast chromosomes III, II and XI will render information on the genomic organisation and the structures of about 1000 new genes. Together with the 1000 sequences in the databanks and data from activities outside the EC, the repertoire of known yeast genes should amount to some 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 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. Furthermore, techniques experienced along with 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 and the coordinator 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) continuously updated maps of chromosome II; (2) a 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 the Yeast Industrial Platform (YIP) in Brugge, September 22-24, 1991, where any relevant information concerning the project was exchanged and future strategies were discussed.

LIST OF JOINT PUBLICATIONS/PATENTS WITH TRANS-NATIONAL AUTHORSHIP:

Too early at this stage of the project.

OTHER PUBLICATIONS/PATENTS:

Too early at this stage of the project.

TITLE: SEQUENCING OF YEAST CHROMOSOME XI
BIOT-0167

OFFICIAL STARTING DATE: 01/01/91

COORDINATOR: BERNARD DUJON, INSTITUT PASTEUR, PARIS, FR

PARTICIPANTS: W. ANSORGE, EMBL, HEIDELBERG, DE
B. DUJON, INSTITUT PASTEUR, PARIS, FR
M. BOLOTIN-FUKUHARA, U. PARIS XI, ORSAY, FR
F. FOURY, U. C. LOUVAIN, LOUVAIN-LA-NEUVE, BE
H. FUKUHARA, INSTITUT CURIE, ORSAY, FR
F. HILGER, FAC. SC. AGRO. GEMBLOUX, BE
C. HOLLENBERG, U. DÜSSELDORF, DÜSSELDORF, DE
J.C. JAUNIAUX, U. L. B. BRUXELLES, BE
A. JIMENEZ, U. AUTONOMA, MADRID, ES
S. OLIVER, UMIST, MANCHESTER, GB
P. PHILIPPSEN, U. GIESSEN, GIESSEN, DE
R.J. PLANTA, VRIJE U., AMSTERDAM, NL
F. POHL, U. KONSTANZ, KONSTANZ, DE
M. RIEGER, BIOT. M. BIO. FORSCH. WILHELMSFELD, DE
C. RODRIGUES, INSTITUTO GULBENKIAN, OEIRAS, PT
G. THIREOS, IMBB, IRAKLIO, GR
D. VON WETTSTEIN, CARLSBERG LAB., KØBENHAVN, DK

OBJECTIVES SET FOR THE REPORTING PERIOD:
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 physical map of chromosome XI. Distribution of clones to participants for sequencing. Determination of the complete sequence of chromosome XI from the set of overlapping clones over the period of three years i.e. determination of ca. 240 kb for 1991.

MAJOR PROBLEMS ENCOUNTERED:
Actual starting dates for sequencing: Due to the time necessary to built up the cosmid libraries and establish the physical map of chromosome XI, cosmid clones could only be distributed to the participants on january 29th, 1991.
All distributed cosmids proved suitable for sequence determination except for three (DNA instability, erroneous map assignments).

Therefore, three participants (F. HILGER, S. OLIVER and C. RODRIGUES) have received new cosmids on march 7th, 1991 and could only start sequencing at this date.

RESULTS

Coordination:

Three cosmid libraries covering, altogether, 380 times the yeast genome, have been constructed and stored. They have been sorted in parallel for chromosome 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.

Colinearity between inserts of all cosmids of chromosome XI with the native chromosome XI itself has been checked by restriction mapping and hybridization.

The set of chromosome XI sorted cosmid has been mapped by four different criteria: fingerprinting, terminal RNA probe hybridizations, EcoRI digestions, cosmid to cosmid cross hybridizations. In addition, the physical map has been directly verified using the nested chromosome fragmentation strategy based on the I-*Sce* I endonuclease (see below).

The physical map has a 3 kb average resolution and shows complete internal consistency. Cosmids covering almost the entire chromosome have been distributed (see annex 4).

Sequencing: Each participant (including the coordinator) has received one cosmid clone with an insert of 30-40 kb. Each participant has the choice of its sequencing strategy, sequencing methods and internal organization of his/her own sequencing team. Most participants have subcloned the cosmid into smaller pieces prior to sequencing. It follows that the number of contigs of final sequence is at present larger than the number of cosmid distributed. Assembly of those contigs will have to be done in the future. 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 and encouraged to exchange information. The table given in annex 3 shows contribution of each participant, calculated as the number of base pair of final sequence submitted to MIPS as of february 18th, 1992. Participants having submitted more than 25000 bp or having terminated their assigned

fragment have sent detailed strategy protocols and quality statements to the coordinator. When necessary, they have been sent new cosmid clones or DNA material in february 1992.

HIGHLIGHTS / MILESTONES:
Cosmid libraries: Three cosmid libraries covering, altogether 380 times the yeast genome, have been constructed and stored and can be used for continuation of the sequencing project on other yeast chromosomes.

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 potential application to other organisms as well.

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:
A total of 45 % of all sequences from chromosome XI have been determined in final form.

WIDER CONSIDERATIONS:
A high resolution physical map of chromosome XI has been constructed from overlapping cosmid clones of three complete genomic libraries of yeast, constructed for this purpose. The coordinated effort of 17 labs from 9 different E.C. countries has resulted in 45 % of the entire chromosome XI sequence being already determined in final form and stored at MIPS. More than 200 putative protein coding genes have already been discovered among which only 22 genes where known from classical genetic and molecular studies. Ca. 70% of the new genes have no clear homolog in data bases, hence represent new type of genes, not discovered before in any other organism and not belonging

to any of the known protein families.

COOPERATIVE ACTIVITIES:
Plenary meeting: First BRIDGE meeting at Brugge (BE) September 22-24th, 1991: All participants of the sequencing program for yeast chromosomes XI and II.

Information exchange: Two reports with ongoing results were prepared for YIP. Three "newsletters" with maps, protocols, progress reports and other relevant data were sent to all participants.

Staff exchange:
Martina Haaseman from MIPS visited B. Dujon's lab for one day for coordination of sequence analysis
Bernard Dujon visited MIPS, Martinsried, DE for two days for coordination of sequence analysis
Muus de Haan from L. Grivell's lab, Amsterdam, NL visited B. Dujon's lab for a week for random subcloning of cosmids
Bénédicte Purnelle from F. Foury's lab visited J.C. Jauniaux's lab for three weeks to learn and import automatic sequencing technique, shotgun strategy and software utilisation.
A.M. Becam from P. Slonimski's lab, Gif sur Yvette FR visited J.C. Jauniaux's lab for three days for sequencing double stranded DNA with the ALF (EMBL) fluorescent sequencer.
Micheline Vandebol from F. Hilger's lab visited J.C. Jauniaux's lab for sequencing with ALF (EMBL) fluorescent sequencer and software utilisation.
Kathrein Hamberg from P. Phillipsen's lab visited B. Dujon's lab for two days for chromosome fragmentation.
Jean Claude Jauniaux visited several times F. Foury's lab for discussion about DNA sequencing strategies and software analysis.
Laurent Gaillon from B. Dujon's lab participated in the two-weeks "Sequencing course" organized by W. Ansorge at EMBL, in November, 1991. Bernard Dujon gave a lecture at this course.
Two technical assistants from D. Von Wettstein's lab participated in the following training courses: *i*- Advanced training course in nucleotide sequencing, February 3-9, 1992, ABI, Foster City, CA, USA.; *ii*- Kebo Users Course, January 15th,1992, Stockholm, SE.
Corien Maat from L. Grivell's lab visited H. Fukuhara's lab for

discussions on automatic sequencing and software analysis.

Materials exchanged: Cosmid clones and other DNA and strain materials were sent by the coordinator to all other participants. Unpublished sequences from several participants were sent to the coordinator for mapping or other applications.

LIST OF JOINT PUBLICATIONS / PATENTS WITH TRANS-NATIONAL AUTHORSHIP:

None as yet.

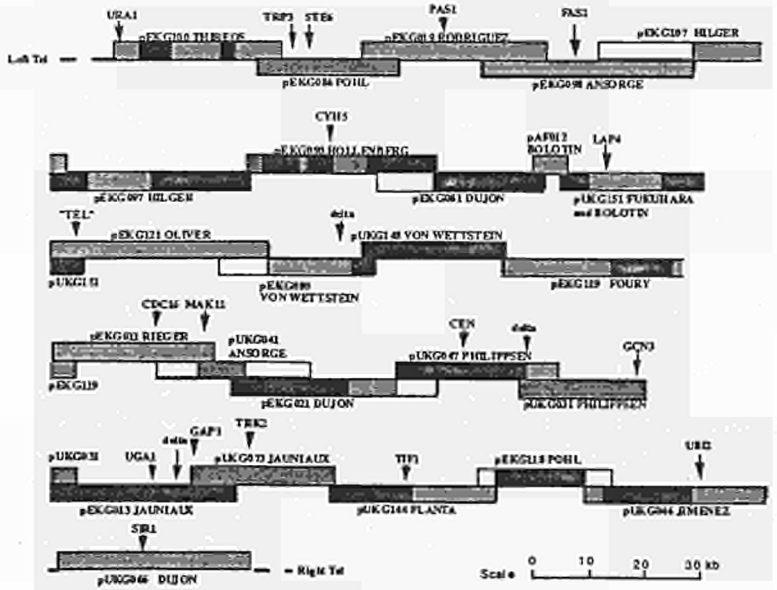
OTHER PUBLICATIONS / PATENTS:

A. Jacquier, P. Legrain, B. Dujon. YEAST (1992) 8, 121-132

Additional publications from different participants are *in press* or *submitted*

ANNEX 4

THE OVERALL ORGANIZATION OF THE SEQUENCING EFFORT



CHROMOSOME XI COSMID DISTRIBUTION Release of March 24th, 1992

Sequences entered in MIPS in final form (indication of sizes are only approximative)

Sequences assigned to contractor

Part of clone not assigned to contractor (overlaps)

The Generation of a Cosmid Bank of S. cerevisiae Chromosome VIII.

S G Oliver, Manchester Biotechnology Centre, UMIST, PO Box 88, Manchester M60 1QD, UK.

Chromosome V and VIII co-migrate as band 5 in pulsed-field electrophoretograms of chromosomal DNA from S. cerevisiae S288C and its close relatives. The EC Yeast Genome Sequencing Consortium has taken responsibility for sequencing chromosome VIII, whereas chromosome V will be sequenced in Stanford by the Botstein/Davis Group. At UMIST, we are adopting two parallel strategies in order to create a cosmid clone bank of chromosome VIII DNA: A total genomic bank of S. cerevisiae S288C has been prepared in the cosmid vector pWE15. This bank is being screened for chromosome VIII clones using specific gene probes from that chromosome (e.g. CUP1 and SOD1) and 'walking' out with the clones so identified to cover the rest of the chromosome. At the same time, chromosome VIII DNA from strain YP148, which carries a polymorphism permitting the separation of chromosomes V and VIII on pulsed-field gels, is being used as a probe to identify chromosome VIII cosmids directly. Unfortunately the chromosome VIII in this strain carries a 45 kb deletion and so not all such clones can be identified by this route. The second major strategy being employed is the creation of a band-5-specific clone bank in pWE15 using DNA from S288C. A collection of chromosome V-specific lambda clones has been obtained from Prof Isono (Kobe, Japan) and these are being used in the first stage to exclude chromosome V clones from this cosmid bank of the two chromosomes.

BUILDING A COSMID CONTIG OF CHROMOSOME X

Meng-Er Huang[1], Jean-Claude Chuat[1], Agnès Thierry[2], Bernard Dujon[2] and Francis Galibert[1]

[1] Laboratoire de Recombinaisons Génétiques (UPR 41-CNRS), Centre Hayem, Hôpital Saint-Louis, 1 avenue Claude Vellefaux, F-75475 Paris cedex 10, France.

[2] Unité de Génétique Moléculaire des Levures (URA 1149-CNRS), Institut Pasteur, 25 rue du Docteur Roux, F-75724 Paris cedex 15, France.

Total yeast DNA was obtained from a diploid strain (FY 1679) obtained by selfcrossing of S288C after switching mating type. Fragments resulting from partial digestion with *Sau*3AI were ligated to linearized pWE15, a cosmid vector with T3 and T7 promoters. Two other libraries were made in the cosmid vector pOU61 COS. The insert size averaged *ca* 25 kb. The yeast genome size was covered 380 times by the 3 libraries taken as a whole. Pools of clones covering 12-25 times the genome size were stored at -80°.

Recombinant colonies were grown on two sets of 137-mm nylon filters (120 per filter). After lysis of the colonies by NaOH, chromosome-X-specific clones were sorted out by hybridization with radiolabeled PFG-purified chromosome X. Two hundred ninety-two clones out of a total of 3940 colonies were derived from the pWE15 library (7.4 %). A hundred and four additional clones were sorted out from the pOU61 COS library.

The clones were subcultured, dispensed into several sets of 96-well plates and streaked onto several sets of nylon filters. Clones taken at random were independently digested with *Hae*III and *Alu*I. The digests were mixed to either T3 or T7 promotors. Radiolabeled RNA probes were obtained after addition of T3 or T7 RNA polymerase and [α^{32}P] UTP. The filters were independently hybridized with either T3 or T7 probes. Clones overlapping with the T3 or T7 probes were further used as probe templates. The procedure was discontinued when 350 clones were aligned in one contig of over 600 kb, a figure to be compared with the size of chromosome X (760 kb $\pm$ 5 %).

Ten sequences taken from databases and corresponding to genes mapped to chromosome X were used to make oligonucleotide probes that were hybridized with the clones. All the clones labelled by a given gene probe were found to map to a unique position in the contig. The distances between the genes on the physical and on the genetic maps were consistent. *Dal*5 was located very close to one end of the contig, as on the genetic map. No sequenced gene marker close to the other end was available. However, since the physical map and the genetic map appear to be colinear, and since the distance between the last gene (*cdc*6) and the end of both maps seems roughly proportional, the contig most probably encompasses the whole of chromosome X (except for the telomeric region, that will be dealt with later).

*Eco*RI digests of selected groups of overlapping clones were comigrated in agarose gels. Southern blots were prepared and within each group each clone was successively used as a probe. The interpretation of these results gives the exact number and size of *Eco*RI fragments and allows to drawn an *Eco*RI restriction map of the contig.

Construction of a chromosome XIV library from S.cerevisiae

Progress Report to the Commission of the European Communities (March 1992)

Kathrein Hamberg and Peter Philippsen

The scientific goal of our project is the physical mapping of cosmid clones covering the complete chromosome XIV (820 Kb) of S.cerevisiae by using a series of new mapping vectors. A DNA segment cloned into these so-called pMACS vectors will direct a gene conversion starting at the homologous sequence of a chromosome and finishing at the end of this chromosome, thereby generating a chromosome fragment. Due to the genetic outfit of the pMACS vectors, in 50% of the conversion events genetically stable chromosome fragments are established. The size of these new chromosomes is a direct measure of the distance between the chromosomal location of the cloned DNA and the end of the chromosome. The assignment to the left or right arm of the chromosome can be done prior to the actual mapping experiments (see below).

We started with a cosmid library of total DNA from strain FY1676 (isogenic to S288C) obtained from Dr. Bernard Dujon, Paris. This library is based on the ligation of size-fractionated partial Sau3A fragments into BamHI cleaved pWE15 and is the same library used for mapping clones of chromosomes X and XI. 2400 colonies were screened with chromosome XIV DNA which was isolated from pulse field gels and radioactively labelled by random priming. The DNA of 625 colonies hybridized to this probe which corresponds to 26% of the clones. This is much higher than expected since chromosome XIV (820 Kb) represents only 6% of the S.cerevisiae genome. One reason is most likely an unavoidable contamination of the probe with chromosome fragments of larger chromosomes. EcoRI digestions of cosmids from colonies showing strong hybridization signals revealed an unusually high percentage of clones carrying solely ribosomal DNA (chromosome XII). It was therefore necessary to rescreen all clones with a ribosomal DNA probe.

267 of the total 625 putative chromosome XIV clones were finally analysed by Southern hybridization of EcoRI digested cosmid DNA, again using gel purified chromosome XIV as probe. With this method we found 86 cosmid clones all EcoRI fragments of which hybridized with the chromosome XIV probe. In the other cosmids only some of the EcoRI fragments hybridized probably due to repetitive DNA or to inserts consisting of fragments from chromosome XIV and other chromosomes. The 86 chromosome XIV clones carry inserts that are 30 - 35 Kb in length representing 3 - 3.5 times the chromosome XIV.

We are just separating the clones into two parts representing the two chromosome arms. By fragmentation at CEN 14 we constructed a new haploid yeast strain carrying the right arm (190 Kb) and the left arm (630 Kb) as genetically stable chromosomes. DNA of both arms was isolated from pulse field gels and used as hybridization probes. Till now we could clearly map 7 clones to the right arm and 27 clones to the left arm of chromosome XIV. In parallel, fragments of the cosmids were cloned into pMACS vectors to allow precise mapping.

As stated last fall at the Brugge-Meeting we expect to finish most of the mapping by May 1992.

TITLE:	Informatics Network (Sequencing the Yeast Genome)
CONTRACT NUMBER:	BIOT-CT90-161
OFFICIAL STARTING DATE:	01/01/1991
COORDINATOR:	H.W. Mewes, Max-Planck-Institut f. Biochemie W-8033 Martinsried, Germany

OBJECTIVES SET FOR THE REPORTING PERIOD:

MAJOR PROBLEMS ENCOUNTERED:

A major problem encountered was the lack of manpower to serve the sequencing community in a timely manner. During 1991 a serious effort had to be undertaken to verify the experimental results of sequencing chromosome III and to finish their publication. In addition, the amount of data to be processed increased significantly, thereby increasing the workload even more.

The conflict of interest between the creators of the data, i.e., the sequencing laboratories, and the recipient scientific community has not yet been solved. The rules for the release of data to the public databases turned out to be unsatisfactory. A better solution to provide public access to the data in time has to be worked out.

RESULTS:

As the informatics coordinator, MIPS has been responsible for the collection, storage and analysis of all sequence data submitted by the 35 laboratories involved. Since January 1991, two other yeast chromosomes, II and XI, are under sequencing. As of February 18, 1992, 200 kb from chromosome II and 290 kb from chromosome XI had been sequenced; the total length comprises 620 and 830 kb, respectively.

Data were submitted by e-mail or on magnetic media. 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. A detailed plot displaying the clones used in sequencing, all open reading frames as well as previously mapped genes, and other elements (Ty, delta, tRNA) was constructed.

The processing scheme is summarized below. 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.

A novel software tool that greatly facilitates data handling has been developed (S. Liebl). Using an X-windows interface, the program allows the rapid display of chromosome clones as well as restriction sites and open reading frames (see Fig. 1). It is capable of scaling up a selected region, handling all restriction enzymes available, and marking sequenced fragments within clones.

The following databases have been created and are maintained for the yeast project:

- YeastProt yeast protein sequence database. All entries already existing in databases were checked for errors by comparing to the nucleic acid sequence, where this was available. Errors were corrected accordingly and partial sequences merged to elimi-

nate redundancy of information. All YeastProt entries are fully annotated. The database is continuously updated using data from the scientific literature as well as direct submissions of data to the EMBL Data Library that are forwarded to MIPS on a daily basis. YeastProt is maintained by M. Haasemann. The current release includes 978 entries comprising 431,867 residues.

- YeastNuc yeast nucleic acid sequence database. This is a merged dataset, i.e., assembled using yeast sequences from several databases and eliminating entries containing identical sequences. However, entries differing in at least one nucleotide are present in YeastNuc. This has the advantage of providing an up-to-date dataset for fast searches at the cost of partially redundant information and entry format diversity (each YeastNuc entry inherits the format of the parent database). The data used to assemble YeastNuc are obtained from EMBL, GenBank, EMBLKEEP and EMBLNEW. The latter two databases contain most recent data forwarded from EMBL to MIPS prior to checking. YeastNuc is maintained by J. G. Sgouros. The current release includes 1,889 entries comprising 3,261,167 bases.

An on-line computing facility was established that enables the participating laboratories to analyze their data and perform database queries. P. Kreisl is responsible for this service.

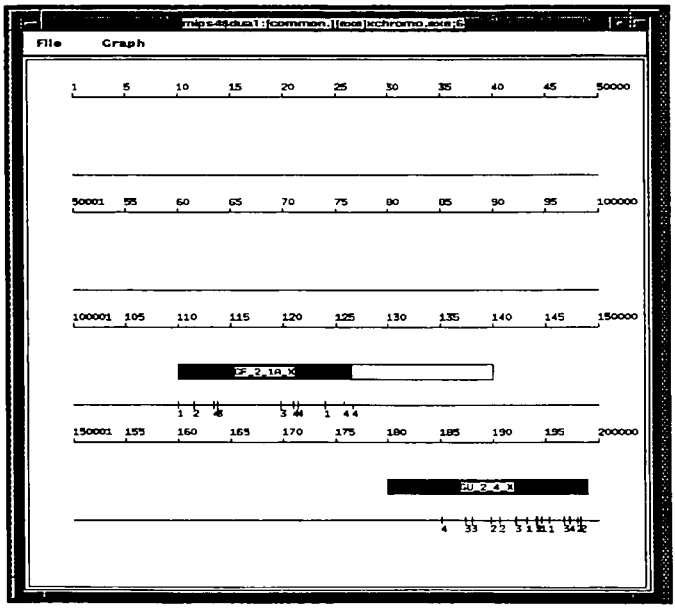


Figure 1 Display from the program Xchromo

Summary of sequence data processing scheme:

A sequence submitted to MIPS is analyzed using the following scheme:

1. Construction of a restriction map based on the sequence. This map is compared to the map of the clone library to verify clone orientation.
2. The sequence is used to check for overlaps:
   - with vectors, using the VecBase vector database
   - with known yeast sequences (identification of known genes as well as Ty and delta sequences)
   - with other clones of the same chromosome
3. Pattern search is then performed at the DNA level in order to detect:
   - promotors
   - upstream activating sequences (UAS)
   - autonomously replicating sequences (ARS)
   - introns
   - tRNA genes
   - other yeast-specific regulatory sequences
   - repeats
4. Extraction of open reading frames (ORFs) using a cutoff value (100 amino acid residues). The locations of these ORFs are correlated with the positions of promotors and terminators in order to assess the probability of expression. Furthermore, a codon frequency algorithm is employed in localizing potential coding regions.
5. For the analysis of ORFs, the following steps are employed:
   - FASTA comparison with the MIPSX merged protein sequence database. This step is essential not only for detecting similarities of ORFs to other sequences in the databases, but also for reidentifying known genes detected already at the DNA level and thus exclude frameshift errors.
   - for FASTA scores in the "twilight zone", the Interactive Sensitive Sequence Comparison (ISSC) algorithm (P. Argos, EMBL) is employed in order to evaluate the significance of the result and optimize the alignment.
   - pattern search is then performed at the protein level using the ProSite Dictionary of Protein Sites and Patterns (A. Bairoch, Geneva). This allows the detection of possible posttranslationally modified sites and may assist in assigning a function to an otherwise unknown ORF.
   - the ORFs are scanned for internal repeats; search for putative transmembrane segments is performed if the methods outlined above give indications for a membrane protein.

6. The chromosome contig is gradually assembled based on the clone overlap data. The locations of previously mapped genes are correlated with the existing physical map of the chromosome. The new physical map is compared to the genetic map.

Work in progress

A major problem that has to be faced and circumvented in the yeast sequencing project is that sequences submitted by the contractors stay private for a long period and cannot be analyzed be any other participating laboratory. We propose to store these sequences in a database that is build by INQ (a transaction oriented database system) and frequently updated. As long as access to data is restricted, queries to this database will result in a sequence code containing the chromosome number, the submission date and the submitting laboratory. Only when these data become public, entry-code, title and sequence alignment will be displayed.

A drawback of the present sequence database query systems is their passive behavior, i.e. queries about sequence data entries or comparisons of test sequences against the database must be repeated frequently. Unless the user repeats a query very often, he will miss the appearance of the information he searches for. We plan to establish an automatic system to distribute electronic messages whenever information associated with a new sequence is found that matches the query. For example, one could ask for all yeast sequences that are new in the database. Such a service would be highly interesting for the laboratories participating in genome analysis.

HIGHLIGHTS/MILESTONES

Up to march 1992, 320,423 bases submitted from chromosome XI and 228,383 bases from chromosome II have been analyzed. MIPS has analyzed all open reading frames by comparison to the latest available sequence databanks. Results have been forwarded to the laboratories. With the help of careful sequence analysis, sequencing errors have been corrected and a number of problems has been solved.

WIDER CONSIDERATIONS

The task of efficiently analyzing, handling, and assembling chromosome data greatly benefits from the experience of the protein sequence databank. The informatics coordination of a large sequencing project will not be successful when done by a 'stand-alone' type of group. The continuous cooperation and information exchange between the yeast and databank groups within MIPS on one hand and between MIPS and the sequencing laboratories on the other is of great importance for the successful completion of the project.

The experience with the informatics coordination of the chromosome III project made clear that a change of policy is necessary in order to guarantee timely access of the scientific community to project data, while at the same time giving contractors the opportunity to publish their results.

COOPERATIVE ACTIVITIES

The coordination of the data processing and evaluation is a highly interactive process. Frequent communication with each of the laboratories by electronic mail, phone and meetings with the DNA-distributors took place. M. Haasemann visited B. Dujon's laboratory, H. Feldmann is a frequent visitor at MIPS. B. Dujon was guest for a seminar and work on the chromosome XI data.

LIST OF JOINT PUBLICATIONS

A detailed report for the Yeast Industry platform is issued bimonthly. Two 'Chromosome' Newsletters were distributed to the contractors in collaboration with the DNA-coordinators.

T-PROJECT

MOLECULAR IDENTIFICATION OF NEW PLANT GENES

TITLE: Development and use of Arabidopsis thaliana as a tool for isolating genes of agronomic importance

CONTRACT NUMBER: BIOT CT90 0207

OFFICIAL STARTING DATE: 1st February

COORDINATOR: M. Bevan, AFRC, Norwich, UK

PARTICIPANTS: see pages 376-401

The first year of the Arabidopsis T project has seen major successes both in terms of the scientific goals achieved, and in the organisation and effective operation of a large interdependent network of scientists. This has been achieved primarily through the willingness of the individual scientists to link their efforts with others, in order to achieve the required effort to push European Arabidopsis science to higher achievement, and through the assistance of EC staff. I am convinced that the success of the T-programmes will encourage the formation of other networks of scientists, under the auspices of the EC. In this way the variety and creativity of European scientists can be fostered in a long term manner.

The main Contract was signed on 1st February, 1991 and the appointment of 30 positions took place soon after this. We held our first Meeting in Perpignan, France, with Michel Delseny (CNRS University of Perpignan) and his colleagues as hosts. This was the first time that the full scope of the programme of the T programme was apparent to many participants, and interesting presentations describing the activities of other large EC-sponsored scientific programmes were given by Jean Guern, who described our sister T programme in Regeneration, and Andre Goffeau, who described the EC-sponsored effort to sequence chromosome III of yeast. Specialist working groups then convened to plan in more detail their interactions after the general presentations. The next general meeting will be in Gent, Belgium on Sept 9-11, 1992, hosted by Marc Van Montagu and colleagues, while the third and final meeting is planned to be held in Cambridge in September 1993.

Databases and genome analysis

In June Caroline Dean, Jeff Dangl, George Murphy and I attended an EC Conference on Genome Sequencing at Elounda, Crete, at which we presented the relevant work of the T programme. Much benefit was gained by interactions with leaders in the field such as John Sulston, and we departed convinced that the time was ripe for the beginnings of an Arabidopsis sequencing effort. A substantial part of the rest of the year was devoted to this aim. This included forming a committee to investigate the "bioinformatics" needs of a putative Arabidopsis sequencing programme, consisting of Jerome Giraudat (F), Dirk Inze (B), Marc Zabeau (NL), and Jeff Dangl (D). We met in Koln on Dec 12 1991 to form our ideas, and again on January 6, 1992, in Boston (as guests of Howard Goodman), to discuss an international effort to coordinate the sequencing effort. Considerable goodwill exists on both sides to harmonise our efforts, particularly in respect to databases and data sharing. An important outcome of the Committee's work was the inclusion of elements of cDNA sequencing in the forthcoming Biotech programme, and the provision of a framework for large-scale sequencing. While in Boston the AATDB database system, adapted for use in Arabidopsis work by Howard Goodman, Mike Cherry and colleagues at MGH in Boston, was demonstrated. This database will soon be available to those with the appropriate hardware, and its primary function will be to assemble physical and genetic map information in a very convenient format. This will be available to the T programme after April 1992.

Interaction with other T programmes and with Industry

In November 1991 I gave a presentation of the Arabidopsis T programme to the participants of the Regeneration T programme. This meeting was a pleasant and important occasion, as it also involved a first meeting with representatives from industry involved or interested in fundamental plant sciences. This meeting lead to a further meeting of interested industrialists in Basel and it now appears that a group of them is willing to form an industrial platform which will serve both the Regeneration and Arabidopsis T projects. A further meeting between the Coordinators of the two programmes and industrial representatives, with the aim to set up the Industrial Platform, will take place in Lyon in May 1992. It is hoped that greater industrial participation will lead to further funding opportunities and to a degree of closer integration of fundamental work with the long-term goals of European industry.

Development of Resource Centres

This was seen as a major objective during the first year of the programme. Together with the AFRC and the University of Nottingham (GB), a large and efficient operation has sprung up, directed by Mary Anderson and Bernard Mulligan. Collections of mutants and ecotypes have been assembled, characterised and catalogued, including collections that would otherwise have been lost. Significantly, the Nottingham collection has also serviced the new US Stock Centre in Ohio by providing them with the entire collection to start their operation. The two Centres will continue to develop their operations in a coordinated way, and will soon use a common database and ordering system called AIMS, developed specifically for that task by scientists at Michigan State University. In addition, the Nottingham Centre is providing the AATDB database system with information on mutants etc. Finally, collections of recombinant inbred and T-DNA-tagged lines will be deposited, which should provide the *Arabidopsis* community with valuable tools for mapping. The DNA Resource Centre in Koln has made a good start to collecting recombinant libraries and probes for use by participants. As its role becomes more widely known and more libraries are prepared it will serve an increasingly important role, especially in relation to the proposed sequencing work in the Biotech programme. Securing long-term funding for a Resource Centre will be a preeminent goal in the coming years.

Scientific Highlights

Brief descriptions of the progress achieved in all of the programmes can be found in the relevant section of this Report. More details of scientific progress, including scientific interactions, can be obtained in a comprehensive report available from the Coordinator. I wish to present some of the major scientific goals met in the first year of the Programme, and discuss the importance of the trans-European scientific network in achieving these.

The accomplishments of the Resource Centre in centralising and collating mutant and ecotype collections, and distributing these are especially significant, as activities such as these require the concerted action of several groups. Their success is a concrete demonstration of the value and effectiveness of coordinated actions such as T programmes. The Physical Mapping Programme, lead by Caroline Dean, has made enormous strides towards establishing a physical map of the genome. Together with colleagues in an international collaboration, about 1/3 of the genome has been mapped, techniques for rapidly screening YAC libraries developed, and the AATDB database established for collating mapping and genetic data. Lothar Willmitzer's lab, part of the Gene Replacement Group, has made significant advances in a difficult area by demonstrating, for the first time, useful frequencies of homologous recombination in *Arabidopsis* using the reconstruction of a hygromycin resistance gene as a selection for these rare events. These constructs are being made available to others in the Group for testing their recombination methods and DNA delivery systems. The T programme has aided collaborations that have enabled these important tools to be distributed for further refinement and testing. Further examples of such collaborations are evident in the Seeds Programme. In the group of Maarten Koornneef, important new mutations affecting abscisic acid action have been isolated, and the effect of these on gene expression, lipid and 2sugar metabolism, and interactions with other mutants are being investigated by this group. A major achievement by the group of Jerome Giraudat has been the identification of an 11kb piece of DNA, using chromosome walking, that complement abi3 mutations. With this gene identified, its sequence will help reveal the mode of action of a key growth regulator in plants. In the Embryogenesis Group, a large effort in the lab. of Keith Lindsey has identified T-DNA tagged loci that affect the tissue-specificity of GUS expression. Several of these effect embryo-specific patterns of expression, and the coming year will probably see a some of the genes directing these patterns of expression identified. Impressive progress has been made in the Floral Induction Programme. A gene encoding the *APETALATA2* product has been tagged using T-DNA and isolated in the group of Marc van Montagu. This gene, which determines floral morphology together with a small group of other homeotic genes, has been further characterised by sequencing. In the group of George Coupland, a walk using YACs of about 1,600kb has covered the *co* locus, which conditions flowering time. Several recombinants have narrowed down the region containing the gene, and complementation experiments are underway to isolate clones containing the gene. Similar walks are progressing towards the *fca*, *fwa*, and *fve* loci. In addition,

epistatic interactions between the different classes of mutants are being studied. In the Transposon Tagging Programme several groups have developed systems that drive high frequency excision and methods have been developed for selecting reintegration events. Notably, the groups of Lothar Willmitzer and George Coupland are at the stage of screening large populations of plants for mutations. Future work will involve making derivatives that can detect gene fusions.

Cooperative Activities.

These have been too numerous to mention in detail, but interactions have occurred in all of the programmes that have lead to collaborations beyond those expected at the beginning of the project. In addition to the annual meetings of the T project, in April 1992 there will be a joint EMBO/EC Workshop on *Arabidopsis* Molecular Genetics, organised by Csaba Koncz and colleagues in Koln. Finally, international collaborations have been fostered and it is hoped these will continue to develop, particularly in the field of genome analysis.

Publications

1. Swinburne, J., Balcells, L.L., Scofield, S., Jones, J. and Coupland, G. (1992). Elevated levels of *Ac* transposase mRNA are associated with high frequencies of *Ds* excision in *Arabidopsis*. Plant Cell, in press.
2. Coupland, G. (1992). Transposon tagging in *Arabidopsis*. In Methods in *Arabidopsis* Research, eds. Koncz, C., Schell, J. and Chua, N-H., in press.
3. Halfter, U., Morris, P.C. and Willmitzer, L. (1992). Gene targeting in *Arabidopsis thaliana*. Mol. Gen. Genet., 231: 186-193.
4. Hwang, I., Kohchi, T., Hauge, B., Goodman, H., Schmidt, R., Cnops, G., Dean, C., Gibson, S., Iba, K., Arondel, V., Danhoff, L. and Sommerville, C. (1991). Identification and map positions of YAC clones comprising one third of the *Arabidopsis* genome. The Plant Journal, 1: 367-74.
5. Schmidt, R., Cnops, G., Bancroft, I. and Dean, C. (1992). Construction of an overlapping YAC library of the *Arabidopsis thaliana* genome. Aust. J. Plant Physiol., 19, in press.
6. Martinez-Zapater, J.M., Capel, M., Cruz-Alvarez, J.A., Jarillo, A., Leyva, Roldan, M. and Salinas, J. (1991). Genetic and molecular analysis of floral induction in *Arabidopsis thaliana*. Flowering Newslett., 12: 12-14.
7. Goto, N., Kumagai, T. and Koornneef, M. (1991). Flowering responses to night-breaks in photomorphogenetic mutants of *Arabidopsis thaliana*, a long day plant. Physiol. Plant, 83: 209-215.
8. Koornneef, M., Hanhart, C.J. and van der Veen, J.H. (1991). A genetic and physiological analysis of late flowering mutants in *Arabidopsis thaliana*. Mol. Gen. Genet., 229: 57-66.
9. Topping, J.F., Wei, W. and Lindsey, K. (1991). Functional tagging of regulatory elements in the plant genome. Development, 112: 1009-1019.
10. Clarke, M.C., Wei, W. and Lindsey, K. (1991). *Arabidopsis* transformation: silver thiosulphate as an aid to increased efficiency. In: *Arabidopsis*: The Complete Guide, eds. Flanders, D. and Dean, C., AFRC PMB Programme.
11. Lindsey, K., Topping, J.F., Clarke, M.C., Wei, W., McArdle, H.F. and Rooke, L.M. (1991). Functional tagging of plant genes by modified T-DNA insertional mutagenesis. J. Exp. Bot., 42 (suppl.), 35.
12. Lindsey, K., Topping, J.F., Clarke, M.C., Wei, W., McArdle, H.F. and Rooke, L.M. (1991). Identification of tissue-specific genes by T-DNA tagging. Abstr. Third Int. Congr. ISPMB No. 500, University of Arizona.
13. Clarke, M.C., Wei, W. and Lindsey, K. (1992). High frequency transformation of *Arabidopsis thaliana* by *Agrobacterium tumefaciens* (submitted).

COORDINATOR

Co-ordinator
M. Bevan 1
S. Bright 2

PARTICIPANTS:

Tagging

*G. Coupland	1	Ac
L. Willmitzer	39	Ac
R. Masterson	23	Ac
C. Konez	19	T-DNA
M. van Montagu	36	Tam3/Ac
A. Pereira	42	En-1

Gene Replacement

*P. van den Elzen	35	Agro.
L. Willmitzer	38	DNA
B. Reiss	32	recA
P. Meyer	24	YACs
J. Paskowski	28	Deletions
P. Hooykaas	14	Agro.

Physical Mapping

*C. Dean	9	YAC
Keygene	40	RFLPs
T. Kavanagh	17	Sfi1
Zaadunie	41	QTL

Resource Centres

*B. Mulligan	26	Seeds
J. Dangl	7	Clones
A. Kranz	-	Seeds

Floral Induction

*J. Martinez	22	fve
G. Coupland	5	fg
C. Dean	10	fca
M. Koornneef	21	various
M. van Montagu	37	ap-2
P.G.S.	47	ap-2

Seed Development

*M. Koornneef	20	aba
C. Karssen	16	aba
M. Pages	27	proteins
M. Delseny	11	proteins
J. Giraudat	12	abi

Embryogenesis

*K. Lindsey	43	T-DNA
P. Gallois	44	proteins
Danisco	45	in-situs

*Co-ordinators

Title: Transposon tagging in *Arabidopsis thaliana*

Principal Investigator(s): M. van Montagu, Rijksuniversiteit Gent, Laboratorium Genetika, Ledeganckstraat 35, Gent, B-9000, Belgium.

Participant No: 36

Expression patterns of pollen-specific promoters were only described in general (e.g. anther specific) or expression was observed in specific anther cell layers and not in the pollen itself (e.g. tapetum). Therefore it was unlikely that transposase constructs driven by these promoters would result in higher germinal excision rates. However, in our laboratory two promoters were characterised which show strong pollen specific expression (mns-1, mns-2) patterns. Currently these promoters are used to drive Ac transposase cDNA.

We established a two-element transposon mutagenesis system in *Arabidopsis thaliana* using the well-characterised maize Activator/Dissociation (Ac/Ds) transposon family.

Our system consists of three elements:

1. Ds elements: Ds elements have been made that carry a chimeric **bar** gene conferring resistance to the herbicide phosphinotricin. These elements can thus be followed genetically. One hundred fifty independent transformed lines of *Arabidopsis* were generated.
2. Activator elements: Ac elements that deliver transposase activity have been cloned and transformed into *Arabidopsis*. These elements bear terminal deletions and are unable to transpose themselves (Ac "wings clipped" elements). In order to enhance transposase expression we constructed an Ac transposase CDNA under the control of the strong Agrobacterium Tr2' promoter. An Ac transposase CDNA under the control of pollen specific promoters (mns-1, mns-2) is under construction.
   In addition, all activator constructs bear the **Gus** gene and a hygromycin resistance gene which allow to score for their presence.
3. Excision assay: All Ds elements are cloned in the 5' leader sequence of a chimeric **NptII** gene. Ds excision will result in resistance to Km .

   In order to circumvent the problem of low germinal excision using Ac in heterologous systems, transactivation of Ds in transformed lines was done using Agrobacterium transformation followed by selection on restoration of the NptII resistance after transposition. In that way plants can be regenerated that are clonal for the excision event.
   Ds transactivation measured by the excision assay, ranged from 1 to 50% in the first generation depending on the Ds transgenic line transformed by Ac.
   Segregation of different DS and Ac elements in transactivated lines is followed in detail. Mapping of different stabilized Ds lines is going on. Transposition could be shown using the marker genes, PCR and Southern analysis.

Title: Enhancer detection with the *En-I* transposable element system.

Principal Investigator(s): M. Aarts and A. Pereira.

Participant No: 42

This project was initiated to develop the maize *En-I* transposable element system to use it for enhancer detection in the *Arabidopsis* genome. First it was necessary to demonstrate that the deletion derivative *I* was able to transpose in *Arabidopsis*. For this experiment we used plants transformed with a construct containing an *I*-element within the reading frame of a TR 1'-NPTII gene, as well as a 'wing-clipped' *En* (i.e. without border sequences) controlled by a CaMV 35S promoter. Imprecise excision of the *I*-element could restore the reading frame of the NPTII gene, giving kanamycin resistance. Assuming that only one in three excisions will restore the NPTII reading frame, this suggests a first

generation excision frequency of 0 to 60 %. Polymerase Chain Reactions were performed on DNA of T_2 plants. Excision of the transposon would give an empty donor site on the integrated T-DNA. The PCR analysis and subsequent sequence analysis revealed that also in a family not showing any kanamycin resistant progeny, excision had taken place. It also confirmed restoration of the reading frame of NPTII in kanamycin resistant plants. The sequence contained footprints typical for *I* element excision. Experiments are underway to determine the reinsertion frequency of the transposon.

To use the *En-I* transposable element system for enhancer detection in *Arabidopsis thaliana*, *En* had to be modified to get a better control over the activity of the element. For this reason we separated the transposase functions from the actual transposon parts by constructing two types of T-DNA binary plasmids. The transposon type contains a marker gene for selection for transformation (kanamycin resistance, NPTII) and a marker gene for selection for excision of the transposon (streptomycin resistance, SPT). The enhancer detector unit is formed by the 5' border fragment of *En* together with the GUS gene. In total 14 constructs, of which the efficiency in enhancer detection use has to be tested. The 9 most promising constructs have been transferred to *Arabidopsis* by root transformation with *Agrobacterium tumefaciens*. Transgenic progeny of plants containing one of these constructs, have been assayed histochemically for GUS activity. The transposase type of T-DNA construct consists of a hygromycin resistance gene (HPT) for selection for transformation, cDNAs coding for the two proteins (TnpA and TnpD) necessary for transposition and the *tms2* gene to select against plants containing the transposase construct after crossing to transposon containing plants. The *tnpA* gene is under transcriptional control of the TR 2' promoter and the *tnpD* gene is controlled by the *chiB* promoter, which is tapetum specific. Transgenic plants have been obtained containing this type of construct, which will be analysed for copy number and T-DNA loci. Crosses have been made with transposon lines to test for expression of the transposase genes and ability to induce excision of the transposon.

Joint activities of the insertional mutagenesis group.

All six members of the insertional mutagenesis sub-group met at the BRIDGE meeting in Perpignan, which was an important opportunity for detailed discussions to set the agenda for the first year of the programme. Since then detailed discussions between members have occurred on several occasions and the programme has fostered a general spirit of co-operation and collaboration. For example, plasmid constructions have been exchanged between the Berlin and Norwich groups along with detailed information on experimental protocols. Members of the group have had detailed discussions at International meetings such as at Tucson in October 1991 (members of the groups from Ghent, Berlin, Köln and Norwich) and in Zürich in November 1991 (members from Berlin and Norwich). Four of the six members will meet at the EMBO course on *Arabidopsis* which is to take place in Köln in April 1992.

Title: Establishing a transposon-tagging system in *Arabidopsis* by use of the maize transposon *Ac*.

Principal Investigator(s): G. Coupland, D. Long, L. Balcells and J. Swinburne, Molecular Genetics Department, Cambridge Laboratory, John Innes Centre, Colney Lane, Norwich, Norfolk, NR4 7UJ.

Participant No: 1

1. Establish use of streptomycin resistance gene to select excisions of *Ds*.
2. Increase rates of germinal excision by the use of different promoters expressing *Ac* transposase. It was previously shown that the *Ac* element transposes at low frequency in *Arabidopsis*. We have introduced into *Arabidopsis* constructs which contain fusions of different promoters to the *Ac* transposase (TPase) gene. The abundance of the *Ac* mRNA in transformants containing each of the fusions was then examined. Between the most active 35S::TPase transformant and the least active *Ac*::TPase transformant the abundance of the mRNA varied around 1,000 fold. Moreover, the mRNA

level varied between transformants containing the same fusion; for example the five 35S::TPase transformants varied across a range of 15 fold. To test the activity of each of these fusions all nineteen transformants were crossed to a single line containing *Ds* inserted within the SPT gene. All of the populations of hybrid seedlings contained some which were variegated: the frequency with which variegated seedlings arose and the numbers of sectors present on the cotyledons varied between TPase transformants. In general those which contained most mRNA produced most variegated seedlings with a high density of resistant sectors. For example the 35S::TPase transformants which contained most mRNA were extremely active: approximately 100% of F1 seedlings were variegated and these contained many green sectors.
In general the 35S::TPase fusion gives rise to very high frequencies of germinal excision: in excess of 35% of F2 plants inherited an active streptomycin resistance gene. This is very much higher than was previously shown for wild-type *Ac* or indeed than we detected with our *Ac*::TPase transformants. Only 5 of the 27 contained *Ds* inserted at a new location. This low ratio would greatly reduce the number of streptomycin-resistant F2 plants which could carry mutations induced by *Ds* insertion. To overcome this problem we have constructed a *Ds* which contains a hygromycin-resistance gene. By activating this with the 35S::TPase fusion we can select for F2 plants which are resistant to both antibiotics and therefore probably contain a transposed *Ds*. Preliminary data obtained using this construct support the earlier experiments: most streptomycin-resistant F2 plants do not contain *Ds*. However, we find that around 50% of F2 families contain at least one seedling which is resistant to both antibiotics. This will form the basis of a transposon tagging strategy and most of our effort during the second year of this project will be expended in testing the efficiency with which mutations arise in the F2 individuals.

Title: Gene tagging

Principal Investigator(s): L. Willmitzer, H. Pena-Cortes and T. Altmann, Institut fur Genbiologische Forschung Berlin gmBH, 63, Ihnestrasse, Berlin 1000, Germany.

Participant No: 39

1. Carry out large scale mutagenesis programme with wild-type *Ac*.

2. Use of herbicide resistance to monitor *Ac* excision.

3. Demonstrate new positions of *Ac* in genome.

In a large scale experiment about 40,000 F3 plants originating from 8 independent transgenic *Arabidopsis thaliana* lines carrying the *Ac*-element between the Tr-promoter and the NPT II gene were assayed for kanamycin-resistance in the greenhouse by kanamycin spraying. 58 (0.15%) kanamycin-resistant plants were detected in the population of the 40,000 plants sown out in the greenhouse. DNA-blot analysis performed on 40 resistant plants demonstrated that in 33 of these plants the expected excision band appeared indicating the excision of *Ac* and that in 22 of the 33 plants new fragments hybridizing with *Ac* were present and thus indicating integration.
To establish a more rapid procedure to select for *Ac* excision under greenhouse conditions, transgenic *Arabidopsis thaliana* lines carrying a construct with the wild-type *Ac* located between the Tr-promoter and the bar-gene were constructed. Upon excision of *Ac* from this construct the bar gene will be expressed and lead to resistance of the plant against the herbicide phosphinotricin. F2-plants derived from phosphinotricin-sensitive F1-plants of 37 independent transgenic lines were examined for resistance by spraying phosphinotricin in the greenhouse. Phosphinotricin-resistant plants were further tested by Southern analysis and 6 independent germinal excision events were identified offspring of these plants showed a mendelian segregation for the resistance marker.
In a third approach, experiments were performed trying to determine whether or not procedures could

be developed allowing a higher proportion of the somatic excisions/transpositions to be fixed in the germ-line. To this end F3-seeds of transgenic *Arabidopsis thaliana* lines carrying the above mentioned Tr-*Ac* NPT II construct were germinated under axenic conditions in the presence of kanamycin. Though large parts of the plantlets bleached and turned white, in 10-68% of the plants green sectors developed which organized into shoot-like structures and finally a plant which flowered and set seed. 85% of the fully resistant plants showed the expected excision band and 55% showed new bands indicative for reintegration of *Ac* thus proving that in this way somatic events could become fixed in the germ-line.

Title: Characterization of an *activator/dissociation (Ac/Ds)* transposable element system in *Arabidopsis thaliana*

Principal Investigator(s): R. Masterson, Max Planck Institut fur Zuchtungsforschung, 5000, Koln 30, Germany.

Participant No: 23

The main objectives were to demonstrate efficient transposition of *Ds* receptor elements suitable for mutagenesis, show the utility of methotrexate selection for the detection of transposed *DsDHFR* elements, and to begin assigning the location of single copy *DsDHFR* T-DNA insertions on the RFLP map. We have successfully demonstrated germinal transposition of our marked *Ds* element, *DSDHFR*, which contain a dihydrofolate reductase (DHFR) gene element by a CaMV promoter. The DHFR marker confers resistance to methotrexate and selection conditions have been established in order to transform *Arabidopsis* and to follow the *DsDHFR* element in progeny generations. Selection of seedlings on medium containing kanamycin has allowed the determination of plantlets in which *DsDHFR* elements have excised from the leader sequence of an NPTII gene. The over-expression of the *Ac* transposase gene, using CaMV-Ac fusions, has resulted in a minimal germinal transposition frequency of 27% in which independent *Ds* insertions were observed in progeny plants. High frequencies of somatic tranposition have been observed and independent germinal events were identified in F-2 generation progeny plants. Similar transposition rates have been determined using reciprocal *Ac/Ds* crosses. In addition, the CaMV-*Ac* fusions were able to activate *Ds* excisions in transgenic plants containing single or multiple *Ds* copies.

Title: T-DNA insertion mutagenesis
Principal Investigators: (C. Koncz, Max-Planck-Institut für Züchtungsforschung, D-5000 Köln 30, Carl-von Linné-Weg 10, Germany)
Participant No: 19

When T-DNA vectors carrying promoterless reporter genes (such as the neomycin phosphotransferase (*aph*(3')II), ß-glucuronidase (*gus*) and fused bacterial luciferase (*lux*F) genes) at their termini are used for transformation, T-DNA inserts in plant genes can simply be identified by screening or selection for the expression of reporter genes in diverse tissues of transgenic plants. This *gene fusion technique* combined with high frequency transformation facilitates the identification of gene mutations. In addition, transformation with gene fusion T-DNA vectors yields a great number of transcriptional plant gene-reporter gene fusions which permit the *in situ* analysis of regulation of plant gene expression *in vitro* or *in vivo*. By determination of the frequency of T-DNA induced gene fusions in diverse plant species it was observed that T-DNA frequently integrates into potentially transcribed chromosomal loci. On average over 30% of T-DNA inserts generate transcriptional gene fusions in *Arabidopsis*. Isolation of gene fusions from transgenic plants permits further studies of *cis*-acting promoter elements and transcriptional factors regulating the expression of T-DNA tagged

genes. To simplify the recovery of T-DNA inserts from the plant genome, T-DNA vectors used in *Arabidopsis* carry a bacterial selectable marker gene and a plasmid replicon. After physical mapping of insertion mutant loci by Southern DNA hybridisation, T-DNA inserts and flanking plant DNA sequences (i.e. promoter and coding regions of tagged plant genes) are rescued by transformation in *E. coli*. Before exploiting the T-DNA for large scale insertion mutagenesis in *Arabidopsis*, it was necessary to study the integration events in detail. Analysis of plant DNA target sites before, and the T-DNA-plant DNA functions after T-DNA integration revealed that T-DNA integration into the plant nuclear genome occurs via *illegitimate recombination*, a process aided by a T-DNA linked bacterial virulence protein VirD2. A viable yellow-pale mutant, designated as *cs*, was identified which was demonstrated to be allelic with a known lethal photosynthetic mutation, *ch*-42 located on chromosome 4. Molecular analysis of the *cs* insertional mutant demonstrated that a T-DNA insertion in the *ch*-42 locus did not result in lethality because the insertion has generated a gene fusion that encodes a partially active C-terminal fusion protein. The *ch*-42 locus encodes a novel chloroplast protein which has recently been shown to mediate the chelation of Mg^{2+}-ions into protoporphyrin IX, a key precursor of chlorophyll. To provide the growing *European Arabidopsis Community* with a collection of T-DNA tagged *Arabidopsis* lines, generation of 2500 individual transformants has been proposed within the frame of Bridge T project. This proposal has been fulfilled in 1991. These lines were derived from a collaborative effort with Prof. G.P. Rédei (Univ. Missouri-Columbia), who has recently added to the collection further ca. 300 transgenic lines. From the collection M_2 seeds from 480 families have already been provided for the *Arabidopsis* Seed Stock Center in Nottingham in 1991. Upon enrichment of available seed material, the remaining lines will be similarly delivered.

Genetic characterisation of the collection has been initiated during the last year.

Title: Site directed mutagenesis of endogenous genes in *Arabidopsis thaliana*

Principal Investigator(s): P. van den Elzen and S. Ohl, Mogen International NV, Einsteinweg 97, 2333 CB Leiden, The Netherlands.

Participant No: 35

1. Construct vectors for *Agrobacterium*-mediated gene replacement

2. Initiate transformation and selection for gene fusion

We decided to establish a targeting system for the gene encoding the large subunit of RNA polymerase II (AtrpII) for the following reasons:

- The gene encompasses 8 kbp and thus offers a large region of homology which is an important factor for targeting efficiency in animal systems. The low targeting frequencies obtained in plants so far (10^{-4}-10^{-5}) might be due to a limited length of homology.
- Gene targeting had been demonstrated in mouse ES cells based on a selection for resistance to amanitin. A small specific change in the highly conserved enzyme confers resistance to the toxin by reducing its binding affinity.
- Experimental evidence suggests that in order to introduce a small (1 nucleotide) change an additional mechanism (DNA repair of a transient heteroduplex) might further increase the frequency of gene targeting.

Genomic clones for AtrpII were provided by C. Koncz, MPI Cologne.

Initially two constructs were cloned, a control construct required to establish selection conditions and a targeting construct The amanitin resistance-conferring mutation was introduced in both constructs by recombinant PCR. The control construct contains the entire coding region and 3'end of the AtrpII gene fused to the CaMV 35s promoter, the targeting construct lacks the first 4 exons of the gene (1 kbp) and should give rise to a functional amanitin resistant enzyme only upon targeted integration into the homologous locus. Both constructs were cloned into a small vector for direct DNA transfer via PEG and into a binary vector for *Agrobacterium*-mediated transformation.
To determine selection conditions protoplasts were transformed with the control construct and regenerating calli selected on amanitin.
Selection on amanitin unexpectedly revealed that *Arabidopsis* protoplasts are at least a factor 100 less sensitive to the toxin than mouse ES cells. Since in vitro sensitivity of plant and animal RNA polymerase II is in the same range and spectrophotometric measurements indicate that amanitin is not rapidly degraded in the culture medium the difference is probably due to a slower uptake of the toxin in plant cells.Therefore a number of experiments were conducted trying to increase the uptake of amanitin.A number of parameters such as regeneration stage and pH of the medium, and permeability of the membranes were modified but amanitin sensitivity was not significantly increased under any of the tested conditions.
Current experiments are aimed at making use of the specific and rapid uptake of biotin and biotin-protein conjugates into plant cells.If this strategy turns out not to improve sensitivity of the calli sufficiently to make a selection feasible, an alternative approach will be to start screening pooled calli for recombinants via PCR. This approach will be more time consuming than a selection scheme and its success will depend on the targeting frequency which we expect to be higher than in previous studies.

Title: Site directed mutagenesis in *Arabidopsis thaliana* using *Agrobacterium* as a gene delivery system

Principal Investigator(s): P.J.J. Hooykaas and A.C. Vergunst, Department of Plant Molecular Biology, Clusius Laboratories, Wassenaarseweg 64, 2333, AL Leiden, THe Netherlands.

Participant No: 14

1. Construct *Agrobacterium* strains and host plants with defective hph-gene.

2. Start transformation work using protoplasts.

In Prof. Willmitzers' group, (participant no. 38) gene targeting was obtained in *Arabidopsis* hph-defective lines using a method of direct gene transfer. Since gene targeting in plants occurs at very low frequencies, a very efficient transformation system is necessary.
The main focus of the project has been to work out a protocol for the efficient transformation of *Arabidopsis* protoplasts via *Agrobacterium*. The first step was to establish a way to co-cultivate protoplasts and bacteria. In the established protocol for *Arabidopsis* regeneration protoplasts are embedded in an alginate matrix directly after isolation. It appeared that the dense alginate matrix makes passage of the bacteria and thus transformation of plant cells impossible.
Two alternative directions were followed:

1. Liquid culture of *Arabidopsis* protoplasts: Most convenient would be to culture protoplasts as well as co-cultivate with bacteria in liquid medium. Culturing of protoplasts in liquid medium however gives rise to partial cell wall formation and only very low cell division rates with as a consequence that the cells die within two weeks of culture. For division complete cell wall formation is necessary.
   It was suggested we use a different hormone regime (high kinetin), to obtain division in liquid medium. This worked, although the frequency of division was still low. Experiments will be done to optimize this.

2. Experiments have been initiated to test whether 2,6-dichlorobenzonitril (DB), a cellulose biosynthesis inhibitor, can be used to extend the time protoplasts survive in liquid culture. We found that regeneration from *Arabidopsis* protoplasts is not impaired after a 3 to 4 day treatment of protoplasts in liquid medium supplied with 2,6-DB, followed by embedding in alginate. Experiments are being performed to test whether 2,6-DB will be helpful in *Agrobacterium*-mediated transformation of *Arabidopsis* protoplasts.

Title: Transfer of homologous DNA-fragments into *Arabidopsis thaliana* using recombinant YAC-clones

Principal Investigator(s): P. Meyer, Max Delbruck Laboratorium, Carl von Linne Weg, 10, D-5000, Koln 30, Germany.

Participant No: 24

The goals of the first year was the construction of two types of vectors: a vector which could be transferred into *Arabidopsis* to establish a number of receptor plants for gene replacement because they carry a negative selectable marker and vectors based on YACs which contain positive selection markers for spheroplast/protoplast fusion experiments and which carry a negative selection marker to allow for selection against non-homologous recombination events. The second goal was the initiation of transformation experiments, the test of selection conditions for a negative marker gene and the initiation of fusion experiments between yeast spheroplasts and plant protoplasts.

A plant transformation vector p1'2'tk has been constructed for the establishment of transgenic *Arabidopsis* lines which serve as receptors for gene replacement experiments. The plasmid contains a positive selectable NPTII gene and the two negative selectable marker gene tms2 of Agrobacterium and TK of herpes simplex virus which are both driven by the bidirectional 1'2'promoter.Two YAC-based transformation vectors have been constructed. pYKHG (20kb) serves as a model vector for gene transfer from yeast into protoplasts. Based on pYAC4 it contains HPT. and NPTII-genes as selection markers and a unique EcoRI- cloning site. pYAC-G2 (14kb) serves as a vector for selection against non-homologous integration events as it carries a tms2 gene inserted into the SalI-site of pYAC45. The technique of DNA transfer into *Arabidopsis* via PEG mediated transfer was established in our lab after a training visit to the lab of Lothar Willmitzer. Primary tests of tms2-function as a negative selectable marker were conducted with a transgenic line R 4018 derived from Agrobacterium-mediated transformation of Landsberg erecta. Southern analysis revealed that R4018 contains two copies of a tms2 gene which are both located on a 12kb NotI-fragment and a 70-80 kb XbaI-fragment.
Functionality of tms2 as a negative marker was tested on medium containing 3uM Naphthaleneacetamide which should be converted into Naphthaleneacetic acid by tms2. While no obvious effect could be observed for wild-type plants R4018 showed an inhibition of root development in germinating seeds and the formation of adventitious roots on seedlings.

Direct transfer of p1'2'tk into C24 was initiated, resulting in numerous transgenic calli which still have to be regenerated and tested for tms2- and tk-activity. pYKHG was transferred into yeast as a linear vector. Pilot experiments for PEG-mediated fusion of YKHG containing yeast and *Arabidopsis* protoplasts were initiated.

Title: Gene Replacement

Principal Investigator(s): L. Willmitzer and P. Morris, Institut fur Genbiologische, 63, Ihnestrasse, Berlin, 1000, Germany.

Participant No: 38

1. Construct vectors and host plants with defective hpt gene.

2. Start transformation work using protoplasts.

In order to establish a system to monitor the success of gene targeting in *Arabidopsis thaliana*, a non-functional hygromycin phosphotransferase (*hpt*) gene was inserted into *Arabidopsis* to supply a chromosomally located target for homologous recombination experiments. A chimaeric gene consisting of the CaMV 35S promoter, the polyadenylation signal of the octopine synthase (*ocs*) gene, and the coding region of the bacterial *hpt* gene with a 19 bp deletion, including a PstI restriction site, was introduced by *Agrobacterium*-mediated transfer into *Arabidopsis*. The deletion resulted in a frame-shift and a premature stop in the translated region.
Protoplasts were prepared from the transgenic *Arabidopsis* lines and transformed by PEG-stimulated direct DNA uptake with DNA containing the promoter-less, intact coding region of the *hpt* gene, both in the single stranded and in the double stranded form. The expectation was that a recombination event between the defective chromosomal target and the intact targeting DNA would repair the chromosomal deletion and thus restore hygromycin resistance. In total 3.46×10^8 protoplasts were transformed and 150 hygromycin resistant calli were found. Since these could also arise from a fortuitous insertion of the intact *hpt* coding region next to an endogenous promoter. Of the 150 calli, 4 gave a positive signal by PCR analysis. That the target gene had indeed been repaired was confirmed by the fact that the PstI site in the deletion was now cleavable in amplified DNA from the corrected lines, whereas in the parental lines, this was not the case. A frequency of homologous recombination of 3×10^{-4} was observed, comparable to the frequencies observed in homologous

transformation studies on tobacco. In order to increase the recombination frequency in *Arabidopsis*, a strategy was adopted to increase the available length of homology above 1050bp.
To this end, one of the recombinant lines which by Southern analysis was known to contain a single repaired chromosomal target gene was chosen. This gene was known to lie within an 11kb genomic fragment. A genomic library was prepared from HindIII digested DNA and positive plaques obtained were further characterised. This clone is currently being sequenced to analyse the events which occurred during the repair of the *hpt* gene, and a deletion is being introduced at the 3'end to ensure non-functionality of the targeting gene.
This 11 kb sequence will then be retransformed into protoplasts of the original parental line, in parallel with the original *hpt* targeting sequence of 1050 bp. This will allow us to make a comparison and assessment of the influence of length of homology on the frequency of homologous recombination in *Arabidopsis thaliana*.

Title: Development and use of *Arabidopsis thaliana* as a tool for isolating genes of agronomic importance.

Pricnipal Investigator(s): B. Reiss, Abteilung Genetische Grundlagen der Pflanzenzuchtung, Max Planck Institut fur Zuchtungsforschung, Carl von Linne weg, 10, D-5000, Koln 30 (Vogelsang), Germany.

Participant No: 32

Stimulation of recombination by recA

1. Construction of expression vectors and plant transformation

The proposed objective of this research was to improve the frequency of gene targeting in *Arabidopsis* by the use of the recombination promoting enzyme recA. A dual strategy was proposed to reach this goal: 1(a) expression of recA enzyme under the control of plant expression signals and determination of its effect on the frequency of gene targeting and 1(b) 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.
1(a) RecA was clonedfrom an E. coli source and its coding region was fused with a nuclear targeting signal at the amino terminus of the recA protein in order to direct it to the nucleus. This was transferred into plants.
1(b) The formation of recA protein/DNA complexes under conditions for plant transformation was established. For this purpose, recA protein was purified and single stranded DNA, the complements of our target genes, were obtained from M13 vectors. To obtain nts-recA in sufficient quantity for biochemical characterisation and complex formation, the nts-recA gene was cloned into an E.coli expression vector. RecA protein complexes were formed with ssDNA and it was showen that these are stable under conditions used for plant transformation.

2. Establish expression levels and localisation of recA

Plants were tested for the presence of recA. However,in none of over 40 plants tested, a recA specific signal was detected. To investigate this lack of expression further, the integration events were analysed by Southern blots. Of the plants tested, only 3 posessed an intact recA transgene. Instead, most plants contained deleted or rearranged recA gene fragments.
recA protein/DNA complexes in a concentation optimal for recombination in vitro were formed and plant cells transformed. However, none of the protoplasts in a series of experiments survived selection and, moreover, around 80% of the protoplasts died without selection when comared to

control cells which had not been treated with recA. At present, strategies are developed to covercome this problem.

Title: Application of intra chromosomal recombination for *Arabidopsis* chromosomes mapping.

Principal Investigator(s): J. Paskowski and J. Masson, Institute of Plant Sciences, ETH Zentrum, Universitatstrasse 2, Zurich, 8092 Switzerland.

Participant No: 28

The aim of this project is to use homologous recombination mechanism between genomic repeats introduced in plant genome to generate deletion mutants in *Arabidopsis*.

It was shown that such recombination events do occur during mitosis and recombinant clones can be selected from appropriate transgenic plants derived protoplasts at a frequency of 10^{-4} in tobacco. The utilization of mitotic homologous recombination in *Arabidopsis thaliana* requires:

1. An efficient plant regeneration procedure from *Arabidopsis thaliana* protoplasts.

2. The evaluation of the appropriate markers necessary for the selection of transformants and recombinants.

3. The construction of recombination substrates and their introduction in *Arabidopsis* plants.

1. Improvement of plant regeneration efficiency from *Arabidopsis* protoplasts
Procedures previously established for plant regeneration from mesophyll II protoplasts of *Arabidopsis thaliana* have been optimized. The protoplast plating efficiency (number of microcalli per number of planted protoplasts) is strongly dependent on the growth conditions of the donor plant, especially medium composition and illumination conditions. The yield of microcalli is markedly influenced by the nitrogen source in the protoplast culture medium. The developed procedure gives reproducible plating efficiencies of 7-10% i.e. 15-20 times higher than previously published (0.5%) for *Arabidopsis thaliana* race C24. The time required for all procedure, from protoplast to shoots has been reduced from 108 to 50 days.

2. Evaluation of appropriate markers for the selection of protoplast derived colonies
The different coding sequences of the genes conferring resistance to Kanamycin (1), Hygromycin (2), Phosphinotrycin (3), Chlorsulfuron (4), Methotrexate (5), Stretomycin (6), Streptomycin (7) will be cloned in a pUC derivative plasmid under the control of 35S promoter and termination sequences. Plasmids 1, 2, 3 were existing, 4 has been cloned and 5 and 6 are under progress.

The establishment of precise dose-response for the different selectable markers on protoplast derived colonies, obtained with our optimized procedure, is under review.

1Title: Physical mapping of the *Arabidopsis thaliana* genome.

Principal Investigator(s): C. Dean, R. Schmidt and G. Cnops, Molecular Genetics Department, Cambridge Laboratory, John Innes Centre, Colney Lane, Norwich, Norfolk, NR4 7UJ.

Participant No: 9

1. Establish an international collaboration in YAC mapping;
2. Construct a YAC library with larger insert sizes;
3. Devise rapid screening methods and techniques to generate end-probes from the YAC clones eg. inverse PCR;
4. Hybridize RFLP probes mapping to the tops of chromosomes 4 and 5 to YAC libraries;
5. Contribute data to the international database;
6. Establish methods of subcloning YAC clones into vectors suitable for plant transformation.

An international collaboration has been established with the initial aim of hybridizing all the currently mapped RFLP markers to the YAC libraries. The next stage of the project will be to walk from each YAC clone until the one hybridizing to the next RFLP marker is reached. Different laboratories have agreed to focus on specific regions of the genome and the current division of the work is:
chr 1 Ecker (USA), Scolnik (USA); chr 2 Goodman (USA); chr 3 Goodman (USA); chr 4 Dean (UK), Somerville (USA); chr 5 Dean (UK), Somerville (USA).

Three different YAC libraries were obtained sonn after starting the programme. These libraries were each composed of 2300 clones with an average insert size of 150kb and each represented > 3 genome equivalents. We have also received an additional two libraries, one from Dr J Ecker and another from Dr C Somerville. Since the library provided by J. Ecker has significantly larger inserts than the other libraries, with an average size of 250kb, we chose not continue our efforts in constructing more YAC clones but focused on overlapping the YAC clones in the available libraries.

Based on protocols used in the *C. elegans* project obtained from Dr A. Coulson (MRC Cambridge) we have made the screening of the libraries as efficient as possible. We can now do a 25 offset plating which enables us to get one whole library on one plate and thus on one 11x8cm filter. This is many times more clones/filter than most robotic machines can achieve. Up to 30 replica copies of each master filter can be made and these are processed using streamlined methods.

All 33 RFLP markers mapping to the tops of chromosomes 4 and 5 have now been hybridized to at least two of the YAC libraries. All the positively hybridizing YAC colonies have been confirmed by Southern blot analysis. This effort, together with others in the International programme, resulted in approximately 30% of the *Arabidopsis* genome being represented in mapped YAC clones.

Linking of the YAC clones hybridizing to adjacent RFLP markers is being achieved by chromosome walking experiments. We have so far concentrated on a 10 cM chromosomal region on chromosome 4 lying between RFLP markers 210 and 226. Currently, three contiguous regions of 920, 820 and 420kb lie within this region, involving 59 YAC clones.
We are establishing Norwich as a node for the *Arabidopsis* database (AATDB) which is being

assembled in the laboratory of Dr Howard Goodman. This will carry all current information on the *Arabidopsis* physical map, current visible marker and RFLP maps, bibliography etc.

Title: Resource Centre

Principal Investigator(s): B. Mulligan, Botany Department, Nottingham University, University Park, Nottingham.

Participant No: 26

1. Set up sub contract with AIS

We have set up the subcontract with Professor Kranz (Frankfurt) for the transfer of the AIS seed collection to Nottingham. The form mutants have been received and re-characterized. The new information gained from this exercise will be incorporated into an updated seed list which will be sent to *Arabidopsis* T-Project participants. The colour mutants and biochemical mutants will be transferred to Nottingham shortly. The AIS database has been received.

2. Continue to collect and catalogue mutants

a) Collection

We have had a major publicity drive to inform people about the Stock Centre and to call for seed donations. As part of this initiative, seed lists etc have been sent to European agroindustries and participants in the T-Project "Regeneration".

(b) Cataloguing

We have characterized the AIS form mutants of Professor Kranz and are now awaiting the delivery of the next batches of mutants. We have also started to characterize several other donated sets of mutants. The catalogued information will, following discussion with the donors, be incorporated into *Arabidopsis* databases as described below. Much of the information will also be included in a seed list to be circulated to T-Project participants in July/August 1992.

(c) Distribution of seed samples

1991=791 seed lines distributed. This number includes the transfer of all our stocks to the *Arabidopsis* Resource Centre at Ohio, USA. Geographical breakdown: Europe 60 requests, 284 lines distributed; rest of the world 63 requests, 507 lines distributed.

1992 (to the end of February) = 215 lines distributed. Geographical breakdown: Europe 15 requests, 73 lines distributed; rest of the world 23 requests, 140 lines distributed.

In total, approximately 600 seed lists have now been distributed around the world.

3. Set up international database and distribution system.

Two American databases, which are independently funded, have been established for the development and administration of international *Arabidopsis* resources. Both databases will share the same information, but the emphasis placed on different pieces of information will be different. These developments mean that it would be redundant to establish a separate database at Nottingham. As a more efficient alternative, therefore, good working links between Nottingham and both American database projects have been set up and the appropriate communications systems (eg.E-mail) have been established. The AIMS database being developed in the US should become a master database for both Ohio and Nottingham. The second database is AATDB ("an *Arabidopsis thaliana* database"). At the moment AATDB is a stand alone system. This database already carries a copy of the Nottingham *Arabidopsis* Stock Centre Seed List, our subscribers list and scanned images of several of our mutant stocks. The first release will be in the Spring 1992. The program will be distributed free of charge and will be available via anonymous ftp from several computers.

Title: Resource Centre

Principal Investigator(s): J. Dangl, Max Delbruk Laboratorium, Carl von Linne weg 10, D-5000, Koln 30, Germany.

Participant No: 7

In the course of the last year, the DNA Resource Center has met several of its initial goals. We assembled, in the first year of the BRIDGE program, "cloning tools" of general use to members of the *Arabidopsis* research community. These tools fall into three categories: RFLP mapping markers (both phage and cosmid), YAC libraries, and phage libraries containing both genomic and cDNA inserts. We developed a useful routine for storage and distribution of these tools. Finally, we began distribution of cloning tools to researchers around the world. Our goals have also included communication with the newly established AIMS center at the Ohio State University. As a prelude to a larger *Arabidopsis* sequencing effort, we placed a very high emphasis on the collection of relevant cDNA and genomic *Arabidopsis* phage banks. Our efforts included a "call for deposition" of phage banks advertised in the AFRC newsletter and international meetings. Several deposits were made; they are listed below:

Genomic banks: From ecotype Col-0 (2 sources, in two different vectors)
From Ecotype Landsberg-*erecta*

cDNA banks: From mature, sterile seedlings
From aerial tissue (no roots)
From flowers

We have also amplified and made available the 90 random phage genomic clones which are the basis of the Meyerowitz laboratory RFLP map. Recently, Dr. Meyerowitz has made available 48 more phage and cosmid mapping probes, which have been added to the collection. In the last few weeks, we have made arrangements to receive the 96 cosmid clones which form the basis of the Goodman lab RFLP map. Two complementary YAC libraries, those most commonly used by the international *Arabidopsis* community, are also stocked and available from the DNA Resource Center. An *Arabidopsis* library in a T-DNA transfer vector "carried" in Agrobacterium has been made and will be deposited in the collection. Direct utilization of this library during chromosome walking may obviate, and will certainly complement, use of cosmid clones.

Title: Floral Induction in *Arabidopsis thaliana*

Principal Investigator(s): M. Koornneef and A.J.M. Peeters (EC Post-doc), Department of Genetics, Wageningen Agricultural University, 2 Dreijenlaan, 6703 HA, Wageningen, The Netherlands.

Participant No: 21

The initial goal of the project was:

1. The construction and physiological analysis of double mutants of the eleven late flowering genes identified in our laboratory (Koornneef et al. 1991).
2. The study of the interaction between phytochrome and flowering by the combined analysis of flowering time and phytochrome deficient mutants.
3. The genetical and physiological analysis of new flowering time loci. 4. The cloning of the *fwa* locus.

1. Late flowering double mutants:

For six combinations this process is already completed and it is expected that at the end of 1992 all possible double mutants are available for physiological analysis. This will give further insight in the epistatic relations of the various loci, thereby allowing a further elaboration of the tentative models for floral induction as presented by Martinez-Zapater and Somerville (1990) and Koornneef et al. 1991.

2. The interaction between phytochrome and floral initiation:

Mutants defective in phytochrome chromophore biosynthesis (*hy1* and *hy2*) and in the light stable PHYB (*hy3*) were found to be early, especially in short day conditions (Goto et al. 1991). Apparently phytochrome promotes inhibitors of floral induction under these conditions. The *hy2/hy3* double mutant, which is more reduced in phytochrome content flowers even earlier. Double mutants between representatives of the various phenotypic late flowering mutant group (*co,gi,fwa* and *fca3*) have been made and triple mutants (*hy2/hy3* with the various late flowering mutants) are being made. These genotypes will be analysed in daylength and light quality experiments (in collaboration with Dr Whitelam, Leicester, UK).

3. New flowering time genes:

Several mutants in genetic backgrounds different than Landsberg erecta (Ler) have been obtained. These included previously described mutants such as Rédei's *ld*, McKelvie's *F, f2* and *f5*. Their often extreme lateness and the dominant nature of several of these mutants suggested that some of these represent new loci. Thus far 3 and possibly 4 loci in addition to the 11 loci have been identified of which 2 have been mapped on the top arm of chromosome 5, where some 4 late flowering genes are located at a relatively short distance.

4. The cloning of *fwa*.

Mutants at the *fwa* locus are dominant and are relatively insensitive to vernalization but responsive to daylength. This gene was mapped at position 51 on chromosome 4 in the vicinity of the *abi1* locus (Koornneef et al. 1991). Genomic subtraction was applied to an irradiated induced mutant allele and has yielded some fragments which are being tested for their genetic relation with the locus. In addition a map based cloning approach was started. YAC's have been identified by Jeff Leung in Giraudat's group (walking to *abi1*), which seems to cover the *fwa* region.

Title: MOLECULAR GENETIC ANALYSIS OF THE VERNALIZATION REQUIREMENT IN *ARABIDOPSIS THALIANA*

Principal Investigator(s): C. Dean and L. Westphal, Molecular Genetics Department, Cambridge Laboratory, John Innes Centre, Colney Lane, Norwich, Norfolk, NR4 7UJ, UK.

Participant No: 10

1. Initiate high-resolution mapping around *fca*;

2. Initiate YAC walking to *fca* from adjacent RFLP markers.

Our first step in walking to the *fca* gene was to map the locus with respect to the RFLP markers. The *fca* mutant, which is in the Landsberg *erecta* background, was crossed with each of the polymorphic parents, Columbia and Niederzenz.These experiments enabled us to map the *fca* locus between the two RFLP markers LEM 580 and 226 (approximately 1.2cM from the RFLP marker LEM 226). These RFLP markers were hybridized to the different YAC libraries. End-probes from the different YAC clones were then generated using inverse PCR or plasmid rescue and were hybridized back to the YAC libraries. In this way, YAC contigs were built up on either side of the *fca* locus. The recombination break points on either side of *fca* were used to direct these chromosome walks. us. Many plants are being screened for recombination break-points lying very close to the *fca* locus using the end-probes isolated from the YAC clones closest to the *fca* locus. We should soon obtain recombinants every 0.1cM with this number of plants allowing the fine mapping of the *fca* gene to within approximately 14kb. The chromosome walk over the *fca* locus, joining the two contigs has proved to be difficult due to the presence of repeated DNA sequences flanking the locus. This gap is likely to be a small physical distance however, based on the genetic distance.

Title: Molecular characterization of the APETALA2 gene

Principal Investigator(s): M. van Montagu, Rijksuniversiteit Gent, Laboratorium Genetika, Ledeganckstraat 35, Gent, B-9000, Belgium.

Participant No: 37

1. Obtain sequence of the *APETALA2* from T-DNA-tagged gene

2. Start to examine the role of the encoded protein

The *APETALA2* gene has been isolated. To isolate the T-DNA and flanking AP2 plant sequences we generated a genomic library from the tagged Ap2 mutant. A T-DNA-containing region was isolated. Sequence analysis of the flanking plant sequences revealed an open reading frame. These flanking sequences were subsequently used to screen a flower-specific cDNA library as well as two genomic libraries. One of Ap2-1, a weak *ap2* allele and one genomic library of wild-type Landsberg erecta.

Several overlapping wild-type *AP2* gene regions were used to rescue two alleles, *ap2-1* and our tagged mutant. These experiments allowed us to confine the *AP2* gene to a 7.2-kb gene region.

The wild-type *AP2* gene, the *ap2-1* allele and several *AP2* cDNA clones have all been sequenced. Based on the sequence data we can say the AP2 protein has a molecular mass of 47 kDa. There is a repeated motif of two amphipatic helices present within the AP2 protein. Comparison of the *AP2* sequence with that of other proteins did not reveal any significant homology to known proteins.

The repeated motif seems to be present in a family of related genes in Arabidopsis as well as in petunia and tomato (results based on Southern hybridization data). In fact, we have isolated cross-hybridizing clones from a flower-specific cDNA library from petunia. Preliminary sequence data indicate that these clones contain at least one and possibly both amphipatic helices that are present in the AP2 protein.

Title: Isolation of the *co* (*fg*) locus and identification of other mutations affecting flowering- time in *Arabidopsis*

Principal Investigator(s): G. Coupland, J. Putterill, F. Robson, K. Ingle and S. Dash, Molecular Genetics Department, Cambridge Laboratory, John Innes Centre, Colney Lane, Norwich, Norfolk, NR4 7UJ, UK.

Participant No: 5

1. Initiate YAC walking to *fg* (*co*) using RFLP probes

2. Isolate recombinants around *fg*(*co*)

We are interested in the *co* mutation since this dramatically delays flowering of *Arabidopsis* under long days. *co* is located on the upper arm of chromosome 5 and is 2cm proximal to tt4. The latter mutation prevents the synthesis of anthocyanin and the gene encodes the enzyme chalcone synthase. The average physical distance between thse loci is likely to be about 300kb.
We started a walk to this locus by hybridising the 4 RFLP markers which are closely linked (within ca. 2cM) to chalcone synthase to the EG and EW YAC libraries. This allowed us to deduce how the YACs lay in relation to each other. In most cases this arrangement was later confirmed by the isolation of inverse polymerase chain reaction generated fragments which are located at the ends of the *Arabidopsis* DNA inserted within the YAC, and hybridisation of these to the appropriate YACs. The short contigs around the RFLP markers were then extended. Two sets of overlapping cosmid clones from this area were obtained from others and the appropriate ones were probed against the YAC libraries. This identified two new YACs. End probes derived from most of the 20 YACs we had identified were then used to screen the libraries and new YACs extending the cloned region in both directions were isolated. It has allowed us to assemble one contiguous segment of *Arabidopsis* DNA which includes RFLP markers 6833, CHS, pCIT1243 and 5962 and is approximately 1550kb long.
The location of *co* within the contig was determined by detailed RFLP analysis after the isolation of recombinants containing cross-overs very closely linked to *co*.

Title: Floral induction: Identification and characterization of genes evolved in floral induction.

Principal Investigator(s): J.M. Martinez-Zapater, Dpto. de Proteccion Vegetal, CIT-INIA, Ctra. de la Coruna, Km 7, 28040, Madrid, Spain.

Participant No: 22

1. Initiate YAC walking to fve from adjacent RFLP markers.

2. Initiate high resolution mapping around fve.

Information provided by the International Mapping effort allowed us to find appropriate markers and recombinant YACs to begin our walk to fve. The right ends of YACs EW6A7, EW15H11, and EW20F11 have been amplified using IPCR and the left ends are currently under cloning by plasmid rescue in the laboratory of George Coupland. These ends will be used to detect polymorphisms around the fve locus and to map them and orientate the YACs.

Progress towards the second objective is currently more advanced. A Landsberg fve homozygous line marked with the cp2 mutation and other mutations on chromosome 2 was obtained. This line was crossed with Niedersenz and Columbia ecotypes and recombinants between the two loci, fve and cp2, were isolated in the F2. Sixty three recombinants have been isolated in this short region of 6cM that will allow the construction of a 0.1 cM resolution map at this side of fve. These DNA preparations are currently being used for Southern hybridization and a high resolution RFLP map at this side of fve will be ready soon. For mapping the other side of fve, we have constructed a line homozygous for fve and sti, a mutation that produce non-branched trichomes. We think this collection of recombinants around the fve locus will be invaluable in the following steps of chromosome walking. Finally, a physiological and morphological characterization of the fve alleles has been performed. The purpose of this analysis was to get more information about the function of the locus and to identify the best conditions to perform the complementation of the mutant phenotype. From this study we have concluded that mutations at the fve locus produce not only a delay in the transition from the vegetative to the reproductive phase, but also affect elongation of the internodes in the floral stem.

Title: Expression of aba-response genes in *Arabidopsis* seeds

Principal Investigator(s): M. Pages, CSIC, Departamento de Genetica Molecular, Jorge Girona, Salgado, 18-16, 08034, Barcelona, Spain.

Participant No: 27

1. Screen libraries with maize clones known to be ABA-induced.

2. Initiate constructions of hybrid promoter reporter genes

3. Initiate studies of ABA-regulated genes in mutant lines

Developmental regulation of the ABA-responsive *rab*-17 promoter in *Arabidopsis thaliana* and ABA deficient and insensitive mutants.

To elucidate the mechanisms by which abscisic acid (ABA) regulates specific expression of the *rab* 17 gene we have obtained transgenic plants of *Arabidopsis thaliana* (L.) ecotype Landsberg *erecta* and ABA-deficient (abi) and ABA-insensitive (aba) mutants derived from it, carrying a large 5' upstream fragment of *rab* 17 (-1330/+29) and the reporter gene GUS.
The plasmid containing the *rab* 17 /GUS gene fusion was transferred to *Arabidopsis thaliana* by root transformation. Lines homozygous for the T-DNA insertion were initiated from T2 plants that gave 100% of kanamycin resistant seedlings after selfing. Six independent insertions were fixed in this way. The integration of the *rab*17/Gus construct was confirmed by Southern analysis.
The effect of ABA-insensitivity or deficiency on the activity of the *rab*17 promoter was analyzed by constructing lines homozygous for aba and abi mutations and carrying the T-DNA insertions.
The genotype of these plants was confirmed by analyzing the phenotypes of the F3 derived plants.
To investigate developmentally regulated expression of the *rab*17/Gus chimeric gene, Gus activity was measured during embryo development in wild type and mutants. Localization of Gus activity was revealed by histochemical staining. Developing embryos stained blue when they were dissected around 15 d.a.f. and between 18 and 25 d.a.f. a rapid accumulation of Gus activity occurred reaching a maximum in wild type dry embryos. This profile mimics the normal time course of *rab*17 mRNA accumulation in the developing maize embryo. Analysis of Gus expression in seed sections of both transgenic wild type and mutants revealed that the highest level of Gus activity was in the endosperm. This result was unexpected because of the absence of expression of *rab*17 mRNA in maize endosperm tissues. It has been reported that various ABA-induced responses are decreased or eliminated in *Arabidopsis* mutants carrying ABA-deficiency and insensitivity mutations. However, the induction of the 1.3kb *rab*17 promoter fragment in transgenic wild type and mutants has been shown to display the same kinetics. These results were expected and correlate well with the observed accumulation of the *rab*17 mRNA of the endogenous maize gene during embryo development in the maize *viviparous* mutants where only lower levels of expression were detected in the mutants. Work is in progress to elucidate the promoter sequences involved in the induction of *rab* genes by ABA in embryo and endosperm both in wild type and mutant plants.

Title: Seed development and Abscisic Acid

Principal Investigator:(s) M. Koornneef, K.M. Léon-Kloosterziel (PhD student) and E.P. van Loenen-Martinet (part-time technician), Department of Genetics, Wageningen Agricultural University, 2 Dreijenlaan, 6703 HA, Wageningen, The Netherlands.

Participant No: 20

The goal of the project is the isolation and characterization of mutants affecting abscisic acid (ABA) metabolism and seed development.

Dormancy mutants:
Mutants without dormancy were found by isolating seeds, which germinated within 1 -3 days after incubation on water. Freshly harvested M2 derived from mutagen (EMS or gamma irradiation) treated *abi3* or wild type Landsberg erecta seeds. This screen yielded approximately 20 mutants that showed this phenotype upon rescreening in the M3 generation. The most dramatic phenotype was represented by a mutant that in addition to a lack of dormancy, a green colour of mature seeds, extreme insensitivity to ABA and a reduced resistance to desiccation. Genetic analysis indicated that this mutant was an allele of the *abi3* locus, which recently has been cloned by Giraudat. This result indicates that the previously isolated alleles of *abi3* were "leaky" and that this gene is probably the most crucial gene that regulates seed development. This mutant and 3 additional new less extreme alleles were sent to Dr. Giraudat for further analysis at the DNA level. The physiological analysis of this extreme *abi3* allele with emphasis on the relation between desiccation tolerance and sugar metabolism is being performed in collaboration with the group of Dr Karssen.
Among the non-dormant mutants 5 are characterized by excessive wilting indicating a lack or non functioning of ABA synthesis or action. Genetic analysis of these mutants is in progress.

Abscisic acid insensitive mutants.
Mutants have been isolated from and *abi3-1* M2 population , which are resistant to 100 μM ABA. These mutants do not show symptoms of wilting and genetic analysis is in progress.

A seed shape mutant.
A mutant has been isolated with a heart shaped seed instead of the oval shape of a wild type seed. Reciprocal crosses and the analysis of segregating generations indicated that this aberrant seed shape is determined by the maternal genotype. This indicates the importance of the testa the determination of the seed shape, despite the fact that the embryo makes up most of the seed volume in the mature seed.

The characterization of the *mat* mutant (in collaboration with Valvekens)
The *mat* mutant has been isolated by Dr. Valvekens in the van Montagu lab in Gent and is characterized by a premature collapse of growth and development including seed development. We have mapped this gene on the lower arm of chromosome 1 and will analyse in detail seed development in this mutant to understand the lack of dormancy observed when this mutant grows in vitro.

The expression of seed specific and ABA inducible genes in ABA related mutants.
We obtained from Dr. Pages (Barcelona) an *Arabidopsis* genotype containing the GUS gene under control of the RAB17 promoter and from Dr Delseny (Perpignan) a genotype with GUS under control of the EM2 promoter. We are combining these GUS constructs with the various *aba* and *abi* mutants by crossing to obtain isogenic genotypes, which can be studied for the expression of these genes during seed development and after ABA and stress application.

Title: Acquisition of desiccation tolerance in developing seeds of *Arabidopsis thaliana*.

Principal Investigator(s): C.M. Karssen, Department of Plant Physiology, Agricultural University, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands.

Participant number: 16

Assist in mutant screening affect seed development and desiccation tolerance.

Results:
Desiccation intolerance is thought to be due to insufficient protection of membranes, resulting in serious leakage of intracellular constituents upon seed imbibition. Three mechanisms have been proposed by which leakage can be avoided; 1) protection of membranes by sugars, preferentially by di- or trisaccharides, 2) changes in composition of phospholipids and/or chain length and saturation level of acyl chains, 3) protection by specific, possibly ABA induced, proteins. Thus far, all three possible mechanisms have received attention in our group.
A) Sugar composition. Sugars were determined in wild-type and double-mutant seeds throughout development. The following differences were found in sugar composition: 1) Only the wild-type seeds accumulate stachyose. 2) Glucose, fructose and sucrose accumulate in higher amounts in the double-mutant seeds than in wild-type. However these differences do not seem to be a major factor in desiccation tolerance as it was found that: 1) Developing wildtype seeds are already fully desiccation tolerant at 13-14 days after pollination (dap), whereas stachyose becomes only detectable after 16 dap. 2) The tri-saccharide raffinose is synthesised in relatively small amounts during seed development, but decreases at the later stage of seed ripening in both wildtype and double-mutant seeds. 3) The amount of sucrose in the desiccation intolerant double mutant seeds is about 2-3 times higher than the amount found in the wildtype seeds. 4) Double mutant plants treated with LAB 173 711 (an ABA analog) develop phenotypically as wild type plants do and form desiccation tolerant seeds. However, no changes in sugar composition are evident as a result of treatment within LAB 173 711.
B) Lipid analysis. Three major differences were found between wild-type and double mutant seed phospholipids: 1) During development, in wild-type seeds the relative amount of 18:1 increases, whereas this does not occur in double mutant seeds. Moreover, the absolute amount of 18:1 is lower than in the wildtype seeds. 2) Wildtype seeds contain 20:1 which is absent in the double mutant seeds. 3) The green double mutant seeds contain far more 18:3 than wild-type seeds..This might be due to the fact that the double mutant seeds contain a lot of MGDG and DGDG which are important constituents of the chloroplast membranes.
C) Protein analysis. Differences have been found earlier (C. Meurs et al. Plant Physiol. (1992), in press), the significance of which will be further explored in a separate line of research.
D) These preliminary data form an interesting starting point for further investigations in the relationship between desiccation tolerance and the membrane protecting characteristics of the above mentioned factors.

Title: Isolation and characterization of *Arabidopsis* genes involved in abscisic acid actions

Principal Investigator(s): J. Giraudat, Institut des Sciences Végétales, CNRS, 91198 Gif sur Yvette, France.

Participant No: 12

Koornneef et al. have identified three distinct abscisic acid-insensitive mutants in *Arabidopsis thaliana* (abi1, abi2, abi3) that can germinate in the presence of toxic levels of ABA.We have undertaken to clone the abi1 and abi3 loci which, on the basis of the mutant phenotypes, are likely to encode elements of two partially distinct ABA-signal transduction pathways. The strategy used is that of chromosome walking, by taking advantage of the available RFLP maps, physical maps and YAC libraries of the *Arabidopsis* genome. The **abi1** locus had been previously mapped to position 44.7 on chromosome 4. We have constructed a high resolution RFLP map in the vicinity of this locus. This was accomplished by crossing Landsberg mutants bearing both the abi1 and flanking visual markers (cer4 or cer2) to wild-type polymorphic Columbia strain. By such an approach, we have determined that the closest RFLP marker (in the interval between cer4 and abi1) maps approximately 2.5 centimorgans from the target gene, separated by six recombinant break points. Since no existing

contigs were available in this area, we have used this RFLP as a starting probe to initiate a walk using available YAC banks. In successive steps, we have so far isolated a total of 13 overlapping clones extending towards abi1. Conservative estimates suggest that this set of YACs spans approximately 500 kb of contiguous sequences in the region. The ends of the new YAC clones were used as probes for RFLP mapping; this revealed that three of the six recombinant break points have been crossed. In terms of genetic distance, this is translated roughly into 1.25 centimorgans walked.
The **abi3** locus had been previously mapped to position 23.5 on chromosome. By a strategy similar to that described above for abi1, we have constructed a high resolution RFLP map in the vicinity of the abi3 locus, using the hy2 and gl1 flanking visual markers. This initially allowed us to identify an RFLP site located at approximately 0.25 cM from the abi3-1 mutation. This RFLP probe belonged to a contig of overlapping cosmids. Further RFLP analysis, using the various members of this contig, revealed that the original marker actually detected several polymorphic sites located on both sides of the abi3-1 mutation. We then established that these flanking polymorphic sites were located within the insert of the cosmid, which was thus likely to contain the abi3 gene. Overlapping restriction fragments of this wild-type genomic clone were tested for their ability to complement the abi3-1 mutation, that is to restore wild-type sensitivity to exogenous ABA at the germination stage. An 11 kb fragment did achieve functional complementation. This fragment contains at least one gene which is expressed in green siliques, as expected for abi3 since all available mutations at this locus affect various aspects of seed development. We are presently analyzing various abi3 mutant alleles induced by γ-radiation in order to firmly establish the identity of the abi3 gene within the complementing fragment.
When subjected to progressive **drought**, *Arabidopsis* plants develop over weeks an adaptive response which includes new root morphogenesis. This characteristic new root system (short tuberized roots) is able to survive in a desiccated soil and to rapidly resume growth upon rehydration. Bidimensional gel electrophoresis has revealed numerous proteins the expression of which is modulated during this adaptive process. We are attempting to clone the corresponding genes, initially by using a differential (watered versus drought adapted) screening approach on cDNA libraries. The *Arabidopsis* Columbia ecotype has been selected as displaying a strong tolerance to desiccation and a well developed root system. Root systems from over 100 drought-adapted and 50 control watered plants (grown individually) have been harvested at a stage (7 weeks) when drought rhizogenesis is still a dynamic process. Poly(A) RNA has been purified from roots and is presently being used for constructing the cDNA libraries.

Title: Research area Seed Development

Principal Investigator(s): M. Delseny, University of Perpignan, Plant Physiology and Molecular Biology, URA 565 CNRS, Avenue de Villeneuve, 66860, Perignan-cedex, France.

Participant No: 11

1. Isolate genes induced by aba in late seed development.

2. Characterise expression in mutant lines made by participant 20 and 16

1. The aim of our programme was to isolate new genes from *Arabidopsis* ≥, regulated by ABA and expressed during seed maturation using probes previously isolated in our group from a dry seed radish cDNA library.

During this year, we have concentrated our efforts on Em-like genes. Two genes have been isolated from an *Arabidopsis* genomic library and completely sequenced on ca 3500 bp. The two genes do not seem to be closely linked. Sequence of the two genes revealed that one of them is similar to the classical Em described in wheat and cotton, but that the second encodes a much longer protein

because a motif of 20 amino-acids is repeated 4 times instead of being present once. We have established that the two genes are expressed by using genes specific probes and by isolating 14 cDNA clones which have been sequenced. Initiation site for transcription of both genes has been determined. Sequencing of the cDNA revealed that polyadenylation was not precise since 3 or 4 sites were found for each gene. Northern blot experiments established that the two genes were not expressed exactly at the same time.
In situ hybridization experiments were carried out with one of the two genes, in collaboration with Montserrat PAGES (BARCELONA) and they revealed preferential expression of gene 1 in the provascular tissues.
Fusion of the full length promoter with the GUS reporter gene have been made, as well as fusions with serial deletions. Constructions have been transferred to transgenic tobacco and *Arabidopsis* and in both case embryo specific expression has been observed. Seeds from GUS-expressing *Arabidopsis* have been forwarded to Marteen KOORNNEEF so that the construction be transferred in aba, abi 3 and aba/abi3 background .

We have also used 2 other radish probes to select *Arabidopsis* genes for a RAB 17 homologue (dehydrin)and for a new gene coding for a protein with a cystein rich motif in the C-terminus (4F11). Two different genomic clones have been purified for RAB 17 like, and a single 4F11 gene.

Title: Gene expression during embryogenesis studied by *in situ* hybridisation

Principal Investigator(s): F. T. Okkels, K. Henriksen and J.E. Nielsen, DANISCO A/S, Copenhagen, Denmark

Participant No: 45

Goal: Development of an in situ hybridization (ISH) method for detecting GUS mRNA in GUS transgenic *Arabidopsis* embryos.

Work has been carried out with biotin labelled 24 bases pair oligo-nucleotides against a number of mRNA's in paraffin sections of various fixations of various plant tissues and *Arabidopsis* embryos. Although we have obtained good results with some of these probes it seems that the GUS mRNA is just at the detection level of the used methods.
A biotinylated poly-T oligo was used to detect the poly-A tails of the mRNA's available for ISH in the sections. Poly-T ISH hybridization together with Acridin Orange proved to be good tools for evaluation of fixations, proteinase treatment and other variations in the ISH procedures used. The Poly-T ISH results show a very strong reaction to the nuclei and a less strong reaction in the cytoplasm of all types of plant tissues used.
To be able to optimize the ISH procedure further we have tested three different oligo probes against the GUS mRNA in leaves of double-haploid beets (with at least 2 copies of the introduced GUS genes from E. coli) and analogue material from non-transgenic leaves as control. Promising results were achieved with reactions in GUS-positive leaves a little bit over the background reactions seen in the GUS-negative leaves, but no clear positive results were obtained when increased stringency in hybridization and washing steps were introduced. Each and every step in the ISH procedure has been tested for its possible contribution to the background reactions. No single step had any effect on the background level. Therefore we must conclude that GUS mRNA was at the detection level of the used methods. The best oligo probe against GUS mRNA was hybridized to a hybond nylon filter with GUS transgenic beet DNA, a good hybridization was found, only one band in each lane.
Therefore we have turned to cloned ribo probes, biotin, digoxigenin and ^{35}S-labelled, transcripts of the whole GUS mRNA. These probes were then hydrolyzed to pieces of approx. 100-250 bp. We have tested a number of ISH methods and variations of these without any clear positive results on sectioned material. But with the digoxigenin labelled probe against GUS mRNA we have a clear reaction above background on small pieces of double transgenic leaves.

Title: Molecular Identification of New Plant Genes: Insertional Mutagenesis to Isolate Genes Controlling Embryogenesis in *Arabidopsis*.

Principal Investigator(s): K. Lindsey, University of Leicester, Leicester, UK

Participant No: 43

1. To construct a T-DNA vector for generating GUS fusions;

2. To generate multiple transformants of *Arabidopsis*;

3. To initiate GUS assays in siliques of T1 (F0) plants, to identify GUS expression in early embryos.

1. T-DNA vector construction
Work to construct a T-DNA vector which could be used to create fusions between a promoterless *gus*A gene and native gene promoters, following random T-DNA insertion in populations of *Arabidopsis* plants, was initiated previously. The vector comprises a promoterless *gus*A coding region

plus *nos* termination sequence, inserted into the multiple cloning site of the Bin19 binary transformation vector, for *Agrobacterium tumefaciens*-mediated gene transfer. The *gus*A coding region has its own translation initiation ATG codon, which is oriented with the 5' end adjacent to the T-DNA left border. The *npt*-II selectable marker of Bin19 allows selection for transformants on kanamycin. Since the sequence between the *gus*A ATG and the left border contains translation stop codons in all reading frames, this construct is expected to generate primarily transcriptional fusions with native gene promoters, but translational fusions may be possible if left border sequences become deleted during integration. This vector, pΔgusBin19, was provided to the participants in Perpignan, for the beginning of the project.

2. Transformation of *Arabidopsis*

Our early attempts to transform *Arabidopsis* by *Agrobacterium tumefaciens* used the root explant transformation system as described by Valvekens *et al.* (1988: *PNAS* 85, 5536). By following this procedure, we obtained transformants at a relatively low frequency, and have developed a modified protocol which improves the efficiency of recovery of transformants by between 10-100-fold.

3. Screening of transformants

The following screening protocol was established. Primary transformants (T1 plants) are assayed for activation of the promoterless *gus*A gene by fluorimetric GUS enzyme assays in extracts of whole siliques. Of the GUS-positive lines, T2 seed is bulked up and both quantitative GUS assays and histochemical GUS localizations are carried out in developing T3 embryos. T-DNA copy numbers are determined by genetic segregation analysis and Southern blot/hybridization, using T-DNA sequences as probes. The segregation of mutant phenotypes is looked for in the T2 and T3 progeny. To date, 423 independent primary transformants have been screened for GUS enzyme activity in siliques. Of these, 74 (17.5%) were GUS-positive. Preliminary studies of GUS expression in the T2 plants have revealed diverse spatial and temporal patterns. Of 30 T2 lines which exhibited GUS-positive siliques and which have been studied to date, 11 have been found to express in the immature seed. One line, for example, shows strong expression in the root apical region of the T3 embryo, and a developmental study is in progress to determine the timing of initiation of GUS activation.

Preliminary work has also been carried out to identify putative T-DNA insertional mutations that affect embryogenesis or seed development. We have identified putative mutants which fall into three classes: embryonic lethals, which fail to complete embryogenesis; altered pigmentation, including albinos and a 'transparent testa' line; and seedling defectives, which may affect tissue pattern formation. We currently are carrying out some genetic studies to establish co-segregation of aberrant phenotype and T-DNA.

Title: T-DNA insertional mutagenesis to isolate genes regulating embryogenesis in *Arabidopsis thaliana*

Principal Investigator(s): P. Gallois and M. Devic, Laboratoire de physiologie et biologie moleculaire (CNRS URA 565), Université de Perpignan, Perpignan, France.

Participant No: 44

1. Assist in vector construction at Leicester

2. Make multiple transformants together with participant 43.

3. Initiate screen of transformants for late embryo expression of GUS

The aim of the project is to tag and isolate genes expressed during embryogenesis in plants by promoter trapping and/or T-DNA insertional mutagenesis. The strategy consists of introducing a T-DNA containing a promoterless GUS reporter gene into a population of *Arabidopsis* plants (C24). The insertion of the T-DNA may lead to expression of the GUS gene by transcriptional fusion and/or to

gene inactivation.

1. During the course of the first year 750 transformants were regenerated. Initial transformants were kept *in vitro* until they set seeds. With the number of transformants increasing, a protocol was developed which allowed the transfer into soil of the *in vitro* plantlets as soon as they started to root.
2. A first screening was carried out to assess the validity of the promoter trapping approach. 100 transformants were assayed by fluorometry for GUS activity in 5 different organs: root, developed cotyledon, leaf, flower and seed. Seeds, (the primary interest for the present bridge contract), were screened at 4 stages: from fertilisation to torpedo stage, from torpedo stage to late cotyledon stage, from late cotyledon stage to the end of maturation and dry seeds. Whole siliques containing the seeds were analysed. 53% of the transformants expressed GUS in at least one organ and all organs tested expressed GUS in at least one transformant. More importantly, 51% of the plants expressing GUS did so in silique+seeds including 14% which showed GUS activity specifically in silique+seeds and not in others organs. These plants are currently being tested in a second round of screening at the T2 generation in order to establish the stability of T-DNA integration. These results demonstrate the validity of the promoter trapping strategy to identify temporal and tissue specific genes expressed during embryogenesis in *Arabidopsis*.

More transformants are now being screened for GUS expression in silique+seed.

Mutant phenotypes (albinos, dwarfs) have been observed during the progeny analysis of the transformants. Co-segregation in the progeny of the T-DNA insertions and the mutations is being studied.

A screening for embryo lethal mutants has been initiated and is expected to be completed during the course of second year.

T-PROJECT

LIPASES

TITLE: CHARACTERISTICS OF LIPASES FOR INDUSTRIAL APPLICATIONS THREE DIMENSIONAL STRUCTURE AND CATALYTIC MECHANISM

CONTRACT NUMBERS: BIOT 0274, 0180, 0258, 0194 and 0272. See relevant reports and names of participants on pages 410, 414, 417, 421, 424.

OFFICIAL STARTING DATE: 1st March 1991

COORDINATOR: R. Verger, C.N.R.S., Marseille (F)

A general overview

Objectives set for the reporting period

Lipases (glycerol ester hydrolases EC 3.1.1.3.) are enzymes that catalyze the hydrolysis of ester bonds of long chain glycerides. First of all, one of the most intriguing and unique features of lipases is the way in which they are "activated" by interfaces. This phenomenon was reported very early. The fact that it is the substrate that forms an insoluble lipid-water interface makes lipolysis a very attractive system for studies on interfacial enzyme kinetics. One molecule of enzyme can catalyze the release of up to 9 10^5 molecules of fatty acid per minute under optimal conditions; this value is one of the highest turnover numbers to have been measured in catalyzed biochemical reactions. Generally speaking, lipases are assumed to be suitable models for complex membrane-bound enzymes which are more difficult to investigate experimentally. No specific and definite molecular mechanism has been proposed to account for this interfacial behaviour.

On the other hand lipases are becoming of increasing importance for European Biotechnology. Lipases are a class of enzymes with high commercial potential, but so far only few industrial applications have been developed. In order to optimize lipase catalyzed reactions, it is essential to have access to the 3-dimensional structure of the protein itself as well as controlling the physico-chemical parameters of the substrate.

The aim of the project is the structural and functional characterisation of the 10-15 lipases in order to establish the basic understanding of their catalytic functions. New knowledge on a number of these enzymes will make it possible to understand why they are lipases and how they functions as such.

1. Triacylglycerol lipases and colipases of the digestive tract and exocellular fungal lipases: structure, interfacial binding and catalysis. Coordinator: R. Verger, CNRS, Marseille, FR. BIOT-CT91-0274 (DTEE), starting from 1/03/91.
2. Three-dimensional Structure and catalytic mechanism of 2-3 selected lipases of industrial relevance. Coordinator: L. Thim, Novo-Nordisk, Copenhagen, DK. BIOT-CT90-0181 (SMA) starting from 1/02/91.
3. Molecular structure and specificity relationship of microbial triacylglycerol lipases. Coordinator: R.D. Schmid, GBF, Braunschweig, DE. BIOT-CT91-0258 (LNBE) starting from 1/06/91.
4. Characterization of lipases from procaryotic origin. Coordinator: M. Egmond, Unilever Research Laboratorium, Vlaardingen, NL. BIOT-CT90-0194 (EDB) starting from 1/04/91.
5. Structure-function relationship of *Pseudomonas* and *Bacillus* lipases. Coordinator: O. Misset, Royal Gist Brocades, Delft, NL. BIOT-CT 91-0272, starting from 1/08/91.

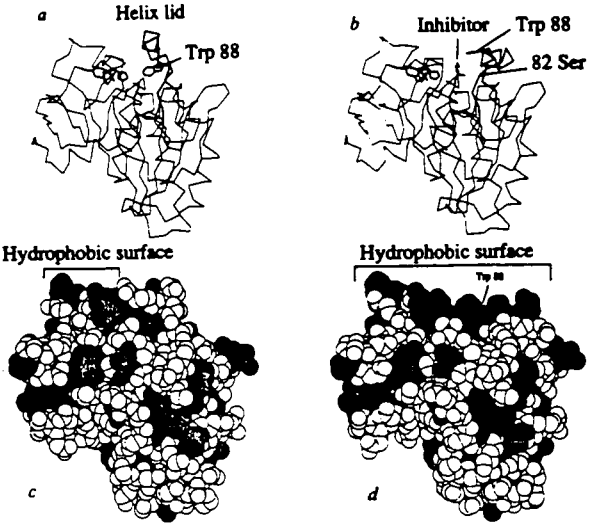
MAJOR PROBLEMS ENCOUNTERED.

The initial budget was received with a delay of nearly one year compared to the work planning chart. Thus, the bar chart in the project proposal should be understood with a starting date of January 1992 instead of January 1991. In the absence of funding, initial work was carried out with internal resources of the laboratories participating in the project.

MAJOR RESULTS.

Three-dimensional structures.

1. *Mucor Miehei* Lipase, structure refined.
2. A model for interfacial activation in lipases was proposed from the structure of a fungal lipase-inhibitor complex.
3. *Fusarium solani* cutinase structure, a lipolytic enzyme with a catalytic serine accessible to solvent, has been solved at 1.25 Å resolution.
4. An almost complete native data set has been obtained at 3 Å resolution of *Pseudomonas glumae* lipase.
5. The modelling of the 3-D structure of *Pseudomonas aeruginosa* was performed using existing lipase and other hydrolase structures as well as amino acid sequence homologies.

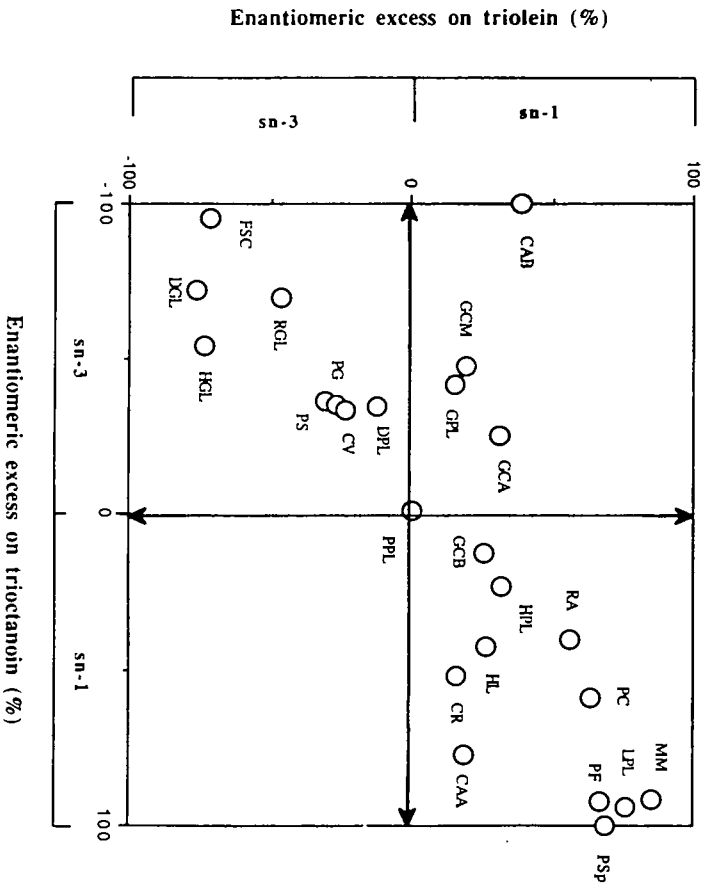


The interfacial activation of lipase. Diagrammatic representation of the conformational change in *Mucor Miehei* Lipase. The native enzyme (**a**) and the complex (**b**) are drawn with their Cα backbone. The catalytic triad, the tryptophan 88 and the lid, and the inhibitor are drawn with all atoms and highlighted with thick bonds. The complete atomic structures drawn with their van der Waals' radii of the native molecule (**c**) and the complex (**d**). The nonpolar atoms are shaded. The increase in the extent of the nonpolar surface is readily seen in this view.

Stereoselectivity of lipases selected by the partners of the BRIDGE T-project: Lipases.

The laboratory in Marseilles received about 20 purified lipases of microbial and animal origin thanks to the collaborative network set-up within the framework of the EEC T-Lipase Program. This gave a valuable opportunity of comparing the catalytic activities and stereopreferences of a group of enzymes which is sufficiently large to be presumably representative of the whole lipase family.

The stereoselectivity of twenty four lipases towards homogeneous prochiral triglycerides was determined. All the lipases tested catalyze the hydrolysis of the chemically alike but sterically nonequivalent ester groups in trioctanoin and triolein with different degrees of stereobias, depending on the fatty acyl chain length of the substrate. Hydrolysis of the *sn*-2 ester group is catalyzed by very few lipases and only CAA shows a clear preference for this position. Most of the lipases investigated (11 with trioctanoin and 16 with triolein) show a stereopreference for the *sn*-1 position. Using trioctanoin as substrate we observed a total stereospecificity for positions *sn*-1 and *sn*-3 with PSp and CAB, respectively. Among the twenty four lipases studied here, fifteen show a higher stereoselectivity with trioctanoin than with triolein. With three mold lipases as well as GPL, the stereopreference switches from *sn*-3 to *sn*-1 when increasing the acyl chain length from 8 to 18 carbon atoms. The main conclusion to emerge from the present study was that the specific stereopreference of each lipase for a given substrate and under given lipolytic conditions can be said to be its fingerprint.



Abbreviations: **HL**, *Humicola lanuginosa* lipase; **PF**, *Pseudomonas fluorescens* lipase; **CR**, *Candida rugosa* lipase; **MM**, *Mucor miehei* lipase; **GPL**, Guinea pig pancreatic lipase; **PG**, *Pseudomonas glumae* lipase; **PPL**, Porcine pancreatic lipase; **CAA**, *Candida antarctica* A lipase; **GCM**, *Geotrichum candidum* lipase (Münster); **RA**, *Rhizopus arrhizus* lipase; **FSC**, *Fusarium solani* cutinase; **PSp**, *Pseudomonas species* lipase; **HPL**, Human pancreatic lipase; **PC**, *Penicillium camemberti* lipase; **DPL**, Dog pancreatic lipase; **HGL**, Human gastric lipase; **DGL**, Dog gastric lipase; **LPL**, Lipoprotein lipase (bovine milk); **PS**, *Penicillium simplicissimum* lipase; **RGL**, Rabbit gastric lipase; **CAB**, *Candida antarctica* B lipase; **GCA**, *Geotrichum candidum* A lipase; **GCB**, *Geotrichum candidum* B lipase; **CV**, *Chromobacterium viscosum* lipase.

COOPERATIVE ACTIVITIES.

- **Utrecht**, November 21st-22nd (1988). Preliminary meeting "Lipases and phospholipases" organized by Dr. H.M. Verheij and Dr. B. Nieuwenhuis.

- **Brussels**, April 26th (1989). Invitation to the national experts meeting on "Multidisciplinary characterization of (phospho)lipases" organized by Dr. B. Nieuwenhuis.

- **Troia** (Portugal), November 14th-17th (1989). Closing meeting of the Biotechnology Action Programme of the EEC. Planing discussions about BRIDGE-T-Lipase project with S. Petersen, J. Cabral, F. Götz, H.M. Verheij, M.D. Legoy, G. Dodson, O. Misset, B. Nieuwenhuis and R. Verger.

- **Brussels**, June 5th (1990). Participation to a meeting of the project leaders of the selected BRIDGE-T-lipase proposals with M. Egmond, R. Schmid, L. Thim, W. Quax, A. Albert, M. Soares, B. Nieuwenhuis and R. Verger.

- **Brussels**, July 3rd (1990). Participation to the lipase industrial platform, organized by Dr. Nieuwenhuis.

- **Braunschweig**, September 12th-15th (1990). Co-organization with Prof. R.D. Schmid, Prof. L. Alberghina and Dr. R. Verger of a G.B.F./E.E.C. workshop entitled "Lipases: Structure, Mechanisms and Genetic Engineering".

- **Marseilles**, January 23rd (1991). Working meeting with B. Nieuwenhuis and R. Verger about the elaboration of the rules and guidelines of the BRIDGE-T-lipase project.

- **Brussels**, February 27th (1991). Meeting of the monitoring unit of the BRIDGE-T-lipase project with Dr. B. Nieuwenhuis, Prof. A. Albert and Dr. R. Verger.

- **Copenhagen**, April 23rd (1991). Meetings of the coordinators and the monitoring unit at Novo-Nordisk with B. Nieuwenhuis, A. Albert, L. Thim, G. Dodson, M. Egmond, O. Misset, R.D. Schmid and R. Verger.

- **Delft**, April 7th (1992). Meetings of the coordinators and the monitoring unit at Gist Brocades with B. Nieuwenhuis, A. Albert, L. Thim, O. Misset, M. Egmond, F. Spener and R. Verger.

TITLE : "Triacylglycerol lipases and colipases of the digestive tract and exocellular fungal lipases : structure, interfacial binding and catalysis".

CONTRACT NUMBER : BIOT-CT91-0274 (DTEE)

OFFICIAL STARTING DATE : 1/03/1991

COORDINATOR : Dr. Robert VERGER, CNRS, MARSEILLE, FR.

PARTICIPANTS :
- Prof. R. Kaptein, University of Utrecht, Utrecth, NL.
- Prof. L. Sarda, Université de Provence, Marseille, FR.
- Dr. G. Matthyssens, Corvas International , Gent, BE.
- Prof. M.D. Legoy, Université Technologique de Compiègne, Compiègne, FR.
- Prof. J.M.S. Cabral, Instituto Supérior Técnico, Lisboa, PT.
- Dr. C. Cambillau, CNRS, Marseille, FR.
- Prof. G.H. de Haas, University of Utrecht, Utrecht, NL.
- Prof. P. Cozzone, Faculté de Médecine, Marseille, FR.
- Dr. R. Verger, CNRS, Marseille, FR.

OBJECTIVES FOR THE REPORTING PERIOD :

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.

MAJOR PROBLEMS ENCOUNTERED : Nothing to report.

RESULTS : CUTINASE

In order to obtain useful heavy atom derivatives, all 14 serine to cysteine single mutants were constructed. Four of these mutants turned out to crystallize in the same space group as the native enzyme.

The three-dimensional structure of a recombinant cutinase from *F. solani pisi* has been solved at 1.25 Å resolution. Cutinase is an α–β protein (figure 1). Its active site is composed of the triad Ser 120, His 188 and Asp 175. As opposed to other known lipases, the catalytic serine is not buried under surface loops, but is accessible to solvent. This could explain why cutinase does not display interfacial activation.

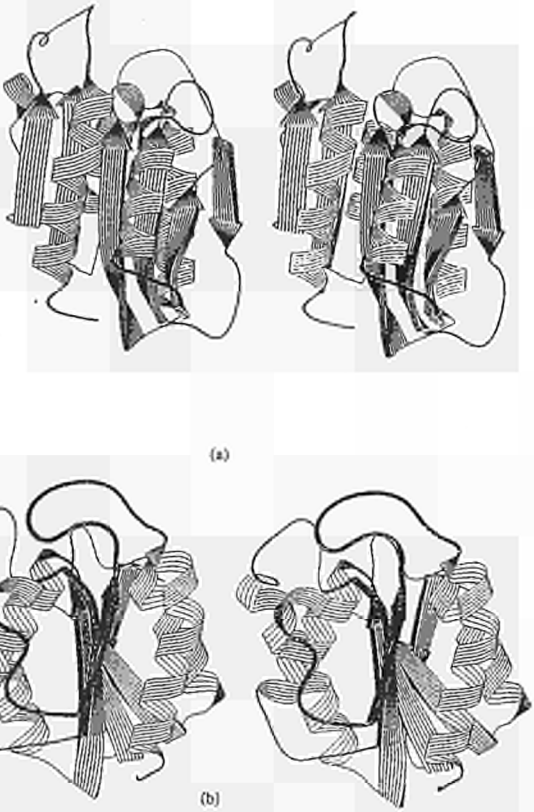


Figure 1 : Stereo views of the backbone tracing of cutinase. Views a and b are drawn 90° from each other.

HIGHLIGHTS/MILETONES :

1°/ Successful construction and production of 16 cutinase mutants for crystallization purposes.
2°/ The 3D-structure of a recombinant cutinase has been solved at 1.25 Å resolution.
3°/ Preparation of 160 mg of pure porcine procolipase and trypsin-activated colipase for NMR and X-ray structural studies.
4°/ Epitope mapping specificity of eight antiporcine procolipase monoclonal antibodies.
5°/ The spatial interactions between histidine and tyrosine residues is a common feature for pig, horse and ox colipases, as shown by high resolution proton NMR.
6°/ A large number of 2D-NMR spectra at 500 and 600 MHz have been recorded for procolipase.
7°/ Isoform purification of gastric lipases was achieved.
8°/ The stability of cutinase in reversed micelles of AOT/isooctane could be increased 45-fold by encapsulating the lipase with hexanol.
9°/ The crucial role of water was evidenced by ^{1}H and ^{13}C-NMR studies of cutinase-catalyzed synthesis of ester in liquid-solid media.
10°/ Transesterification reactions catalyzed by lipases were observed in gas phase.
11°/ Six isomeric pseudoglycerides : didecanoyl-deoxyamino-O-methyl glycerol, were synthesized in optically pure form.
12°/ The stereoselectivity of twenty four lipases of animal and microbial origin towards homogeneous prochiral triglycerides was determined.

WIDER CONSIDERATIONS : Nothing to report.

COOPERATIVE ACTIVITIES :

- Compiègne, November 20th (1989), November 8th (1990) and Gent, June 6th (1990). Scientific discussions of a common lipase project involving PGS and l'Université Technologique de Compiègne.
- Marseilles, September 17th-October 8th (1990). Visit of A.M.T.J. Deveer (Utrecht University) to perform a collaborative study on lipases by using the monomolecular film technique.
- Marseilles, June 14th (1991). Scientific presentations during the starting up meeting with B. Nieuwenhuis, L. Sarda, R. Kaptein, P. Cozzone, M.D. Legoy, J.N. Barbotin, G. Matthyssens, J. Cabral, C. Cambillau and R. Verger.
- From January 6th to March 28th (1992), E. Melo (Instituto Superior Técnico, Lisbon) stayed in Marseilles to perform monolayer experiments.

LIST OF JOINT PUBLICATIONS/PATENTS WITH TRANS-NATIONAL AUTHORSHIP :

1. Crystallization and premiminary X-ray study of recombinant cutinase form *Fusarium solani pisi*. C. Abergel, C. Martinez, J. C. Fontecilla-Camps, P. De Geus and M. Lauwereys. J. Mol. Biol. 215 (1990) 215-216.
2. *Fusarium solani* cutinase is a lipolytic enzyme with a catalytic serine accessible to solvent. C. Martinez, P. de Geus, M. Lauwereys, G. Matthyssens and C. Cambillau. Nature (1992), accepted for publication .
3. Stereoselectivity of lipases. S. Ransac, E. Rogalska, Y. Gargouri, A.M.T.J. Deveer, F. Paltauf, G.H. de Haas and R. Verger. J. Biol. Chem. 265 (1990) 20263-20270.

OTHER PUBLICATIONS/PATENTS :

1. Immunochemical determination of porcine pancreatic colipase : differentiation between procolipase and its activated form. L. de la Fournière, G. Forte, J. Rathelot, G. Piéroni, R. Julien and L. Sarda, Pancreas 6 (1991) 63-69.
2. Antigen specificity of anticolipase monoclonal antibodies : characterization of colipase-Fab complexes using gel filtration HPLC. N. Rugani, C. Dezan, B. Bellon and L. Sarda. Biochem. Biophys. Res. Commun. 177 (1991) 726-733.
3. Antigen specificity and cross-species reactivity of a monoclonal antibody (mAb 72.11) against porcine pancreatic procolipase. C. Dezan, N. Rugani, L. de la Fournière, L. Sarda and B. Bellon. Biochimie 73 (1991) 1417-1425.
4. The hydrophobic-aromatic domain of porcine pancreatic colipase A : complete assignments of tyrosine aromatic resonances by proton 2D-NMR. P. Canioni, C.F. Martin, E. Guittet, J.Y. Lallemand, M. Rovery and P.J. Cozzone. FEBS Lett. (1992) submitted.
5. ^{1}H and ^{13}C NMR studies of lipase-catalyzed synthesis of ester in liquid-solid media : crucial role of water. C. Sarazin, G. Goethals, J.P. Seguin, M.D. Legoy and J.N. Barbotin. Ann. N.Y. Acad. Sci. (1992), in press.
6. Transesterification reactions in gas phase catalyzed by lipolytic enzymes. F. Parvaresh, H. Robert, D. Thomas and M.D. Legoy. Biotechnol. Bioeng. (1992) 39, 467-473.
7. Synthesis of fatty acid esters by a recombinant cutinase in reversed micelles. M.J. Sebastiao, M.R. Aires-Barros and J.M.S. Cabral. Fundamental of Biocatalysis in non-conventional media, Noordwijkerhout (The Netherlands), 26-29 April, 1992.
8. Dog gastric lipase : stimulation of its secretion *in vivo* and cytolocalisation in mucous pit cells. F. Carrière, V. Raphel, H. Moreau, A. Bernadac, M.A. Devaux, R. Grimaud, J.A. Barrowman, C. Bénicourt, J.L. Junien, R. Laugier and R. Verger. Gastroenterology (1992), in press.
9. Inactivation of human pancreatic lipase by dodecyl-dithio-5-(2-nitrobenzoic acid). Y. Gargouri, C. Cudrey, H. Mejdoub and R. Verger. Eur. J. Biochem. 204 (1992) 1063-1067.

TITLE:

3-Dimensional structure and catalytic mechanism of 2-3 selected lipases of industrial relevance.

CONTRACT NUMBER:

BIOT-CT90-0181 (SMA)

OFFICIAL STARTING DATE:

01.02.1991

COORDINATOR:

Lars Thim, Novo Nordisk A/S, DK-2880 Bagsvaerd, Denmark

PARTICIPANTS:

University of York, UK (G.G. Dodson)
Centre National de la Recherche Scientifique, Marseilles, France (R. Verger)
Novo Nordisk A/S, Denmark (L. Thim)

OBJECTIVES:

The project will include the study of 2 or 3 lipases:
A. Guinea Pig Pancreatic Lipase (GPL)
B. Mucor Miehei Lipase (MML) and:
C. Human Bile Salt Activated Lipase (BSAL), if possible within the time period.

MAJOR PROBLEMS ENCOUNTERED:

Nothing to report

RESULTS:

A. Guinea Pig Pancreatic Lipase (GPL)

A total amount of approx. 0.1 mg (~2 nmol) of guinea pig lipase was purified to homogeneity from acetone powder of guinea pig pancreas. The purified GPL was reduced with DTT and derivatized with 4-vinyl pyridine. The resulting PE-Cys-GPL was purified by reverse phase HPLC. By direct sequencing of PE-Cys-GPL it was possible to identify the N-terminal 20 amino acid residues. This sequence showed a large degree of homology to human, dog and pig pancreatic lipases. PE-Cys-GPL was digested with trypsin and tryptic fragment purified by HPLC. Several of these fragments including the C-terminal fragment was sequenced. On the basis of the partial amino acid sequence, two synthetic oligonucleotides were constructed and used for screening in a cDNA library from guinea pig pancreas.

From these studies, it was possible to isolate and sequence a full length of cDNA encoding GPL, and to reduce from these results the complete amino acid sequence of the lipase. The cloned GPL has been expressed in relatively high yield in Aspergillus. Recombinant GPL from the Aspergillus expression system has been purified by affinity chromatography followed by gelfiltration. Several batches of purified, lyophilized rGPL for preliminary crystallographic and enzymatic analysis have been produced.

In general, rGPL proved to be quite susceptible to proteolysis. Freshly dissolved samples consisted almost entirely of intact molecules, although the proportion of the latter decreased upon standing at room temperature. This effect was magnified

in the presence of SDS: a sample incubated at 30°C for 20 min. became totally degraded. This suggests that the samples contained proteolytic activity which was possibly associated with the lipase itself. Perhaps the catalytic triad has proteolytic as well as lipolytic activity.

Despite these problems, crystals were obtained under several conditions, although the best were grown from PEG 10,000 in 10 mM DTT/10 mM $CaCl_2$/20 mM HEPES pH 7.2. DTT was added to prevent oxidation of the free cysteine residue, and $CaCl_2$ was added to ensure full occupancy of a calcium binding site. The largest crystal 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=225.2Å, $\alpha=\beta=\gamma=81{,}4°$ (this can be re-indexed into a hexagonal cell a=b=302.3Å, c=429.2Å, $\alpha=\beta=90°$, $\gamma=120°$). 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 analysed further. Experiments are continuing towards obtaining new crystal forms for X-ray diffraction work using both active and inhibited protein. Use of the latter has the advantage that any proteolytic activity should also be inhibited.

B. Mucor Miehei Lipase (MML)

A series of different potential lipase inhibitors have been synthesized. One of these compounds, n-hexyl phosphonate ethyl ester, has shown to form a stable inhibitor complex with MML. The crystal structure at 3Å resolution of this complex showed that the enzymes active site (Asp, His, Ser - triad) is exposed by the movement of a small helical peptide fragment (the "lid"). It has been proposed that the structure of MML in the lipase-inhibitor complex is equivalent to the activated state generated by the oil-water interface. Thus the structure of the MML-inhibitor complex has formed the basis for a general model of interfacial activation of lipases (ref. 1). The coordinates cf the atoms of MML and the MML-inhibitor complex have been deposited in the Brookhaven protein data bank. The enzymatic properties including the stereoselectivity of MML are, as in the case or rGPL, to be reported elsewhere.

C. Human Bile Salt Activated Lipase (BSAL)

Nothing to report.

HIGHLIGHTS/MILESTONES:

1. Guinea pig pancreatic lipase (GPL) purified to homogeneity
2. Partial sequence of GPL determined on protein level
3. GPL cloned
4. Complete GPL cDNA sequence determined

5. Expression of rGPL in Aspergillus achieved
6. Semilarge scale purification of rGPL started
7. Preliminary crystallographic analysis of rGPL started
8. Stereoselectivity of GLP and rGPL analyzed
9. Mucor Miehei Lipase (MML) structure refined (ref. 2)
10. Co-crystallization of MML with inhibitor achieved
11. Data collection of MML inhibitor complex finished
12. Model for interfacial activation (ref. 1)
13. Stereoselectivity of MML analyzed

WIDER CONSIDERATIONS:

The crystal structure of the Mucor Miehei inhibitor complex (ref. 1) and the complete sequence of GPL have shown that the elucidation of the 3D structure around the active site (Asp, His, Ser) and around the lid is extremely important in understanding the mechanism of action of lipases. As a consequence of these results, it has been decided not to work on human bile salt activated lipase (BSAL), but instead to concentrate the activities on the importance of the lid area for the mechanism of action of lipases (e.g. by site-directed mutagenesis in the lid area of GPL).

COOPERATIVE ACTIVITIES:

Copenhagen, June 2nd, 1988. Preliminary discussions CNRS/Novo Nordisk.
Copenhagen, February 1st, 1989. Discussions CNRS/Novo Nordisk.
Copenhagen, June 6th, 1989. Discussions of a common lipase project between CNRS/University of York/Novo Nordisk.
Copenhagen, November 21st, 1989. Contract discussions CNRS/Novo Nordisk.
Copenhagen, October 9th, 1990. Working seminar. Approx. 20 participants from CNRS, York and Novo Nordisk.
Marseilles, October 17th, 1990. Working visit of Dr. M. Barfoed (Novo Nordisk) to CNRS Marseilles to set up a future collaboration in order to transfer the monolayer know-how (K.S.V. Instrument) to Copenhagen.
Copenhagen, April 22nd, 1991. Scientific meeting between Novo Nordisk, York and CNRS. Approx. 25 participants.
Copenhagen, April 23rd, 1991. Meetings of the Coordinators and the monitoring unit at Novo Nordisk with B. Nieuwenhuis, A. Albert, L. Thim, G. Dodson, M. Egmond, O. Misset, R.D. Schmid and R. Verger.
Marseilles, June 10th, 1991. Scientific discussions and oil drop tensiometer demonstration to Dr. Kim Borch and Dr. Erik Gormsen (Novo Nordisk).

JOINT PUBLICATIONS:

1. Brzozowski, A.M., Derewenda, U., Derewenda, Z.S., Dodson, G.G., Lawson, D.M., Turkenburg, J.P., Björklin, F., Huge-Jensen, B., Patkar, S.A., and Thim, L.
A model for interfacial activation in lipases from the structure of a fungal lipase-inhibitor complex.
Nature 351 (1991) 491-494.

2. Brady, L., Brzozowski, A.M., Derewenda, Z.S., Dodson, E., Dodson, G.G., Tolley, S., Turkenburg, J.P., Christiansen, L., Huge-Jensen, B., Norskov, L., Thim, L., and Menge, U. A serine protease triad forms the catalytic centre of a triacylglycerol lipase.
Nature 343 (1990) 767-770.

TITLE: **Molecular Structure and Specificity Relationship of Microbial Triacylglycerol Lipases**

CONTRACT NUMBER: BIOT-CT91-0258 (LNBE)

OFFICIAL STARTING DATE: June 1st, 1991

COORDINATOR: Prof. R. D. Schmid, GBF, Braunschweig, Germany

PARTICIPANTS: Prof. L. Alberghina, University of Milano, Italy,
Prof. J. Cabral, University of Lisboa, Portugal,
Prof. E. Cernia, University of Roma, Italy,
Ms. C. Davies, Unilever, Colworth, UK,
Prof. F. Paltauf, University of Graz, Austria,
Prof. Spener, University of Münster, Germany,
Dr. R. Verger, C.N.R.S., Marseille,
Prof. Dr. R.D. Schmid, GBF, Germany.

OBJECTIVES SET FOR THE REPORTING PERIOD:

Purification and crystallization of various microbial lipases. Initial studies on their x-ray structure elucidation. Raising of antibodies against various lipases. Cloning, sequencing and expression of selected lipases. Kinetic and inhibition studies aiming at the understanding of their mechanism of action.

MAJOR PROBLEMS ENCOUNTERED:

The initial budget for the project was received late in February, 1992, with a delay of nearly one year compared to the bar chart plan. Thus, the bar chart in the project proposal should be understood with a starting date of Jan. 1992 instead of Jan. 1991.

RESULTS:

The project is focussed on the structure elucidation, protein engineering and mechanistic investigation of various fungal and bacterial lipases. In the absence of funding, initial work was carried out by internal resources of the 7 laboratories participating in the project.

Lipases of Geotrichum candidum

Investigations were carried out on 3 lipases from this organism, namely G. candidum Amano (GCL-4) and two industrial enzymes (GCL-A and GCL-B). Whereas GCL-B is unique with respect to its high specificity, GCL-A, GCL-4 and a G. candidum lipase isolated from the ATCC 34614 strain, whose structure has been published recently by M. Cygler et al., Montréal, are less specific and bear strong resemblance to each other.

Lipase GCL-4: the enzyme, but not the strain, is available from Amano Co., Japan. The Münster group has developed a purification protocol for the preparation of 260 mg pure enzyme from 300g commercial product within <24 hrs. MW was 62 kDa. Heterogeneity during IEF procedures (5 bands) was lost after deglycosylation with endoglycosidase H. The deglycosylated enzyme was crystallized at the GBF and a native x-ray data set was obtained with a resolution of 2.5 Å. Comparison of GCL-4 with the lipase analyzed by Cygler through peptide sequencing at the GBF showed 98% homology (based on 110 from 545 amino acids). Inhibition studies of the Münster group showed that carbidiimide inhibits the enzyme in aqueous solution, but organophosphate inhibits only in the presence of a lipid/water interface or after addition of isopropanol, suggesting that the serine in the catalytic triad is protected from the solvent in aqueous solution.

Lipase GCL-B: the Unilever group has prepared, by fermentation at the 50 l scale, several 100 mg of pure enzyme. Extensive after-treatment and crystallization experiments at the GBF led to a protocol for the generation of long needles which are yet unsuitable for x-ray analysis. The Unilever group has produced antibody sera (rabbits) specific for GCL-B and GCL-A. Both sera cross-reacted. The antibodies were purified using immobilized GCL-A and GCL-B. The Unilever group has constructed a cDNA library for G. candidum CMICC 335426 which produced GCL-A and GCL-B. 19 clones were isolated, 3 of which might contain complete structural genes; 2 of these clones are currently being sequenced in a M13 sequencing vector.
The Unilever group and the Marseille group have jointly examined the specificities of GCL-A and GCL-B and confirmed the high specificity of GCL-B. With selected substrates, V_{max}/K_m values of the immobilized enzymes were determined in a predominantly non-aqueous assay system.

Lipases of Candida

Investigations were carried out on the lipases of Candida cylindracea from Meito Sangyo (CCL-M) and from strain ATCC 14830 (CCL-LIP1-LIP4).

Candida cylindracea, Meito Sangyo: a purification protocol for the commercial product (OF type) was developed by the Münster group. In SDS-PAGE, a homogenous 67 kDa protein was seen, but on IEF, CCL-M was separated into 3 isoforms. Attempts of enzymatic deglycosylation via endoglycosidase H and F were not successful.

Candida cylindracea ATCC 14830: the Rome group succeeded to purify the enzyme (recovery of 80 %, no further details indicated) via affinity chromatography based on a freshly synthesized lauroylated and cross-linked PVA. The Milano group screened a genomic library of the strain with oligonucleotide probes and successfully isolated 3 positive clones containing 4 different lipase sequences, namely CCL-LIP1 to CCL-LIP4. Sequence homology was about 80 % and corresponds to a 57 kDa enzyme. N-glycosylation sites were identified and the consensus sequence around active-site serine-209 is conserved among all isoforms.

Lipases of Rhizopus

Investigations were carried out on 3 Rhizopus lipases, namely the enzyme from Rhizopus niveus (RNL), from Rhizopus arrhizus (RAL) and from Rhizopus oryzae (ROL).

Rhizopus niveus: neither the enzyme nor the producing strain are commercially available. The GBF group has used the patented amino acid sequence of this enzyme to model its 3-D structure on the basis of the Rhizomucor miehei lipase (MML) coordinates, available at the GBF from our own investigations. Sequence homology between MML and RNL is 63 %. The model has been subjected to several steps of molecular dynamics operations but is yet unproven by physicochemical studies.

Rhizopus arrhizus: the enzyme, but not the strain, is available from Sanofi Co., France. The Münster group has elaborated a purification procedure to obtain 2 homogenous lipase fractions (RAL-I, 39 kDa, and RAL-II, 32 kDa).

Rhizopus oryzae: the enzyme was screened by the GBF group from various DSM strains. After production at the GBF, in a 70 l bioreactor, the enzyme was isolated and purified to homogeneity by the Münster group. The pure ROL is a 30 kDa protein which, in Western blotting, reveals immunological crossreactivity with RAL-II (Münster group). N-terminal sequenzing at the GBF led to a complete alignment with amino acid 28-67 of RNL. The GBF group was successful in cloning the ROL gene from a genomic library into E. coli. After sequencing about 50 % of the gene, sequence homology to RNL and to Rhizopus delemar lipase (recently cloned and sequenced by a group at the ERRL, USDA, Philadelphia) was 97 %. Thus, the computer model of RNL may constitute a good approximation to the ROL structure, allowing for the immediate definition of protein engineering experiments.

Lipases of Pseudomonas, Penicillium and Chromobacterium

Investigations were carried out on 3 lipases, namely on the lipase from Pseudomonas spec. (PSL), the lipase

from Penicillium simplicissimum (PIL) and the lipase from Chromobacterium viscosum (CVL).

Pseudomonas spec: the **GBF group** developed a purification protocol for a homogenous enzyme preparation. Crystals were obtained and precession photographs revealed a space group C2 and the lattice constants.

Penicillium simplicissimum: the **GBF group** developed a simple purification protocol resulting in a homogenous enzyme preparation of which diffracting crystals were obtained.

Chromobacterium viscosum: the enzyme, but not the strain, is available from Toyo Jozo, Japan. The **Münster group** developed a purification protocol allowing for the isolation of 400 mg pure lipase from 50 g commercial product within 3 days. In SDS-PAGE, the molecular weight was determined at 30 kDa, but under reducing conditions 2 bands formed at 10 kDa and 20 kDa, respectively. In IEF, considerable heterogeneity was observed. Several isoforms were isolated by chromatofocussing or preparative IEF. The subunits of one isoform were separated and about 50 % of the protein sequence of the tryptic digest was determined by the **GBF group**, confirming the DNA sequence published by Toyo Jozo. This isoform was crystallized by the **GBF group** and a native data set with a resolution of about 2.2 A was obtained. The **Lisboa group** produced monoclonal antibodies for "fraction A" and "fraction B" lipase obtained from crude Chromobacterium viscosum lipase by gel filtration. By applying immunoblotting and electrophoretic techniques, evidence was obtained that "fraction B" lipase (molecular weight around 30 kDa) consists of more than one isoform, whereas "fraction A" lipase is probably a high molecular weight aggregation of "fraction B" lipases.

Other microbial lipases

The **GBF group** screened a number of thermophilic bacteria for lipases. Whereas screening in the genus Thermus did not reveal the presence of lipases, the screening of several thermophilic Bacilli was more successful. First steps for the purification of thermophilic lipase from **Bacillus stearothermophilus** (BSL) were investigated.

Kinetic and mechanistic studies

The **Marseille group** investigated the stereoselectivity, towards prochiral triglycerides, of 24 purified lipases of animal and microbial origin. Most of the lipases showed stereopreference for the sn-1 position; hydrolysis of the sn-2 bond was catalyzed by few lipases. The **Münster group** studied active-site inhibition of Geotrichum candidum lipase (GCL-4) (see above). The **Graz group** attempted to prepare photoaffinity labels for the binding site of Chromobacterium viscosum lipase but the two labels used so far did not lead to positive results. The **Graz group** further synthesized various substrate analogs of triacylglycerols, namely rac-1-hexadecyl-2,3-dioleoylglycerol (HOH) and 2-hexadecyl-1,3-dioleoylglycerol (HOO). Comparison of these substrates with triolein led to the surprising result that both lipases from Rhizopus arrhizus and from Chromobacterium viscosum exhibited higher enantioselectivity if HOH was used as a substrate, i. e. when position 2 of the glyceride was changed to an alkyloxy moiety.

HIGHLIGHTS/MILESTONES

Purification protocols available for most lipases under study. Diffracting crystals obtained for 4, needle-form crystals obtained for 2 more lipases. 1 Lipase structure obtained by modelling. 2 lipases cloned. Pertinent observations as to stereo- and regioselectivity of various lipases obtained.

WIDER CONSIDERATIONS

The project proceeds smoothly and has good chances to benefit European chemical industry on the long term. Severe delays in project funding reduced competitiveness to North American and Japanese efforts, particularily in structure elucidation and cloning.

COOPERATIVE ACTIVITIES: See under results.

LIST OF JOINT PUBLICATIONS/PATENTS WITH TRANSNATIONAL AUTHORSHIP

none

OTHER PUBLICATIONS/PATETNS/COMMUNICATIONS

Alberghina L., Crandori R., Longhi S., Lotti M., Fusetti F. and Vanoni M. (1991). Molecular cloning of a lipase and of a lipase-related gene from *Candida cylindracea*. In Lipases: Structure, Mechanism and Genetic Engineering (Alberghina L., Schmid R.D. and Verger R., eds) GBF Monographs Vol. 16, VCH.

M.A. Taipa, J.M.S. Cabral and M.R. Aires-Barros, "Purification and Characterization of *Chromobacterium viscosum* Lipase", to be presented at the European Research Conference on Bioseparation- New Strategies in Bioseparation Process, 24-28 May 1992, Espinho, Portugal.

Cernia E., Magri A.D., Ortaggi G., Castagnola M., Rabino R., Bartoli F., (1991). Lipolytic enzymes separation and purification through functionalized synthetic polymers. In: Lipases: Structure, Mechanism and Genetic Engineering (Alberghina, L., Schmid, R.D. and Verger R., eds)

Geotrichum candidum produces several lipases with markedly different substrate specificities. Sidebottom et al, (1991) Eur. J. Biochem. 202 485-491.

Substrate specificities of Lipases A and B from *Geotrichum candidum* CMICC 335426. Charton & Macrae (1992) Biochem. Biophys. Acta. 11233, 59-64.

Purification and Substrate Specificities of Lipases from *Geotrichum candidum* in "Lipases, Mechanism and Genetic Engineering" (Alberghina, L.,Schmid, R.D. and Verger R., eds), pp. 335-338.

Patent No. EP90 301457.
"9 cis fatty acid-specific lipase B".

Hedrich, H.C. Spener, F., Menge, U., Hecht, H.J., Schomburg, D. and Schmid, R.D. (1991), Enzyme Microb. Technol., 13, 840-847.

Isobe, K., Nokihara, K., Karas, M., Stahl, B., Hedrich, H.C., Kordel, M., Schmid, R.D., Spener, F. and Hillenkamp, F. (1992) Anal. Biochem., submitted.

Characterization of lipases for industrial application

Contract number Biot-CT-90-0194
Official starting date: 01/04/1991
Coordinator: M.R. Egmond, Unilever Research Laboratory, Vlaardingen (NL)
Participants: A. Cleasby, University of Oxford (UK)
G.H. de Haas, University of Utrecht (NL)
R. Verger, CBBM / CNRS (FR)
Objectives set:
- Obtain structural information of lipases from procaryotic 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
- Pseudomonas lipases are difficult to obtain in pure form, often leading to generation of different crystal forms in crystallization experiments.
- Poor solubility and aggregation hampers exploitation of NMR techniques in structural investigations of lipases

Results

1. Progress of crystallographic studies on Pseudomonas glumae lipase (A. Cleasby)

Since the start of the project an almost complete native dataset (87% completion) has been obtained at 3 Å resolution. Also datasets of three heavy atom derivatives[1] were collected at 3.0 Å, giving rise to isomorphic changes of 13%, 22% and 12%, respectively. The derivatives were solved for the positions of the heavy atoms using a combination of Patterson and difference Fourier techniques. After refinement of their occupancy and positional parameters the derivatives were used in the calculation of phases. The figure of merit at 3.0 Å for the three derivatives is 0.64. By now 75% of the reflections have been phased. The quality of the crystallographic data is summarized in Table I. An electron density map was calculated using these phases. It clearly showed the boundaries of the molecule, along with some helical regions. The Wang method of solvent flattening was applied to the map in a cyclical manner, while the resultant phases were used to calculate a second map. Interpretation of these maps is now in progress. There are four molecules in the asymmetric unit. The known amino acid sequence is being fitted to one of them. The latter effort is currently carried out in collaboration with Unilever Research.

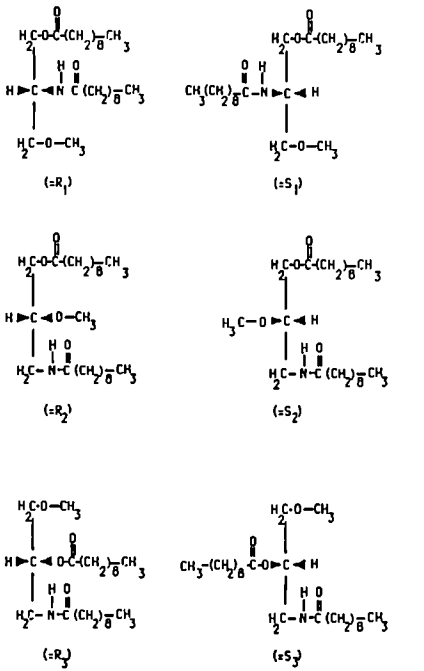
2. Progress of synthetic effort (G.H. de Haas)

Pseudoglycerides containing, respectively, ester, ether and amido linked groups (compounds shown in Figure 1) were synthesized in optically pure form. Obviously, only the ester bond will be hydrolyzed by lipases. These triglyceride analogues have been investigated as substrates for two highly purified lipases, from porcine pancreas and Pseudomonas glumae, respectively. The lengths of the two acyl-groups and the alkylether chain allowed kinetic analyses both by titrimetric as well as surface film techniques.

The findings obtained with the pancreatic enzyme confirmed the well-known properties of this enzyme: hydrolysis takes place exclusively of primary ester bonds and the enzyme possesses no stereopreference for either the 1- or 3- position. The Pseudomonas lipase attacks the 3-ester bond slightly better than the 1-esterbond (see also Verger) , but is able to effectively hydrolyze secondary ester bonds as well. Moreover, in the hydrolysis of the secondary ester bond the Pseudomonas glumae lipase appeared to possess a strong stereopreference: the R-isomer (R_3 in Figure 1) is hydrolyzed 50 times more rapidly than S_3. This property is rather unique and could allow interesting application in stereospecific synthetic reactions. Comparison of lipase activity on pseudo-glycerides (only allowing one site of attack) and

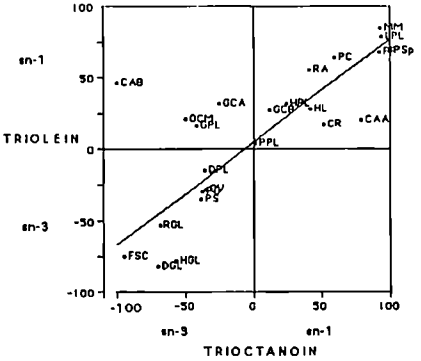
[1] one gold, a mercury and a platinum containing compound, respectively

Figure 1



(R1) stands for (R)-1,2-didecanoyl-2-deoxyamino-3-O-methylglycerol
(R2) stands for (R)-1,3-didecanoyl-3-deoxyamino-2-O-methylglycerol
(R3) stands for: (R)-2,3-didecanoyl-3-deoxyamino-1-O-methylglycerol

Figure 2



Abbreviations:
CAA:Candida AntarcticaA; CAB:Candida AntarcticaB;CR:Candida rugosa; CV:Chromobacterium viscosum;DGL:Dog gastric lipase;DPL: Dog pancreatic lipase;FSC: Fusarium solani cutinase; GCA: Geotrichum candidumA;GCB: Geotrichum candidumB; GCM: Geotrichum candidumM; GPL:guinea pig pancreatic lipase; HGL:Human gastric lipase; HL: Humicola lanuginosa lipase;HPL:Human pancreatic lipase; LPL: Lipoprotein lipase; MM: Rhizomucor miehei lipase; PC: Penicillium camembertii lipase; PF: Pseudomonas fluorescens lipase; PG: Pseudomonas glumae lipase, PPL: Porcine pancreatic lipase; PS: Penicillium simplicissimum lipase; PSp: Pseudomonas species (unknown) lipase; RA: Rhizopus arrhizus lipase; RGL: Rat gastric lipase

Table I. Summary of crystallographic data

Statistics	Native	Gold	Mercury	Platinum
%complete	82	62/46	50/62	68/97
merging R	3.6	3.2	4.8	4.4
Riso	-	13	18/23	12
Nsites	-	3	6/5	5
Fh/E	-	2.21	2.45	1.95
R(Cullis)	-	47.1	43.8	50.0
FOM	-	50.1	49 1	51.0

several triglycerides (see R.Verger) will provide further understanding of the mechanism of preferential attack exerted by lipases.

3. **Progress of kinetic and mechanistic studies on lipases (R. Verger)**

Lipases are able to recognize triglyceride substrates in several ways. In principle, the two chiral forms of triglycerides, or their prochiral forms will be recognized. This recognition will depend on the nature (e.g. chain lengths) as well as on the positions of the acyl chains attached to the glycerol backbone. The overall steric course of the enzymic reaction will provide evidence of reaction pathways and deepen our understanding of the reaction mechanism of lipolytic attack. In particular, this understanding will be obtained by comparing steric preferences of a series of lipases collected within the framework of the EEC T-Lipase programme. The results show stereopreferences ranging from 0% to 100% for some 20 lipases using the method developed at the CNRS laboratory (shown in Figure 2). These studies demonstrate the fine-tuning of interactions involved. Even within lipase families (e.g. based on high amino acid sequence homology) this stereopreference may differ. However, combined with structural information of the active site architecture, a thorough understanding will emerge of the mechanisms underlying substrate recognition and turnover. This information is also of vital importance for application of lipases in synthetic reactions.

HIGHLIGHTS

X-ray analysis: an almost complete native dataset has been obtained at 3 Å resolution.
Synthesis and analysis of pseudoglycerides: when P.glumae lipase is forced to act on the ester at the he 2-position, a strong stereopreference is found for the R-isomer.
Kinetics and mechanism:For triglyceride substrates the stereopreference of a wide range of lipases has been revealed. Some lipases switch preference depending on the acyl chain length.

WIDER CONSIDERATIONS

The first steps on the elucidation of structure function rules for lipases have been taken. It has become clear that only an integrated effort will allow us to be successful in our objective to understand lipolytic action - based on many lipase structures, as well as large series of mechanistic studies. These mechanistic studies require not only pure triglycerides, but also specially designed pseudo-glycerides to track down specific interactions with the enzyme.

COOPERATIVE ACTIVITIES

Copenhagen (DK), April 23rd (1991); Coordinators meeting at Novo Nordisk
with A.Albert, G.Dodson, M.R.Egmond, O.Misset, B.Nieuwenhuis, R.D.Schmid, L.Thim, and R.Verger
Oxford (UK), October 2nd (1991); Scientific meeting with A.Cleasby, M.R.Egmond
Utrecht (NL), October 5th (1991); Scientific meeting with A.M.T.J.Deveer, M.R.Egmond, G.H.de Haas, S.Ransac, H.M.Verheij and R.Verger. Subject "competitive inhibition of lipolytic enzymes"
Oxford (UK), January 21/22nd (1992); Scientific meeting with A.Cleasby, M.R.Egmond

LIST OF JOINT PUBLICATIONS/PATENTS

A.Cleasby, E.Garman, M.R.Egmond, M.Batenburg (1992),J.Mol.Biol.224,pp (*in press*)
Ransac, S., Rogalska, E., Gargouri, Y., Deveer, A.M.T.J., Paltauf, F., de Haas, G.H., and Verger, R. (1990), *J. Biol. Chem.*, 265, 33, 20263-20270
Ransac, S., Deveer, A.M.T.J., Rivière, C., Slotboom,A.J., Gancet, C., Verger, R. and de Haas, G.H. (1992) *Biochim. Biophys. Acta* , 1123, 92-100
Deveer, A.M.T.J., Dijkman, R., Leuveling-Tjeenk, M., van den Berg, B., Ransac, S., Batenburg, M.,Egmond, M., Verheij, H.M., de Haas, G.H., (1991), *Biochem.*, 30, 10034-10042
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. (1991), *Biochim. Biophys. Acta*, in press
Deveer, A.M.T.J., den Ouden, A.T. Vincent M., Gallay, J., Verger, R., Egmond M.R., Verheij H.M. and de Haas, G.H. (1991), *Biochim. Biophys. Acta*, in press

TITLE: EXPLORING THE STRUCTURE-FUNCTION RELATIONSHIP OF PSEUDOMONAS AND BACILLUS LIPASES

CONTRACT NUMBER: BIOT CT91 0272

OFFICIAL STARTING DATE: 01.08.91

COORDINATOR: Dr. O. Misset, Gist-brocades, Delft, NL

PARTICIPANTS: C. Colson, Université Catholique de Louvain-la-Neuve, B
U. Winkler, Ruhr Universität Bochum, D
B. Dijkstra, Rijksuniversiteit Groningen, NL

OBJECTIVES SET FOR THE REPORTING PERIOD

The first reporting period covers the period 01/08/1991 until 31/03/1992 which means the first 8 months of the total 36 month project period.
According to the Working Programme (Annex I of the EC-contract and Annex I of the Consortium agreement) the first 8 months should be used to obtain highly purified lipase preparations and to start the crystallization of this material. Furthermore, a kinetic characterization of the purified enzymes was estimated to start after month six.

MAJOR PROBLEMS ENCOUNTERED

1. Project management problems
   The major problem which was encountered during the execution of the project was the fact that the "Advanced Payment" by the EC was made much too late! It prohibited that the project could start efficiently on the first day of the contract period (01/08/1-991).

2. Scientific problems

   2.a Pseudomonas lipase
   Lipase was isolated from cell-free growth medium obtained from Gist-brocades where Pseudomonas aeruginosa was grown under conditions of maximum lipase production. A significant variability in the chemical composition of the supernatants led to a necessary modification of the purification procedure for every new batch of supernatant (extra work).
   The current expression level (ca. 30-60 mg/liter) is not enough to obtain hundreds of milligrams of highly purified lipase in an easy way. Attempts to obtain overexpression of the structural lipase gene in E. coli failed which was attributed to the requirement of at least one additional gene product termed lip H (Helper gene-unpublished results). Overexpression in the homologeous system (i.e. Pseudomonas aeruginosa) will now be performed by using suitable plasmids.
   An increase in expression level of 5-10 fold is expected to be realized by the end of April 1992.

   2.b Bacillus lipase
   The expression level of Bacillus lipase is estimated to be even lower than that for the Pseudomonas lipase. Also in this case overexpression in Bacillus will be performed by using suitable plasmids. An overexpression strain will be available by the end of April 1992.

RESULTS

It should be noted that the results described here were obtained only partly at the expenditure of the EC-grant(s).

1. Pseudomonas aeruginosa lipase

   1.a Purification of Pseudomonas aeruginosa lipase (RUB)
   The cell-free growth medium obtained from Gist-brocades contained 30-60 μg/ml of lipase. After purification via gel filtration chromatography on a Hiload Superdex 200 FPLC column and preparative electrophoresis (Rotofor) as major steps, two samples of 1 mg each were obtained and given to Dijkstra for crystallization experiments (see below).

   1.b Enzymatic characterization of Pseudomonas aeruginosa lipase (RUB)
   Inhibition studies with phenylmethanesulfonylfluoride (PMSF) and hexadecylsulfonylfluoride (ASF) solubilized in detergent showed that Pseudomonas aeruginosa lipase activity was 10^6 times more sensitive against ASF as compared to PMSF under identical experimental conditions. The presence of a disulfide bridge formed by the two cysteine residues present in the native lipase protein could be demon-

strated by showing (i) an alteration in migration behaviour in gel electrophoresis (SDS-PAGE) of the lipase depending on treatment with dithiothreitol and iodoacetamide, and (ii) the rapid and complete loss of enzymatic activity when lipase was treated with dithiotreitol or β-mercaptoethanol in the presence of 0.1% (w/v) SDS. Digestion of lipase with proteases trypsin and arg C led to formation of fragments of Mr 23 and 25 kDa, respectively, which both contained the NH_2-terminus of the lipase protein as shown by Western-blotting using the appropriate antibodies.

1.c Crystallizaton of Pseudomonas aeruginosa lipase (RUG)
With two different batches of purified Pseudonomas lipase material (each approximately 1 mg) various crystallization conditions were explored. As precipitants $(NH_4)_2SO_4$ (10-60% saturated), polyethylene glycol 4000 (10-40%) and 2-methyl-2,4 pentane diol (10-40% v/v) were used in buffers with a pH value ranging from 5.5-8.5. In some cases precipitants were found, but none of the conditions led so far to suitable crystals. More lipase is required in order to allow for higher protein concentration in the crystallization experiments (now approximately 1.5 mg/ml).

1.d Model building of Pseudomonas aeruginosa lipase (RUG)
Since no suitable crystals of this enzyme were available, a 3D-model was build using the sequence and structural information from the following enzymes:

Enzymes	Source	Sequence	Structure known	Structure available
lipase	Human pancreas	+	+	-
lipase	Rhizomucor miehei	+	+	C_α
lipase	Geotrichum candidum	+	+	C_α (1)
lipase	Bacillus subtilis	+	-	-
lipase	Pseudomonas species	+ (5x)	-	-
Acetylcholinesterase	Torpedo californica	+	+	+ (2)
Serine carboxypeptidase	Wheat germ	+	+	+
Haloalkane dehalogenese	Xanthobacter autotrophica	+	+	+
Diene lactone hydrolase		+	+	+ (3)

(1) gift from dr. M. Cygler, (2) gift from dr. J. Sussmann, (3) gift from dr. D. Ollis

Based on the sequence comparisons and secondary structure analysis, we tried to use the coordinates of known structures to build our model. The Geotrichum candidum lipase was chosen as the source model, since its secondary structure elements are more similar to those of the other enzymes used in this comparison than the R. miehei lipase. However, we have only the coordinates of the alpha carbons of the Geotrichum candidum lipase, but fortunately, the acetylcholinesterase has a structure very similar to that of G. candidum, except in the flap region where a deletion in the sequence is present. The coordinates of the acetylcholinesterase have thus been used, and the structure build. Using FRODO, the amino acids have been replaced in order to correspond to those of the P. aeruginosa lipase sequence. Next, gaps were removed and the structure was energy minimized.
The superposition of the model on the structure of G. candidum and R. miehei lipase is reasonable with respect to the conserved β-strands and conserved α-helices. Also, the side-chains of the active-site residues superimpose quite well.
Currently, we are working towards improvement of the model. We also hope to build a preliminary model for the B. subtilis lipase within the next month.

2. Bacillus subtilis lipase

2.a Amino acid sequence of Bacillus subtilis lipase
The amino acid sequence has now been completed and verified. The gene is coding for a precursor lipase molecule consisting of a leader of 32 amino acids and a mature lipase of (only!) 184 amino acids. This makes the Bacillus lipase the smallest of all lipases. In contrast with other lipase sequences, no consensus

sequence of Gly-X-Ser-X-Gly around the active site Ser could be identified but instead this sequence is likely to be composed of Ala-X-Ser-X-Gly.
Recently, the amino acid sequence of Bacillus pumilis lipase was disclosed in a patent application of Solvay (WO 91/16422). It appeared that both lipases revealed a homology of 74.1%. The B. pumilis lipase does also not contain the Gly-X-Ser-X-Gly sequence but instead the Ala-X-Ser-X-Gly sequence at a position homologous to the one in the Bacillus subtilis lipase.

2.b Mutagenesis of Bacillus subtilis lipase
Lipase mutants A106G and A106I have been constructed on DNA level and will be expressed in Bacillus subtilis. The rationale behind these mutations is to unravel the role of Ala 106, in the consensus sequence.

2.c Purification of Bacillus subtilis lipase
A purification procedure for the lipase from the culture supernatant is presently under investigation. Promising results were obtained with a procedure consisting of ammonium sulphate precipitation and hydroxyapatite chromatography. A 250-fold purification could be established with a recovery of approximately 10%. The final preparation reveals two major bands on SDS-gels of which one (with a MW ≈ 19 kDa) is most likely the lipase. However, there is still not enough material available for crystallization experiments.
The construction of an overexpressing Bacillus strain will facilitate both the purification of lipase as well as achieving the required amount of lipase.

2.d Enzymatic characterization of Bacillus subtilis lipase
The lipase activity was measured with paranitrophenyl palmitate (0.8 mM) in a buffer containing 0.1 M phosphate (pH = 8), 2 mg/ml deoxycholate and 1 mg/ml gum arabic.
Ca^{++} markedly stimulated the activity when added up to 40 mM but became inhibitory beyond that concentration. No effects were observed with Fe^{3+}, Mn^{2+} or EDTA (all at 1 mM). Acetone was found to strongly inhibit the activity (up to 20% v/v). The enzyme could be inhibited by PMSF but only at very high concentrations of the latter (> 4 mM).
The enzyme showed roughly the same activity with 0.8 mM of the following substrates:

$$CH_3\text{-}(CH_2)_n\text{-}\underset{\underset{O}{\|}}{C}\text{-pNP}\ (n = 0, 2, 8, 10, 14, \text{and } 16)$$

HIGHLIGHTS/MILESTONES

1. Pseudomonas aeruginosa lipase has been purified in reasonable amounts of sufficient quality to start the crystallization experiments.
2. The confirmation of the Bacillus subtilis lipase sequence by the work of Solvay (Bacillus pumilis lipase sequence) reinforces the importance of the Bacillus subtilus lipase work in our project. The Bacillus subtilis lipase can be regarded as one of the most unique and interesting lipases of the entire T-project.
3. The modelling of the 3D-structure of Pseudomonas aeruginosa using existing lipase and other hydrolase structures as well as amino acid sequence homologies is very original and stimulating. This modelled structure therefore has to be considered as a serious attempt to achieve the result of the T-project.

WIDER CONSIDERATIONS

Nothing to report.

COOPERATIVE ACTIVITIES

1. Meeting
   The project team had the following project meetings in the reporting period (01/08/91 - 31/03/92):
   * 28/10/91 in Delft (minutes, d.d. 20/11/91)
   * 05/03/92 in Louvain-la-Neuve (minutes in preparations)
   The next meeting is scheduled for 30/06/92 in Groningen.

2. Materials and staff exchanged
   *Gist-brocades received the wild type Pseudomonas aeruginosa and Bacillus subtilis strains for fermentation purposes.
   *Bochum has obtained cell-free supernatants of several Pseudomonas aeruginosa fermentations from Gist-brocades.
   *Gist-brocades has made available plasmids (to allow for overexpression in Bacillus subtilis and Pseudomonas aeruginosa) to Colson and Winkler respectively.
   *Groningen has received in total 2 mgs purified lipase of Pseudomonas aeruginosa.

   *A small amount of purified lipase from Pseudomonas aeruginosa was sent to Robert Verger for kinetic analysis (August 1991).
   *10-21/2/1992, Dr. S. Ransac (RUG) worked in Winkler's laboratory (RUB) on the purification of the Pseudomonas lipase.

3. Lectures
   As a result of the formation of the lipase project teams, the following lectures were presented:
   *February 1, 1991, Ruhr Universität Bochum
   Dr. B. Dijkstra (Groningen): "Structure and function of Phospholipase A_2".
   *April 19, 1991, Ruhr Universität Bochum
   Dr. O. Misset (Gist-brocades): "Protein engineering of industrial enzymes".
   *July 1, 1991, CNRS-Marseille
   Dr. O. Misset (Gist-brocades): "Protein engineering of industrial enzymes".

LIST OF JOINT PUBLICATIONS WITH TRANS-NATIONAL AUTHORSHIP

Dartois, V., Baulard, A., Schanck, K. and Colson, C. (1992) B.B.A. (in press), "Cloning, nucleotide sequence and expression in E. coli of a lipase gene from Bacillus subtilis 168".

OTHER PUBLICATIONS/PATENTS

Nothing to report.

T-PROJECT

BIOTECHNOLOGY OF LACTIC ACID BACTERIA

TITLE: **Improvement and Exploitation of Lactic Acid Bacteria for Biotechnology Purposes.**

CONTRACT NUMBER: BIOT-CT91-0263 (SSMA)

OFFICIAL STARTING DATE: I March 1991

COORDINATOR: Prof Charles Daly, University College, Cork, Ireland.

PARTICIPANTS:

C.Y.Boquien, INRA, Thiveral Grignon, France.

V. Bottazzi, Universita Cattolica del Sacro Cuore, Piacenza, Italy

M.C.Chopin, INRA, Jouy en Josas, France.

T.M.Cogan, Teagasc, Fermoy, Co. Cork, Ireland.

M.A. Collins, Queens University of Belfast, Northern Ireland.

P.E.Hernandez Cruz, Universidad Complutense, Madrid, Spain.

C.Daly/G.F. Fitzgerald, University College Cork, Ireland.

R.Jimenez Diaz, U.E. de I. de Biotecnologia de Alimentos, Sevilla, Spain

A. Mendes Faia, Universidade de Tras-os-Montes e Alto Douro, Vila Real Codex, Portugal.

M.J.Gasson, AFRC Institute of Food Research, Norwich, United Kingdom.

A.Geis, Bundesanstalt für Milchforschung, Kiel, Germany.

W.P.Hammes, Universitat Hohenheim, Stuttgart, Germany.

E.B.Hansen, Chr. Hansen's Laboratorium, Hoersholm, Denmark.

J. Josephsen, Royal Veterinary and Agricultural University, Frederiksberg, Denmark.

A.Joyeaux, Societe Euipharm, Saint Simon, France.

G. Kalantzopoulos, Agricultural University of Athens, Greece.

K. Kersters, Universiteit Ghent, Belgium.

W. N. Konings, University of Groningen, Haren, Netherlands.

A.M. Ledeboer, Unilever Research Laboratorium, Vlaardingen, Netherlands.

V.M. Marshall, Polytechnic of Huddersfield, United Kingdom.

F.Mulholland, AFRC Institue of Food Research, Reading, United Kingdom.

I.F. Nes, Agricultural University, Ås, Norway.

B. Jelle, Chr. Hansen's Laboratorium, Hoersholm, Denmark.

R. Plapp, Universitat Kaiserslautern, Germany.

J.R.Prigent, Texel (Rhone Poulenc Group) Dange Saint Romain, France.

P. Ritzenthaler, I.N.S.A., Toulouse, France.

G. Sakellaris, The National Helenic Research Foundation, Athens, Greece.

M.H. Santos, Centro de Technologia Quimica e Biologicia, Oeiras, Portugal.

K.H. Schleifer, Technische Universitat Munchen, Germany.

J.E. Suarez, Univerversidad de Oviedo, Spain.

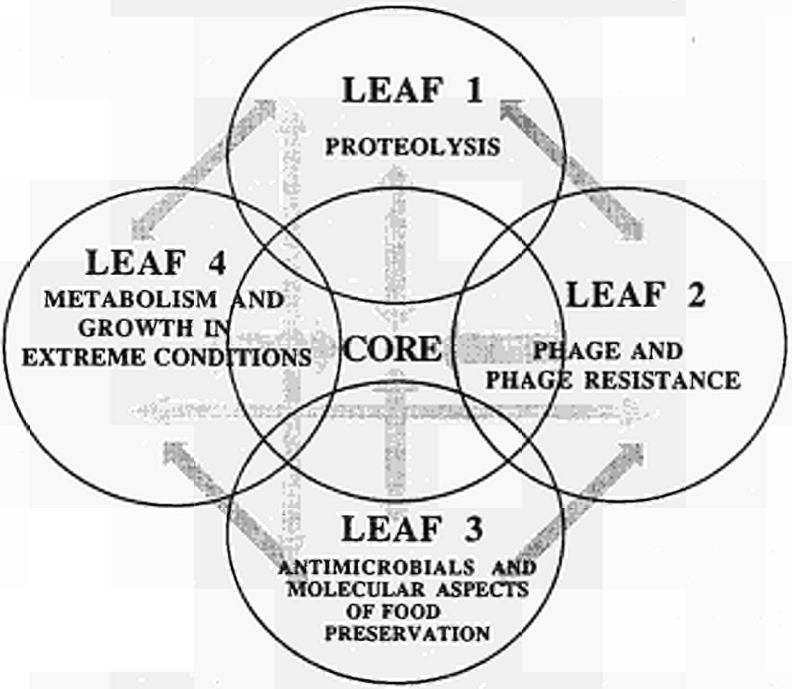
J.H.J. Huis in t'Veld, TNO Nutrition and Food Research, Zeist, Netherlands.

G.Venema, University of Groningen, Haren, Netherlands.
W.M. de Vos, NIZO, Ede, Netherlands.
P.J. Warner, Cranfield Institute of Technology, Bedford, United Kingdom.

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-LEAF INTERACTIONS IN THE T-PROJECT ON LACTIC ACID BACTERIA



CORE TECHNOLOGIES:

OBJECTIVES SET FOR THE REPORTING PERIOD:

1. Gene analysis and the study of gene expression in LAB including mapping of chromosomal genes and elements in *Lactococcus*.
2. Development of genetic tools, i.e. integration/delivery vectors.
3. Study of conjugation mechanisms in *Lactococcus* and *Lactobacillus*.
4. Analysis of recombination systems in *Lactococcus*.
5. Characterisation of plasmid replication, stability and incompatibility functions in *Lactococcus*.

RESULTS:

1. Gene analysis and gene expression

(a) Transcriptional fusions between *lac* promoter DNA fragments of varying sizes and a *cat*86 reporter gene were introduced in *L. lactis* MG5267 containing a single chromosomal copy of the *lac* operon. Determination of the CAT activities in glucose or lactose-grown cells of the resulting strains revealed that the *lac* promoter is strongly affected by LacR repressor and regions flanking the promoter. DNAse footprinting studies showed that the LacR repressor, purified from LacR-overproducing *E.coli* cells, binds to two operator sequences, one located near the 35 region of the *lac* promoter and the other upstream of the *lac*R initiation codon. A *lac*R deficient strain was constructed by replacement recombination. Detailed analysis of the resulting strain showed that expression of the *lac* operon is not only controlled by the LacR repressor but is also subject to catabolite repression.

(b) Translational fusions of the lactose promoter to *E. coli* β-galactosidase and transcriptional fusions to *Vibrio fischerii* luciferase genes were constructed. Induction was demonstrated in both reporter systems and evidence of a glucose repression phenomenon was obtained. Expression of luciferase under control of the promoter for the lactose repressor was also found to be lactose inducible.

(c) Gene replacement experiments were used to target the gene for phospho-β-D-galactosidase. Both Campbell integration and gene replacement events were obtained using selection on glucose-containing media and, unexpectedly, replacement of *lac*G with an erythromycin resistance marker did not cause a lactose negative phenotype. The *lac*F gene is being targeted as an alternative and an explanation for the lactose positive phenotype of the *lac*G mutant is being sought.

(d) The *Lactococcus lactis* chromosomal region containing the seven structural genes required for tryptophan biosynthesis has been characterised by cloning and sequencing. The seven genes are present in the *trp*EGDCFBA order. The *trp* genes are transcribed on a single monocistronic mRNA and its transcription start has been mapped. A region strongly resembling a transcription terminator has been identified

between the transcription start and the 5' end of the *trp* genes. A *trp*E:*lac*Z fusion has been introduced by single cross-over in the *Lactococcus lactis* chromosome. The expression of the fused protein responds to the presence or absence of tryptophan in the medium by a 100-fold factor.The *Lactococcus lactis* chromosomal regions containing the functional genes for histidine and branched-chain amino acids (leucine, isoleucine and valine) biosynthesis have been characterised by cloning and sequencing.The nine genes required for the biosynthesis of histidine are present in a twelve ORFs cluster in the *his* CGDBHAFIE order. The nine genes required for the biosynthesis of branched-chain amino acids are present in a ten ORFs cluster in the *leu*ABCD*ilv*DBNCA order. The region upstream of the *leu* genes has the potential for forming two alternative stem and loop secondary structures, one containing a transcription terminator. This suggests that regulation by transcription attenuation could modulate the expression of the *leu* genes.

(e) Pulsed field gel analysis has led to the identification of various chromosomally integrated elements in *L. lactis* MG1363 including the IS elements IS*S1* and IS *904*, the transposons Tn*919* and Tn*5301*, the temperate bacteriophage øT712 , the sex factor pF1430 and integrated lactose genes . IS*S1* promoted chromosomal integration has been exploited to develop a superior physical mapping strategy.

(f) The genes encoding galactose 1-phosphate uridyltransferase (*gal*T) and galactokinase (*gal*K) were cloned from the chromosone of *Lactobacillus helveticus* 1829 and expressed in *E. coli*. The *gal*K gene encoded a protein with an apparent molecular mass of 49 kDa and was located upstream of *gal*T. *gal*T was sequenced (ORF of 1464 bp, peptide of deduced molecular mass of 55.6 kDa).

(g) The surface layer protein of *Lactobacillus helveticus* was subjected to extensive characterisation. The genetic determinant encoding this protein was cloned in *E. coli*.

2. Development of Genetic Tools

(a) A *Bacillus subtilis* strain in which the *rep* gene of pWVO1 was integrated into the chromosome was constructed. In this host, pWVO1 defective in replication functions, but still carrying the origin of replication (*ori* $^+$), can multiply. This system has now also been developed in lactococci. It was shown that in a *rep*$^-$ *Lactococcus* , the *ori*$^+$ plasmid integrates if it is supplied with a chromosomal segment of lactococcal DNA. The system has been made foodgrade by replacing the antibiotic gene (erm) by sucrose gene(s) of *Pediococcus*, which are expressed in *Lactococcus*. Transformants are easily selected because they grow in sucrose-containing media.

(b) A.4.2 *Hind*II fragment of the conjugative transposon Tn*919* was cloned into a replicating Cm^r plasmid and formed the basis for the construction of a range of integration vectors which were capable of inserting into chromosomally-located transposon DNA. These vectors successfully integrated into Tn*919* present on the

chromosomes of *Enterococcus* and *Lactococcus* by Campbell-type cross-over recombination. These Tn*919*: vector structures could subsequently be transferred to recipient hosts by conjugation. The *abi*829 phage resistance gene was integrated and expressed in the chromosome of *L. lactis* subsp. *lactis* MG1363. Constructs that mediated replacement recombination generated a Cm^r variant of Tn*919*, designated Tn*919* Cm.

(c) A thermosensitive plasmid with a broad host range has been isolated. This plasmid is non-replicative at temperatures above 35°C, thus making it ideal for use as a delivery vector in the mesophilic lactic acid bacteria. The origin of conjugative transfer (*ori*T) of plasmid pIP501 has been characterised. A plasmid vector containing a 1.6 kb *ori*T segment can be mobilized (in the presence of a helper plasmid which supplies transfer functions in trans) into a wide range of LAB recipients. The thermosensitive plasmid has been adapted for transfer to numerous lactic acid bacteria by cloning in *ori*T and has been used as a delivery vector of DNA fragments homologous to the chromosone. Single crossover plasmid integration via homologies of 0.5 and 3 kb between the vector and chromosome have been readily selected in *L. lactis* at 37°C.

3. Conjugation

(a) The mechanism mediating high frequency plasmid conjugal transfer was studied in an *Lb. plantarum* strain. Bacterial cell structures involved in this mechanism were identified. Further aims of this work are the comparison of HF conjugation systems in *Lactobacillus* and *Lactococcus* and the elucidation of roles payed by secreted proteins in the two bacterial genera.

(b) Pulsed-field gel electrophoresis revealed that a 25kb fragment of plasmid pLP712 which was originally integrated into the host chromosome by transduction was maintaned in the same location in a 140KB *Sma*I fragment even following repeated rounds of transduction and conjugal transfer. Analysis of a chromosomally integrated sex-factor suggests that conjugal transfer of the integrated lactose genes reflects their passive transfer during polarized transfer of the whole chromosome. This could lead to a novel strategy for mapping the genome of *L. lactis*. Detailed analysis of the integrated pLP712 DNA precisely determined the region involved as that between co-ordinates 0.26/1.5 and 24.6/27.0.

4. Analysis of Recombination (Rec) Systems in Lactococcus

(a) Cloning of the *L. lactis recA* gene. Degenerate primers were designed, using two highly conserved *Rec*A protein domains as templates determined by aligning previously sequenced *Rec*A proteins. Using Polymerase Chain Reaction, an internal segment of the lactococcal *recA* gene was isolated. This segment was used as a probe to recover the entire *recA* gene of *L. lactis*. Using the same primer pairs, internal segments of 8 other gram-positive *recA* genes were isolated and sequenced (species

include *Lactobacillus, Corynebacterium, Listeria, Leuconostoc and Staphylococcus*). These segments facilitate the isolation and cloning of *rec*A genes which would be difficult to achieve by other means.

5. Studies of inherent plasmid functions.

(a) The replication region of the cryptic 8.7 kb plasmid pCI305 from *L. Lactis* subsp. *lactis* UC317 was cloned and sequenced. A *trans*-acting region, designated *rep*B (ORF of 1158bp, peptide of deduced molecular mass of 46 kDa) was found to be essential for replication as was *rep*A, a replication origin-like region. This contained 22 bp direct repeats repeated three and one half times, in addition to a number of smaller direct and inverted repeat sequences. Studies with pCI305 (which does not produce single-stranded intermediates during replication) indicated that it is incompatible with pAM401 and pIL253 (theta replicating plasmids containing pIP501 and pAMβI replication regions, respectively).

PROTEOLYSIS LEAF

OBJECTIVES SET FOR THE REPORTING PERIOD:

1. Biochemical analysis of proteinase from *Lactobacillus delbruckeii* subsp. *bulgaricus*
2. Molecular analysis of proteinases of lactococci.
3. Biochemical and genetic studies of peptidases of lactococci and lactobacilli.
4. Biochemical and genetic analysis of amino acid and peptide transport systems in lactococci.

RESULTS:

1. Proteinase

Proteinase production by the *Lactobacillus delbrueckii* subsp.*bulgaricus* strain ACADC235 was studied. Maximum yield was obtained at the end of the exponential growth phase in media at pH greater than 5.5. The properties of the extracted enzyme were demonstrated. Plasmid DNA was not detected in the producing strain, suggesting that the proteinase gene is chromosomally located. Hybridisation studies, using lactococcal proteinase genes as probes, have been initiated with a view to localising the proteinase gene on the *Lactobacillus* chromosome.

The proteinase gene complex from pCI301 (from *Lactococcus lactis* sub.*lactis* UC317) was cloned. Two derivatives which encoded a cell-envelope and secreted proteinase were generated. The UC317 proteinase had a hybrid PI/III activity.

Cheddar cheese was made with derivatives of strain UC317 (cured of its native *prt* genes) which produced either the cell envelope-associated or the secreted proteinase and results indicated that the former generated greater proteolytic activity in the cheese. Cheddar cheese was also made with *L. lactis* subsp. *cremoris* SK11 (PII type proteinase), the wild-type UC317 (PI/PIII type) and its Prt^- derivative. Water soluble peptides unique to SK11 and

UC317 were detected by PAGE and HPLC. Sensoric evaluation showed that the flavours of cheese made with Prt^+ strains were similar but those made with the Prt^- isolate lacked flavour.

Control of proteinase production in *L. lactis* SK11 appeared to be host specific and medium dependent. Construction of strains that produced different levels of SK11 proteinase showed that production of the enzyme is rate limiting for growth in milk. The control of proteinase production in SK11 was shown, using *LacZ* fusions, to be less tightly controlled (2-3 fold) than expected.

Expression of *prt* P and *prt* M, using various reporter genes, was measured in several lactococcal strains. In strain MG1363, the activities of the regulatory sequences of both genes were approximately three-fold higher than in strain E8.

2. Peptidase

A novel peptidase assay, using capillary electrophoresis has been developed.

A dipeptidase has been purified from *Lactococcus lactis* subsp. *lactis* NCDO712. The enzyme shows slight differences in substrate specificity when compared to other lactococcal dipeptidases.

A five-step procedure was used for the purification of an aminopeptidase and a dipeptidase from cell-free extracts of *Lactobacillus delbrueckii* subsp. *bulgaricus* B14. Both were metal dependant-enzymes. Suitable substrates for assay of the amino peptidase were L-Lys-NA and N-Ala-L-Arg-NA. The dipeptidase was most active on dipeptides containing an aminoterminal hydrophobic amino acid such as Leu-Leu and Leu-Gly.

The enzymes present in the proteinase/peptidase complex of a strain of *Lactobacillus delbrueckii* subsp. *lactis* have been characterised by elucidation of their physical properties and, particularly, their substrate specifities. A special staining procedure, using chromogenic substrates, was developed to detect peptidase enzymes in colonies of strains containing plasmids from the *L. delbrueckii* gene banks. Three genes, *pep*A for the gene of X-prolyl-amino-peptidase, *pep*B for the gene of an amino peptidase and *pep*C for an iminopeptidase have been isolated. After minimising the genomic DNA, the nucleotide sequence of *pep*A, *pep*B and *pep*C has been determined. Using an alternative approach, a 1.8 kb DNA fragment which encodes the ability to utilise leucine-containing peptides has been isolated. The nucleotide sequence of this gene showed high homology to a gene required for the transport of branched amino acids in *Pseudomonas*.

The gene for lysine-aminopetidase (*lap*, or *pep*N) from the *Lactococcus lactis* subsp. *cremoris* strain Wg2 has been isolated and sequenced. The *lap* gene was demonstrated in several *Lactococcus* strains but not in strain AM2. Comparsion of the deduced amino acid sequences showed that LAP has extensive homology to mammalian aminopeptidases and belongs to the super family of Zn^{2+} metallohydrolases and shows identity to the core deca-peptide concensus sequence for the Zn^{2+} binding motif of these enzymes.

The determinants for the amino-peptidases API and APII from the *Lactococcus lactis*

subsp. *cremoris* strain AM2 have been cloned. The determinant for API was sequenced. The specified protein shares homology with the cysteine proteinases family and was thus designated *Pep*C. No signal sequence was identified, indicating that *Pep*C is intracellularly located, as previously suggested by electron microscopy studies.

Two endopeptides genes from *Lactoccous* strains have been cloned and have been partially sequenced.

Insertional mutagenisis in *pep*X showed that this gene was not essential for casein utilisation and growth of *Lactococcus* strains in milk.

3. Transport

It has previously been shown that the di/tripeptide transport system of *Lactococcus lactis* is essential for the degradation of casein to small peptides and free amino acids. In addition, the di-tripeptide transport system is required for growth of the organism on media containing casein as sole source of amino acids. The transport system delivers di- and tripeptides to the intracellularly located peptidases.

The gene encoding the di/tripeptide transport protein (DtpT) of *L. lactis* has been isolated by complementation of an *Escherichia coli* strain which carried a deletion in the *dppA* gene (encoding the binding protein of the dipeptide transport system) and in which the oligopeptide transport system was inactiviated by random mutagenesis. The origin of the lactococcal di/tripeptide transport gene has been confirmed by Southern analysis, and the functional properties of the protein encoded by the gene have been assessed by transport studies using [^{14} C]-L-ala-L-glu as substrate. The gene has been subcloned in suitable expression vectors and is currently being characterized by DNA sequence analysis.

To establish whether additional peptide transport systems are present in *L. lactis*, mutants have been isolated which lack the DtpT system and/or individual amino acid transport systems. Amino acid (alanine, glycine) negative transport mutants have been isolated in order to exclude possible interference of extracellularly located peptidases in the peptide transport assays. Various single and double mutants and triple mutants lacking the ala/gly, the di-tripeptide and the oligopeptide transport mutants have been characterized with respect to their ability to transport amino acids and peptides using reverse-phase HPLC and radiolabeled substrates. The results, obtained so far, indicate that *L. lactis* possesses an ATP-driven oligopeptide transport system in addition to the proton motive force-driven (DtpT) system. The oligopeptide transport system appears to be highly sensitive to variations in the intracellular pH. The properties of the peptide transport systems in relation to growth of *L. lactis* are currently being analyzed.

PHAGE LEAF

OBJECTIVES SET FOR THE REPORTING PERIOD:

1. Characterisation and molecular analysis of LAB phages
2. Analysis of phage-resistance mechanisms.

RESULTS:

1. Characterisation and Analysis of Phages

Comparsion of nineteen *Leuconostoc mesenteroides* phages isolated from coffee fermentation or dairy products by DNA- hybridisation, morphology and composition of major structural proteins revealed the existence of six genetic groups, four containing only one phage. Twelve phage belonged to a unique group represented by phage øcc59A.

Eight *Leuconsotoc oenos* phages were characterised into two genetic groups containing seven and one phage, respectively.

Lactobacillus plantarum phages isolated from milk and silage samples belonged to the family *Styloviridiae.*

The phage genome of phage TUC2009 (small isometric-headed phage induced from *Lactococcus lactis* subsp. *cremoris* UC507) was mapped and shown to be circularly permuted with terminal redundancy. Early and late expression functions were assigned to specific regions of the TUC2009 map.

The gene for the lysin of the lactococcal phage P001 was cloned using the *E. coli* vector pLG339 and sequenced. An identified open reading frame would encode for a protein of molecular weight 22,327 and was in agreement with an isolated protein with lytic activity. Inside the open reading frame a second putative ribosomal binding site followed by an in-frame start codon at the appropriate distance was found. A lytic protein corresponding to the deduced molecular wight, 8,262, was also isolated. The POOI lysin gene showed a high degree of sequence homology with that of *Lactococcus lactis* subsp. *lactis* phage øvML3.

The genome of the temperate phage mv4, which is representative of a widespread phage genetic group of *Lactobacillus delbrueckii* subsp. *lactis* or *bulgaricus,* is circularly permuted and terminally redundant. A circular map of the 36 kb mv4 genome was established and the location of the *pac* site and the *att* site determined. The genes coding for the two main structural proteins and for a phage associated lysin were also mapped. Phage mv4 is capable of transducing a limited set of pieces of bacterial DNA and several specific attachment sites of mv4 into the cell chromosome were identified. Phage mv4 lysogenic bacteria were shown to be immune to infection by *L. delbrueckii* virulent phages related to mv4.

Despite difficulties to-date in obtaining a high frequency transformation system in *L. delbrueckii* subsp. *bulgaricus*, one early phage promoter has been characterised in the heterologous host, *L. casei* . Using an alternative strategy based on the analysis of the regulatory regions upstream of the phage operons, the nucletodie sequences of two operons have been determined; one contained the endolysin encoding gene and the other included the

genes encoding the phage structural proteins.

While lysogeny is rare among thermophilic lactic acid streptococci, *S. thermophilus* J34 harbours a prophage. Prophage curing and relysogenisation have been achieved. The genome of temperate phage TP-J34 consisted of a circularly permuted and terminally redundant linear DNA ranging in size from 41.5 to 48.7 kbp. On the circular map the *pac*-site and *att*-site were localised. A close relationship between TP-J34 and virulent phages of different subgroups of *S. thermophilus* was shown by DNA-DNA hybridisation and confirmed by immuno blotting and immuno electron microscopy.

2. Phage Resistance

The five abortive infection determinants *abi*-105, *abi*-416, *abi*-420, *abi*-750 and *abi*-829 were shown to be different. The sequenced *abi*-829 gene was found to be identical to the *hsp* gene from pTR2030.

The *abi*-105 and *abi*-416 targets were located on a 2.7 kb DNA fragment from phage bIL66 and on a 8.3 kb DNA fragment from phage bIL41, respectively.

pCI528, a 46kb absorption-blocking plasmid, was found to alter the cell surface characteristics of its host, *L. lactis* subsp. *cremoris* UC503, by directing the production of a galactose and rhamnose containing hydrophilic polymer.

Infection with phages P008 and P416 labeled *in vivo* with the fluoresence dye 4'-6-diamidino-2-phenylindole (DAPI) showed that adsorption and DNA injection were not affected by the lactococcal phage resistance plasmids pBu1-8 and p1149-3. In resitant cells carrying either of the plasmids, phage DNA was replicated over more than five hours post infection but transcription of the phage DNA could not be observed.

Lactococcal plasmids pFNk/094 and pJW563 encode for type II R/M systems. In the case of plasmid pFW094, the methylase gene has been located on a 2.1kb fragment. The endonuclease has been purified and characterised by its ability to digest DNA under different conditions. The recognition sequence of the enzyme, *Lla*II, was found by sequencing. The enzyme is sensitive for *dam*-methylation (isochizomer to *Nde*II and *Mbo*I).

The genes encoding the R/M system of plasmid pJW573 have been located on a 5.0 kb DNA fragment. Two non-homologous genes encoding *Scr*FI methylase enzymes have been cloned from the chromosome of *L. lactis* subsp. *cremoris* UC503 and expressed in *E. coli*. One of the genes has been sequenced and significent homology to the methylase (genes) of *EcoR* II and D*cm* was observed.

Two strains of *Lactobacillus plantarum* produced cell wall bound nucleases that may play a role in phage resistance. Phage resistant mutants of a strain of *Lactobacillus helveticus* and a strain of *Streptococcus thermophilus* were obtained. The resistance was linked to a modification of the phage receptor in the *Lb. helveticus* strains and to a restriction and modification system in the streptococcal strain.

ANTIMICROBIALS LEAF

OBJECTIVES SET FOR THE REPORTING PERIOD:

1. Extend the available range of Lactic Acid Bacteria capable of producing antimicrobials as determined by standardised methodology and agreed target strains.
2. Localise, isolate and characterise the genetic determinants of bacteriocin production.
3. Develop conditons for the production and purification of bacteriocins.
4. Determine the mode action of bacteriocins.
5. Characterise the nisin transposon.

RESULTS:

A test panel of 28 target strains was composed and together with a detailed descripton of methods to be used, distributed to the LEAF participants. This facilitated comparison of bacteriocins produced by strains in different laboratories and ensured that duplication was minimised.

Approximately 70 *Lactobacillus* strains isolated from successful olive fermentations were screened for bacteriocin production. A number of promising strains are being examined in detail. The *Lb. plantarum* strain LPCO-10 produced the bacteriocin designated plantaracin S which was active against strains of *Lactobacillus, Pediococcus, Propionibacterium* and *Clostridium*. The latter two genera are important spoilage agents in olive fermentations. Preliminary experiments suggested that *Lb. plantarum* LPCO-10 produced a compound which cross reacted with antiserum to nisin. However, hybridisation experiments now being finalised suggest that plantaricin S is not the same molecule as nisin. Plantaricin S showed a bacteriocidal mode of action and was active in the pH range 3.0 to 7.0. Ultrafiltration studies suggested a molecular wight in the range 3,000 to 10,000 daltons.

A number of LAB isolated from Spanish dry fermented sausages showed antagonistic activity against spoilage and pathogenic bacteria. For example, *Lactobacillus sake* 148 produced a compound active against various lactobacilli and several Gram-positive foodborne pathogens but not against Gram-negative bacteria tested. The activity was eliminated by treatment with proteases, heat resistant and was bacteriostatic rather than bacteriocidal. Biochemical analysis suggested a molecular weight of 4640 daltons.

A number of LAB isolated from meat environments showed antimicrobial activity against the target indicator strains. One isolate, *Lactobacillus sake* P6, inhibited 18 of 32 *Listeria monocytogenes* strains examined. The antimicrobial involved was sensitive to proteolytic degradation (papain, pronase, trypsin and chymotrypsin) and heat resistant (no effect at 115°C for 15 minutes). While no correlation between plasmid DNA content and the ability to produce the antimicrobial has yet been observed, genetic studies are continuing.

Approximately 20% of 130 LAB isolated from dairy products showed evidence of inhibitory activities and two were chosen for further study on the basis of their broad spectrum of activity and their stability in broth cultures. The activities were heat resistant and sensitive to

proteases. Preliminary characterisation suggests that the apparent size of one of the bacteriocins studied was over 100,000 d., although after SDS-PAGE it was less than 5,000 d, indicating a certain degree of aggregation.

Lactobacillus sake L-45 and *Lactobacillus acidophilus* M247 produced antimicrobials with a very narrow spectrum of activity.

Pediococcus pentosaceus FBB61 produced a plasmid encoded antimicrobial active against several Gram-positive bacteria while *Pediococcus acidilactici* P-2 produced a bacteriocin identical to the one produced by *P. acidilactici* PAC1.0.

Production of the antimicrobial from *Lb. acidophilus* M46 has been optimised by modifying the culture conditons. The use of a very clean production medium (which did not support growth of the culture) and an organic solvent extraction procedure provided an almost pure (>90%) antimicrobial after just one purification step. The molecular weight was estimated at 2400 (SDS-PAGE). The gene(s) encoding this antimicrobial were located within a 4kb fragment of the 14kb plasmid pCV461. Introduction of this fragment into *Lb. plantarum* LP80 resulted in transformants producing antimicrobial activity.

With a view to improved antimicrobial production by *Lactobacillus* strains, gene expression and protein secretion were studied and expression vectors constructed. The applicability of the expression vector for heterologous gene expression has been demonstrated.

For the first time a detailed analysis was made of the mode of action of a lactococcal bacteriocin (lactococcin A). It was convincingly demonstrated that LcnA increases the permeability of the cytoplasmic membrane, and that the immunity protein prevents lactococcin A-induced permeabilization.

A second bacteriocin, lactococcin B was specified by plasmid p9B4-6. This bacteriocin resisted standard purification procedures. A new method, including a rotoforesis and cut off centrifugation-filtration step was successfully developed.

By means of fusing the immunity protein against LcnA to MalE, sufficient fusion product could be purified for raising antibodies against the immunity protein. These will be helpful to investigate the immunity mechanism.

The mode of action of pediocin PA-1, produced by *Pediococcus acidilactici* PAC1.0, was examined and appeared to be comparable to that of Lactococcin A. By means of cloning a DNA fragment carrying the presumptive immunity gene against pediocin in an expression vector, the fragment was shown to carry the immunity gene.

Characterisation of the nisin transposon involved chromosomal walking from the ends of the element using clones from a lambda library. The objective is an ordered collection of lambda clones to cover the entire element. Sequencing from the ends of the element has also begun to build a picture of the arrangement of nisin biosynthetic genes.

METABOLISM AND SCREENING

OBJECTIVES SET FOR THE REPORTING PERIOD:

1. Screening and characterisation of Lactic Acid Bacteria isolated from natural food and feed sources such as wines, olives, meat, sourdough and cheeses.
2. Use of numerical and molecular taxonomic methods to improve classification and identification of LAB.
3. Creation of a deposit and distribution centre for strains of LAB.
4. Biochemical analysis of fermentation pathways and products thereof in LAB
5. Cloning and characterisation of selected key genes relevant to sugar and citrate metabolism.

RESULTS:

1. Screening, Characterisation and Identification of Lactic Acid Bacteria

LAB were screened from Portoguese wines and selected for their ability to perform the malolactic fermentation under conditions present during wine-making.

Twenty-four out of 31 LAB isolates from spoiled port wine formed a single homogenous cluster when analysed by SDS-PAGE and may represent a new species. The determination of 16rRNA of representative isolates is underway. The major fermenting isolates in natural substrates were isolated and included the following:

rye and wheat sourdough:	*Lactobacillus sanfrancisco* and *Lb. brevis*
sorghum sourdough:	*Lb. amylovorus*, *Lb. reuteri* and *Lb. fermentum*
sausage:	*Lb. sake* and *Lb. curvatus*

Over 500 strains from natural food sources were screened for the production of bacteriocins, proteolytic activity and production of exopolysaccharides(EPS). Eight *Lactococcus* strains showed nisin-like antimicrobial production. Based on proteolytic activity, the strains were placed in four groups. Sixteen strains showed a very proteolytic activity. Twenty-seven strains produced exopolysaccarides. A synthetic medium for EPS production has been developed as well as a quantitive isolation and analysis procedure for rapid analysis of different EPS's.

SDS-PAGE fingerprinting identified the majority of LAB isolates from natural sources. Normalised SDS-PAGE protein fingerprints of all the LAB isolates were added to the database developed. Identification to species level was successful for over 80% of the strains obtained. The technique of adding as many strains as possible for every database entry has proved to be extremely valuable for heterogenous taxa.

At least six different protein electrophoretic groups were found within the species *Lactobacillus acidophilus*. Specified r RNA-target oligonucleotide probes for *Lb. acidophilus* and *Lb. gasseri* were developed and excellent agreement was found between the specificity of

the probes and groupings on the basis of protein patterns.

The taxonomic status of *Streptococcus salivarus* subsp. *thermophilus* has been reinvestigated by DNA-DNA hybridisation studies of 24 industrially used strains with the type strains of *S. salivarius* subsp. *salivarius* and *S. salivarius* subsp. *thermophilus*. The two species could be clearly distinguished under stringent hybridisation conditons. These studies, together with phenetic data, led to the proposal to revive the species *S. thermophilus*.

A 23SrRNA targeted oligonucleotide probe for the specific identification of *S. thermophilus* has been developed.

2. Metabolic Studies

The effects of aeration and the presence of malic acid on the metabolism of glucose were monitored in growing and non-growing cells of *Leuconostoc oenos* and revealed considerable alterations to the patterns of end products when aerobic and anaerobic conditions were compared.

Erythritol and glucose were detected as the two major products of glucose metabolism investigated using *in vivo* 18C-NMR. The metabolites detected and the fermentation shift observed in the presence of oxygen indicated that glucose was catabolised heterofermentatively as well as via a novel erythritol-forming pathway.

Strains of *Lb. sanfrancisco* isolated from rye and wheat sourdough grew readily on maltose but required adaption times of up to 150h for growth on glucose. After subsequent transfer of glucose-grown cells to fresh media, the cells were able to grow on glucose or maltose, but lost this ability to utilise glucose upon renewed exposure to maltose. During fermentation of maltose, *Lb. sanfrancisco* strains accumulated glucose in the buffer and produced lactic acid, acetic acid and ethanol.

The utilisation of lactose by *Lactobacillus helveticus* involved the enzyme β-galactosidase whose activity was inducible by lactose and IPTG but repressed by glucose. The basal level of the activity of the enzyme increased 25 fold as the incubation temperature was increased from 37 to 44°C. Investigation of instability of the lac^+ phenotype suggested that the *lac* determinants were not solely plasmid linked.

Hybridisation studies suggested that the β-galactosidase gene of *Lb. bulgaricus* and the lac permease gene of *S. thermophilus* showed little homology to the *lac* genes of *Lb. helveticus*.

An *Eco*RI restriction fragment of chromosomal DNA from *Lb. helveticus* expressing β-galactosidase has been cloned and is being analysed.

Lactate dehydrogenase is a key enzyme in sugar metabolism and offers opportunities to engineer citrate metabolism. From published sequences for bacterial lactate dehydrogenase genes, primers were designed and used to generate a PCR probe for lactococcal lactate dehydrogenase. Clones for this gene were isolated from a lambda gene library of *L. lactis* MG1363 and the lactate dehydrogenase gene was sub-cloned and fully sequenced. The

lactococcal gene was 975 bp encoding a protein of 325 aa. The lactococcal enzyme had homology with other published lactate dehydrogenase sequences including *S. Mutans* (76.4%), *Lb. casei* (68.2%), *Lb. plantarum* (57.5%) and various *Bacillus* species (>50%).

During purification of α-acetolactate decarboxylase, the enzyme was found to be highly unstable. In contrast, purification of diacetyl reductase proceeded without obstacles and presently antibodies are being raised against the purified enzyme.

In growing cells of *Leuconostoc lactis* NCW1, citrate decreased lactose uptake and simultaneously increased the growth rate at pH 4.5, 5.5 and 6.5. No acetoin, ethanol, diacetyl or acetolactate (ALA) were produced but higher than the theoretical amounts of acetate and lactate were formed. Production of ethanol began as soon as citrate consumption decreased to zero. In non-growing cells, little uptake of citrate occurred unless glucose was added at pH 4.5, 5.0 or 5.5. Citrate uptake was more rapid than glucose uptake. ALA was produced in significant amounts from citrate and glucose only when the glucose level was low.

With resting cells of *Leuc. mesenteroides* Fr8/1, the rate of citrate uptake was lower than that of glucose in acetate buffer and faster than that of glucose in phosphate buffer and no citrate was taken up in the absence of sugar. Both citrate and glucose utilisation were non-linear at pH 4.5 and linear at pH 5.0 and 5.5. At the latter two pH values, acetoin and ALA were produced from a mixture of glucose and citrate but only after glucose utilisation was complete.

In contrast to the leuconostocs, resting cells of Cit$^+$ *Lactococcus lactis* subsp. *lactis* 19B metabolised considerable amounts of citrate in the absence of glucose and produced acetoin and ALA in the presence of glucose.

MAJOR PROBLEMS ENCOUNTERED:

Although the Lactic Acid Bacteria are difficult targets for genetic analysis, problems encountered did not inferfere with the specific objectives of the project.

HIGHLIGHTS/MILESTONES:

Successful use of luciferase to monitor controlled gene expression *Lactococcus*. Discovery of a chromosomal mobilisation in *L. lactis* MG1363. Development of a reliable foodgrade integration system for lactococci. Definition of promoter-operator regions and identification of a new catabolite repression control circuit. Identification of the aggregation promoting factor and the binding substance responsible for the high frequency of conjugation in *Lb. plantarum*.. Construction of expression vectors for *Lactobacillus*.

Construction of strains that overproduce the cell envelope located serine proteinase and demonstrate improved growth in milk. Cloning of a range of peptidase genes. The isolation of the DtpT gene representing the first example of a bacterial transport system that is encoded by a single gene, and is driven by a proton motive force. Isolation of amino acid and

peptide transport mutants that allow a full analysis of the role of the individual transport systems in the degredation of caseins.

Characterisation of the temperate phage mv4 of *Lb. delbrueckii* subsp.*bulgaricus*. Identification of the receptor for a *Lactobacillus helveticus* lytic phage. Isolation of nuclease and methylase genes involved in R/M systems. Elucidation of the structure of the regulatory region of a phage late operon.

Isolation of strains with broad spectrum antimicrobial activity. Purification of the antimicrobial from *Lb. acidophilus* M46. Successful transfer of the genes responsible for antimicrobial production from *Lb. acidophilus* M46 to *Lb. plantarum*. Elucidation of the mode of action of Lactococcin A.

Cloning and sequencing of the lactococcal lactate dehydrogenase gene. Isolation of strains of *Lb. sanfrancisco* with an effective but unusual maltose metabolism. Demonstration of a new pathway leading to the formation of erythritol by *Leuconstoc oenos* .

Major advances in the screening and identification of LAB. Excellent correlation between DNA probes and protein profiles of investigated LAB. Development of a synthetic medium in which 1g/litre of exopolysaccharide can be produced.

WIDER CONSIDERATIONS:

The T-Project on Biotechnology of Lactic Acid Bacteria has already, in its first year, demonstrated the success of the approach of forming and fostering a large transnational team to tackle a major research objective. The industrially important LAB include several genera that are vitally important in key indigenous industries throughout Europe e.g. dairy, meat and bread fermentations and winemaking. The present project is making essential advances in the knowledge of the genetics, physiology and biochemistry of a range of LAB.

Considerable advances have been achieved in aspects of the molecular biology of plasmids, transposons, the chromosome, individual genes and their expression, as well as strain improvement and protein engineering. Technologies for chromosome integration, the construction of food grade markers and cell lysis are being developed.

The CORE and LEAF concept of the project ensures that the research findings underpin important aspects of the application of LAB in industry.

The very significant progress achieved in the molecular analysis of the proteolytic activity of LAB has created the basis for understanding fundamental factors concerning growth of these bacteria in milk. It will, in addition, help unravel the complex questions regarding the specific role of starter cultures in ripening and flavour development in a range of fermented dairy products.

Modern analytical techniques have been successfully applied to the study of bacteriophages of LAB and to the elucidation of the molecular basis for their interaction with their hosts. A "food grade" strategy, involving the transfer of phage resistance plasmids by

conjugation to appropriate recipient strains has been used to construct lactococcal strains with enhanced phage resistance. Strains constructed in this manner are currently used in commercial cheesemaking and represent one of the first tangible benefits to industry stemming from genetic analysis of lactic acid bacteria. Furthermore, the imminent availability of self-cloning strategies for these hosts, coupled with knowledge of factors controlling gene expression and regulation, plasmid stability and incompatibility, will soon allow for the provision of an expanded range of phage resistant strains for commercial use.

The discovery of a range of antimicrobials produced by LAB is an exciting development. The research programme being undertaken includes studies on the purification, biochemical characterisation, mode of action and genetics of several bacteriocins and should permit targeted applications in food systems with the objective of enhancing quality and safety.

The metabolism research is focused on the fermentations of lactose, maltose, citrate and malate which are vital to the industrial use of LAB in the dairy, bread and wine industries. Key traits of LAB used in meat fermentations are also targeted. In addition to conventional biochemical assays, the powerful technique of *in vivo* NMR is being exploited. An important aspect of the research is the cloning and analysis of the enzyme genes in the various pathways. As well as adding to the overall characterisation of the important metabolic pathways, this will facilitate a metabolic engineering approach to the elevation of the yield of flavour compounds in food fermentations.

In the screening activity, large numbers of LAB are being tested for a variety of key industrially relevant properties. This activity is backed up by taxonomic studies including the analysis of protein profiles using SDS-PAGE and the use of ribosomal RNA probes. In addition to ensuring the correct characterisation and identification of LAB, these studies will provide valuable "tags" for industrially useful strains.

The T-Project provides a network that continues to keep Europe at the forefront of the industrially important research on LAB. The award of the 1992 Rhone-Poulenc International Award of the American Dairy Science Association to Professor Gerard Venema of Groningen University, who has participated in CEC sponsored LAB research since 1982, is a fitting reflection of the esteem in which European research in this field is held.

COOPERATIVE ACTIVITIES

A plenary meeting of all 34 groups participating in the project was hosted by Prof. Helena Santos at Estoril, Portugal in April 1991. The second plenary meeting is scheduled for Cork in May 1992.

Meetings of the CORE and LEAF subgroups were hosted by INRA, Jouy-en-Josas, F; TNO-Zeist, NL; NIZO, Ede, NL; Unilever Research, Vlaardingen, NL and University of Groningen, Haren, NL in the period October to December, 1991.

Several participants met, in addition, at LAB conferences at Caen, F and Sevilla, E.

There is very extensive collaboration between laboratories, featuring exchange of materials e.g. bacterial strains, DNA probes, cloned genes and antibodies, as well as methodologies. Within the LEAF, Antimicrobials, assay methods have been standardised and a set of target strains have been distributed to all participating laboratories. Close collaboration is a feature of the activities associated with the screening, characterisation and identification of LAB from natural sources.

Staff exchanges have included 9 extended visits totalling 49 man-months and an additional 21 short visits of up to two weeks duration. Three scientists financed under the Training Activity of the CEC BRIDGE Programme are being hosted at Cranfield Institute of Technology, UK; University College, Cork, IRL and University of Groningen, NL.

Thirteen joint publications with transnational authorship have been published. Three other publications featured collaboration between laboratories within individual countries.

LIST OF JOINT PUBLICATIONS/PATENTS WITH TRANS-NATIONAL AUTHORSHIP:

Boizet, B., Mata, M., Mignot, O., Ritzenthaler, P. and Sozzi,T. (1992). Taxonomic characterisation of *Leuconostoc mesenteroides* and *Leuconostoc oenos* bacteriophages FEMS Microbiology Letters 90, 211-216.

Cocconcelli, P.S., Gasson, M.J., Morelli, L., Bottazzi, V. (1991). Single-stranded DNA plasmid, vector construction and cloning of *Bacillus stearothrmophilus* - amylase in *Lactobacillus* Res. Microbiol. 142, 643-652.

Hayes, F., Vos, P., Fitzgerald, G.F., de Vos, W.M. and Daly, C., (1991). Molecular organisation of the minimal replicon of the novel, narrow-host-range, lactococcal plasmid pCI305. Plasmid 25, 16-26.

Jarvis, A.W., Fitzgerald, G.F., Mata, M., Mercenier, A., Neve, H., Powell,I.B., Ronda, C., Sazelin, M., and Teuber, M. (1991). Species and type phage of lactococcal bacteriophages. Intervirology, 32, 2-9.

Law, J. ,Vos, P., Hayes, F., Daly, C., de Vos, W.M. and Fitzgerald , G.F. (1992). Cloning and partial sequencing of the proteinase gene complex from *Lactococcus lactis* subsp. *lactis* UC317 J. Gen. Microbiol. 138, 709-718.

Mayo, B., Kok, J., Venema, K., Bockelman, W., Tueber, M., Reinke, H. and Venema G. (1991). Molecular cloning and sequence analysis of an X-prolyl dipeptidyl aminopeptidase form *Lactococcus lactis* subspec. *cremoris*. Appl. Environ. Microbiol. 57 38-44.

Poolman, B., Molenaar, D. , Smid, E.J., Ubink,T., Abee, T., Renault, P.P.and Konings, W.N. (1991). Malolactic fermentation: electrogenic malate uptake and malate/lactate antiport generate metabolic energy. J. Bacteriol. 173, 6030-6037.

Tan, P.S.T., Chapot-Chartier, M.P., Pos, K.M., Rousseau, M., Boquin, C.Y., Gripon, J.C. and Konings,W.N.(1992). Localization of peptidases in lactococci. Appl. Environ. Microbil. 58, 285-293.

Tichaczek, P., Nixxen-Meyer, J., Nes, I.F., Vogel, R.F. and Hammes, W.P. (1992). Characterization of curvacin A of *Lactobacillus curvatus* LTH1174 and sakacin P. of *L. sake* LTH673 System. Appl. Microbiol. (in press).

van Belkum, M.J., Kok, J., Venema, G., Holo, H., Nes, I.F., Konings, W.N. Abee, T. (1991) The bacteriocin *lactococcin* A specifically increases permeability of lactococcal cytoplasmic membranes in a voltage-independent, protein-mediated manner. J. Bacteriol 173, 7934-7941.

van der Lelie, D., Chavarri, F., Venema,G., and Gasson, M.J.(1991). Identification of a new genetic determinant for cell aggregation associated with lactose plasmid transfer in *Lactococcus lactis* . Appl. Environ. Microbiol 57, 201-206.

van Rooijen, R.J., Gasson, M.J. and de Vos, W.M. (1992) Characterisation of the promoter of the *Lactococcus lactis* lactose operon: contribution of flanking sequences and *lac*R repressor to its activity. J. Bacteriol. 174 (in press).

Winters, D.A., Poolman, B., Hemme, D.,. and Konings, W.N. (1991) Branched-chain amino acid transport in cytoplasmic membranes of *Leuconostoc mesenteroides* subsp. *dextranicum* CNRZ 1273 Appl. Environ. Microbiol. 57:3350-3354.

OTHER PUBLICATIONS/PATENTS:

Bobillo, M. and Marshall, V.M., (1991). Effect of salt and culture aeration on lactate and acetate production by *Lb plantarum* Food Microbiol 8 153-160.

Bobillo, M. and Marshall,V.M., (1992). Effect of acidic pH and salt on acid end products by *Lb plantarum* in aerated, glucose-limited continuous culture. J. Applied Bacteriol. (in press).

Bockelmann, W., Schulz, Y. and Teuber, M. (1992). Purification and characterisation of a dipeptidase from *Lactobacillus delbrueckii* subsp. *bulgaricus*. Int. Dairy J. (in press).

Bruinenberg, P.G., Vos, P., and de Vos, W.M., (1992). Proteinase overproduction in *Lactococcus lactis* strains; regulation and effect on growth and acidification in milk. Appl. Environ. Microbiol. 58, 78-84.

Casey, C., (1991). Molecular and physical characterisation of lysogenic and lytic bacteriophage. Ph.D. Thesis, Cork.

Casey, J., Daly, C., and Fitzgerald, G.F. (1991). Chromosomal integration of plasmid DNA by homologous recombination in *Enterococcus faecalis* and *Lactococcus lactis* subsp. *lactis* hosts harbouring Tn*919*. Appl. Environ. Microbiol., 57, 2677-2682.

Chapot-Chartier M.P., Nardi, M., Chopin, M.C., Chopin, A. and Gripon, J.C. (1992). Cloning and DNA sequencing of *pep*C, a cysteine aminopeptidase gene from *Lactococcus lactis* subsp. *cremoris* AM2 (submitted to Appl. Environ. Microbiol).

Cocconcelli, P.S., Triban, E., Basson, M. and Bottazzi,V. (1991). Use of DNA probes in the study of silage colonization by *Lactobacillus* and *Pediococcus* strains. J. Appl. Bacteriol., 71, 296-301.

Coffey, A., Costello, V., Daly, C. and Fitzgerald, G.F. (1991). Plasmid-encoded bacteriophage insenstitivity in members of the genus *Lactococcus* with special reference to pC1829. Genetics and Molecular Biology of Streptococci, Lactococci and Enterococci, Ed. G.M. Dunny et. al. (American Society for Microbiology) pp 131-135.

Coffey, A.G. (1990). Genetic analysis of plasmid encoded bacteriophage insensitivity in *Lactococcus*.. PhD Thesis Cork.

Coffey, A.G., Fitzgerald, G.F., and Daly, C. (1991). Cloning and characterisation of determinant for abortive infection of bacteriophage from lactococcal plasmid pC1829. J. Gen. Microbiol. 137, 1355-1362.

Collins, M.A. andThompson, J.K. (1992). Lactose metabolism and lactose gene sequence homologies amongst lactobacilli. J. Applied Bacteriology (submitted).

Ehrmann, M., Ludwig, W. and Schleifer, K.H. (1992). Species specific oligonucleotide probe for the identification of *Streptococcus thermophilus*. Syst. Appl. Microbiol. 15 (in press).

Fitzgerald, R.J., Doonan, S., McKay, L.L. and Cogan, T.M. (1992) Intracellular pH and the role of D-lactate dehydrogenase in the production of metabolic products by *Leuconostoc lactis*. J. Dairy Research (in press).

Gasson, M.J., Swindell, S., Maeda, S.M. and Dodd, H.M. (1992) Molecular rearrangement of lactose plasmid DNA associated with high frequency transfer and cell aggregation in *Lactococcus lactis* 712. Mol. Microbiol (submitted).

Geis, A. (1992). Cloning and DNA sequence analysis of a lysin gene of the lactococcal bacteriophage P001 FEMS Microbiol Lett. (submitted).

Geis, A., Janzen, T., Tueber, M. and Wirsching.F., (1992). Mechanism of plasmid mediated bacteriophage resistance in lactococci. FEMS Microbilo. Lett. (submitted).

Goodman ,S.A., Orr, C. and Warner, P.J. (1991) Development of yeast inhibitory compounds for incorporation in silage inoculants NATO ASI Series in press.

Goodman, S.A. and Warner, P.J. (1991). Stabilisation of silage UK Patent application 9017286 7.

Griffin H., Swindell S., and Gasson., M.J. (1992) Cloning and DNA sequence analysis of the gene for 2-lactate dehydrogenase from *Lactococcus lactis* : evolutionary relationships between twenty one different *ldh* genes. Gene (submitted).

Haandrikman, A.J., Kok, J. ,and Venema ,G. (1991). Lactococcal proteinase maturation protein Prt M is a lipoprotein J. Bacteriol. 173, 4517-4525.

Haandrikman, A.J.,Meesters, R.,Laan, H., Konings, W.N., Kok, J. and Venema G. (1991). Processing of the lactococcal extracellular serine proteinase Appl. Environ. Microbiol. 57, 1899-1904.

Hill, C., Daly, C. and Fitzgerald, G.F. (1991). Isolation of chromosomal mutations of *Lactococcus lactis* subsp *lactis* biovar. *diacetylactis* 18-16 after introduction of Tn*919*. FEMS Microbiol. Lett. 81, 135-140.

Kersters, K. and Pot B. (1991). Classification and identification methods for lactic acid bacteria with emphasis on protein gel electrophoresis. Lecture and abstract "Lactic 91", Caen, France, September.

Kok, J., (1990). Genetics of lactococcal proteinases in: Proceeding 6th Internationl Symposium on Genetics of Industrial Microorganisms (ed. H. Heslot, J. Davies, J. Florent, L. Bobichon, G. Durand, L. Penasse) Societe Francaise de Microbiologie, Strassbourg. Vol. 1 pp 459-466.

Kok, J. (1991). Proteinase genes of cheese starter cultures. Biochemical Society Transaction. 19, 670-674.

Kok, J., (1991). Special purpose vectors for lactococci in; Genetics and Molecular Biology of *Streptococci, Lactococci* and *Enterococci* (ed. G. Dunny, P. Cleary, L.L.McKay) ASM, Washington pp 97-102.

Konings,W.N., Smid, J., Laan, H., Driessen,A.J.M. (1991). From casein to cheese: the role of *Lactococcus lactis*. Food Biotechnology. 5, 263-277.

Laan, H. (1991) The extracellular proteinase of *Lactococcus lactis* PhD Thesis University of Groningen.

Laan, H. and Konings, W.N. (1991). Autoproteolysis of the extracellular serine proteinase of *Lactococcus lactis* subsp. *cremoris* Wg2. Appl. Environ. Microbiol. 57, 2586-2590.

Lahbib-Mansais, Y., Boizet, B., Dupont, L., Mata, M., and Ritzenthaler P. (1992) Characterisation of a temperate bacteriophage of *Lactobacillus delbrueckii* subsp. *bulgaricus* and its interactions with the host cell chromosome. Journal of General Microbiology. (In press).

Law, J.M., (1991). Biochemistry and genetics of the proteolytic system in *Lactococcus*. PhD Thesis Cork.

Law, J.M., Fitzgerald,G.F., Daly, C., Fox, P.F., and N.Y. Farkye. (1992). Proteolysis and flavour development in Cheddar cheese made with the single starter strains *L. lactis* subsp. *lactis* UC317 or *L. lactis* subsp.*cremoris* HP. J. Dairy Sci. 75, 1173-1185.

Le Bourgeois, P., Lautier, M., Mata, M. and Ritzenthaler P. (1992). New tools for the physical and genetic mapping of *Lactococcus* strains. Gene 111, 109-114.

Leenhouts, K.J., Gietema, J., Kok, J. and Venema, G. (1991). Chromosomal stabilization of the proteinae genes in *Lactococcus lactis* . Appl. Environ. Microbiol. 57, 2568-2575.

Leenhouts, K.J., Kok, J. and Venema, G. (1991). Lactococcal plasmid pWVO1 as an integration vector for Lactococci, Appl. Environ. Microbiol. 57, 2562-2567.

Leenhouts, K.J., Tolner, B., Bron, S., Kok, J., Venema, G. and Seegers, J.F.M.L., (1991). Nucleotide sequence and characterisation of the broad host-range lactococcal plasmid pWVO1. Plasmid, 26, 55-66.

Leenhouts, K.J., Kok, J. and Venema, G.(1991). Replacement recombination in *Lactococcus lactis*. J. Bacteriol. 173, 4794-4798.

Meyer, A.D. and Warner, P.J. (1991). Induction of the 6-phospho gluconate pathway is correlated with change of colony morphology in *Lactobacillus acidophilus*. Abstracts 'Lactic acid bacteria, applications in the Agro-food Industries' Caen M10. September.

Pimentel, M. S., (1991). Metabolism of citrate and malate by *Leuconostoc oenos*.. Work presented as partial fulfilment of the degree in Enology, Universidade de Tras os Montes e Alto Douro.

Pot B., Deescheemaeker, P., Roeges, N. and Kersters K. (1991). Identification and classification of lactic acid bacteria by SDS-PAGE of whole-cell proteins. Poster and Abstract "Lactic 91" Caen, France, September.

Pot B., Descheemaeker, P., Roeges, N. and Kersters K. (1991). Identification and classification of lactic acid bacteria with emphasis on protein gel electrophoresis. Poster and Abstract BA10 Macromolecular Identification and Classification of Organisms, Antwerpen, Belgium, December 7.

Reniero, R., Cocconcelli, P.S., Bottazzi, V. and Morelli, L. (1992). High frequency of conjugation in *Lactobacillus* mediated by an aggregation-promoting factor. J. Gen. Microbiol. 138 (in press).

Schleifer, K.H., Ehrmann, M., Krusch, U., Neve, H., (1991). Revival of the species *Streptococcus thermophilus* (ex-Orla-Jensen, 1919) nom. rev. Syst. Appl. Microbiol. 14, 386-388.

Schouler, C., Bouet, C., Mata, M., Drouet, X. and Ritzenthaler P. (1992) Characterisation of *Lactococcus lactis* phage antigens. Appl. Environ. Microbiol. (in press).

Schulz, Y. and Bockelmann, W. (1992). Purification and characterisation of a dipeptidase from *Lactobacillus delbrueckii* subsp. *bulgaricus*. Int. Dairy J. (in press).

Seegers, J., Leenhouts, K., Kok, J., Venema, G. and Bron, S . (1991). New type of minus origin in the broad-host-range plasmid pWV01, Book of Abstracts 6th International Conference on Bacilli Stanford. Calif, 28-31 July.

Smid, E. (1991) Physiological implications of peptide transport in lactococci. PhD thesis University of Groningen.

Smid, E.J., Poolman, B. and Konings,W.N. (1991). Casein utilization by lactococci. Appl. Environ. Microbiol. 57, 2447-2452.

Smith, H., Van Dijl, J.M, de Jong, A., Perez-Martinez, G., Kok, J , Venema, G. and Bron, S. (1991). Signal sequences from *B. subtilis* and *L. lactis*, Book of Abstracts First European Meeting on Protein export in *Bacillus subtilis*, Schiermonnkioog, The Netherlands 25-26 April.

Sobrino,O.J., Rodriguez, J.M., Moreira, W.L., Fernandez, M.F., Sanz, B. and Hernandez, P.E. (1991). Antimicrobial activity of *Lactobacillus sake* isolated from dry fermented sausages. International Journal of Food Microbiology. 13, 1-10.

Strøman, P. (1992) Sequence of a gene (*lap*) encoding a 95.3 KDa aminopeptidase from *Lactococcus lactis* subsp. *cremoris* Wg2. Gene 113, 107-112.

Tan, P.S.T., Pos, K.M. and Konings, W.N. (1991). Purification and characterization of an endopeptidase from *Lactococcus lactis* subsp. *cremoris* Wg2. Appl. Environ. Microbiol. 57, 3593-3599.

van Belkum, M.J. (1991). Lactococcal bacteriocins: genetics and mode of action Diss. RUG, Groningen.

van Belkum, M. J., Haijema, B.J., Jeeninga, R.E., Kok, J. and Venema, G. (1991). Organisation and nucleotide sequence of two lactococcal operons. Appl. Environ. Microbiol. 57, 492-498.

van de Guchte, M., (1991). Heterologous gene expression in *Lactococcus lactis*. Diss, RUG, Groningen.

van de Guchte, M., Kok, J. and Venema, G., (1991). Distance-dependent translational coupling and interference in *Lactococcus lactis*, Mol. Gen. Genet. 227, 65-71.

van de Guchte, M., van der Lende, T., Kok, J. and Venema, G., (1991). A possible contribution of mRNA secondary structure to translation initiation efficiency in *Lactococcus lactis*. FEMS Microbiol. Letters 81, 201-208.

van Rooijen, R.J., van Schalkwijk, S., and de Vos, W.M. (1991). Molecular cloning, characterisation and nucelotide sequence of the tagatose 6-phosphate pathway gene cluster of the lactose operon of *Lactococcus lactis* . J. Biol. Chem. 266, 7176-7181.

Vaughan, E., (1991). Characterisation of a bacteriocin produced by *Lactobacillus helveticus* and cloning of chromosomal genes by heterologous completmentation. PhD Thesis Cork.

Veiga da Cunha, M., Firme, P., San Romao, V.S and Santos, H. (1992). Application of 13C-NMR to elucidate the unexpected biosynthesis of erythritol by *Leuconostoc oenos* Appl. Environ. Microbiol. (submitted).

Vos, P., Boerrigter, I.J., Buist, G., Haandrikman, A.J., Nijhuis, M., de Reuver, M.B., Siezen, R.J., Venema, G., de Vos, W.M.and Kok, J. (1991). Engineering of the *Lactococcus lactis* serine proteinase by construction of hybrid enzymes. Protein Engineering 4, 479-484.

Warner, P.J. (1991). Prospects for use of genetically manipulated microorganisms in agriculture SGM Quart 18, 27.

Wolmowska, R., Ceglowski, P., Kok, J. and Venema, G. (1991). Isolation, sequence and expression in *EScherichia coli*, *Bacillus subtilis* and *Lactococcus lactis* of the DNase (streptodornase)-encoding gene from *Streptococcus equismilis* H46A, Gene, 106, 115-119.

T-PROJECT

FACTORS REGULATING PLANT CELL GROWTH AND DIFFERENTIATION

Factors Regulating Growth and Differentiation of Plant Cells
A Basis for the Understanding of Plant Regeneration

Coordinator:	J. Guern Institut des Sciences Végétales CNRS Gif-sur-Yvette (F)
Contract:	BIOT 900206
Sub-Coordinators:	K. Palme, Max-Planck-Inst., Köln, (D) (see page 456) P. Costantino, Univ. di Roma "La Sapienza", (I) (see page 461) P. Pechan, Max-Planck-Inst., Köln, (D) (see page 465) S. de Vries, Agricultural Univ. Wageningen, (NL) (see page 468) M. Hall, Univ. College Wales, Aberysthwyth, (UK) (see page 471)
Contracts:	BIOT 900178, BIOT 900179, BIOT 900160, BIOT 900177, BIOT 900158 (see following reports)
Duration:	36 months from January 1991

After one years work, 25 laboratories structured in 5 groups, working together in the frame of the T-Project, have built a strong network, involving more than 120 researchers, aiming to understand the mechanisms by which growth factors control the differentiation of plant cells.

Well in line with the initial goals of the T-Project, genes and gene products corresponding to putative auxin receptors, genes coding for G-proteins, genes and gene products involved in the regulation of the cell cycle, putative ethylene and fusicoccin receptor binding proteins, have been isolated and characterized. The isolation and characterisation of extracellular glycoproteins involved in the regulation of somatic embryogenesis, the characterisation of cell wall epitopes specific for differentiation steps, the isolation of well defined oligosaccharides with growth regulating activities, the detailed dissection of promoters of the *rol* genes from *Agrobacterium rhizogenes*, the analysis of the function of some of these genes have also been significant achievements of this first phase of the T-Project. More than 80 papers have been published. Powerful tools (c-DNAs, antibodies, oligosaccharides, labelled ligands, transgenic plants, etc) have been built and circulated during this period, offering new languages to describe early events of plant morphogenesis.

An impressive scale of cooperation between the members of the T-Project has been established during this first year. Frequent sectoral meetings, a general meeting, considerable exchange of tools and personnel, efficient cooperation with the industrial partners clearly demonstrate the strength of the multidisciplinary character of the T-Projects. The second phase of the project will certainly accelerate progress of our knowledge of the regulation of growth and differentiation in Plant cells.

TITLE:	Molecular Analysis of Auxin-Specific Signal Transduction in Plant Cell Communication
CONTRACT NUMBER:	BIOT-CT90-0178
OFFICIAL STARTING DATE:	03/01/91
COORDINATOR:	K. Palme, Max-Planck-Institut, Köln, DE (MPI)

PARTICIPANTS:
K. Palme, Max-Planck-Institut, Köln, DE (MPI)
M. Venis, Horticulture Research Int., Kent, UK (HRI)
H. Barbier-Brygoo,Institut Sciences Vegetales CNRS, Gif-sur Yvette, FR (ISV)
D. Inzé, Rijksuniversiteit Gent, Gent BE (RUG)
P.A. Millner, University of Leeds, Leeds, UK (ULeeds)
F. Okkels Danisco/AS, Copenhagen, DK (Danisco)

OBJECTIVES SET FOR THE REPORTING PERIOD:
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 analysis of cell division control genes and genes encoding small GTP-binding proteins (RUG); to identify and study heterotrimeric G proteins (ULeeds); to identify and study novel auxin binding proteins (MPI, Danisco).

MAJOR PROBLEMS ENCOUNTERED:
Difficulties to immunolocalize ERabp in tissue sections with a wide variety of poly- and monoclonal antibodies.

RESULTS:
Research in our subgroup has focused on the (i) identification and characterization of genes encoding putative auxin receptors, (ii) to understand at the molecular level the primary physiological and morphogenic consequences of auxin action, (iii) the characterization of cell cycle control genes, and (iv) the analysis of small and heterotrimeric GTP-binding proteins. Auxin is involved in the control of various aspects of growth and development in higher plants, including cell division, stem elongation, xylem differentiation, gravitropism, and senescence. Receptor like proteins have been assumed to play an important role in recognition and transmission of the auxin signal. Additional members of a gene family encoding maize auxin bindig proteins, termed Zm-ERabp's for Zea mays auxin binding protein located in the lumen of the endoplasmic reticulum (ER), have been isolated and shown to be expressed in specific tissues. *In vitro* studies demonstrated that the Zm-ERabp1 protein was translocated into ER derived microsomes, processed and glycosylated (MPI). A gene encoding the *Arabidopsis* homologue for Zm-ERabp1 was isolated and mapped to a single locus on chromosome 4. To obtain more information on possible *in planta* functions of these proteins, transgenic tobacco plants overproducing Zm-ERabp1 were constructed and analyzed for changes in auxin sensitivity. As expected auxin sensitivity was increased in these transgenic plants. Protoplasts isolated from these plants showed a 30-fold increase in auxin sensitivity of transmembrane potential (ISV, MPI). This increase was selectively abolished using monoclonal antibodies that recognize only Zm-ERabp1, but not endogenous

tobacco ER-abps. These results suggest that at least a fraction of this protein is released from the ER and located at the plasma membrane. It was shown that Zm-ERabp1 stimulated root elongation growth in a novel tobacco mutant identified by T-DNA mutagenesis and impaired in auxin related growth characteristics (MPI). A region of the maize auxin binding protein (Zm-ERabp1) containing several amino acids likely to be present at the active auxin binding site was identified. A polypeptide embracing this region was synthesised, coupled to carrier protein, and polyclonal antisera produced (HRI). Several of these antisera recognised all three isoforms of maize ERabp's on Western blots, as well as ABP homologues in other species, both monocots and dicots, and IgG fractions from two of these sera were shown (ISV, HRI) to hyperpolarise protoplast membrane potential characteristic of auxin action. The anti-peptide antibodies have apparent auxin "agonist" activity, showing that the selected peptide probably is part of the auxin binding site and supporting the role of Zm-ERabp1 as an auxin receptor (ISV, HRI). Using an epitope mapping kit, the epitopes recognised by all these antibodies have been located, providing information for structural and functional analysis (HRI). All anti-ABP sera, including those from MPI (recombinant Zm-ERabp1) or other sources recognise two or three dominant epitopes clustered around the glycosylation site. Two monoclonals are directed at the endoplasmic reticulum (ER) retention sequence KDEL at the C-terminus of ABP. Interestingly, none of these antibodies were able to interfere with the auxin-induced transcriptional activation of the chimeric *rolB* promoter:GUS reporter gene (ISV). This raises the possibility of multiple auxin-perception-transduction pathways in various plant tissues and cells. To probe further into novel auxin binding proteins and their function, a joint PhD project (MPI, Danisco) has recently been started. Immunolocalization studies have been performed to identify the specific localization of some of these proteins. In addition antibodies against recombinant abps were used to search in λgt11 expression libraries for clones that represent potential candidates for novel auxin binding proteins.

As a first step for studying the regulation of cell division in plants, several *Arabidopsis* genes with high homology to cell cycle regulation genes characterized in other organisms were isolated. A *cdc2*-homologous cDNA was cloned from *Arabidopsis thaliana* (RUG) encoding a polypeptide with extensive homology to $p34^{cdc2}$ protein kinases. Complementation experiments of ts-mutants in yeast confirmed that the *Arabidopsis* cDNA encodes a functional homologue for the $p34^{cdc2}$ kinase. Analysis of promoter/ß-glucuronidase fusions in transgenic *Arabidopsis* and tobacco plants is currently in progress and will provide new insights into how plant hormones affect their expression during development. A second class of characterized genes with a key role in cell division are encoded by cyclins and we have shown that there are at least five different cyclin genes in *Arabidopsis*. A second part of work in our group deals with the characterization of small GTP-binding proteins in *Arabidopsis* (RUG). Small GTP-binding proteins constitute a superfamily of eukaryotic proteins which are thought to play a key role in the regulation of diverse cellular processes. *Arabidopsis* cDNAs encoding a GTP-binding protein, called Rha1, has been cloned encoding a small G protein protein with high homology involved in early endocytosis. Antibodies raised against the *Arabidopsis* protein are currently used to determine its subcellular localization. Research in the Leeds laboratory has concentrated on identification of novel heterotrimeric G proteins. This is important as cloning approaches in several laboratories, which were based on homology, resulted in isolation of only one particular class of heterotrimeric G proteins. Heterotrimeric guanine nucleotide binding regulatory proteins (G-proteins) transduce signals across cell membranes by coupling receptors for chemical and sensory stimuli to effectors such as enzymes and channels. Biochemical and immunological

techniques were applied to search for novel members of the G protein family in plants. This will present a basis for molecular cloning and functional analysis of these proteins. Several putative Gα subunit proteins were identified including a 40 kDa protein related to GPA1. Using a Giα specific antiserum a 37 kDa protein was identified in *Arabidopsis* membrane fractions (ULeeds). Currently antisera to peptides corresponding to the Gq class of G protein subunits are being raised, a group of proteins known to act in vertebrates in gating phosphoinositol metabolism. Further biochemical analysis resulted in partial purification of G proteins from peas indicating that some of these plant G proteins significantly differ from their animal counterparts. Understanding these proteins, the receptors and effectors to which they couple will represent an important step towards understanding plant signal transduction and hence control of regeneration.

HIGHLIGHTS/MILESTONES:

Several novel ERabp genes have been cloned from maize and *Arabidopsis* to allow structural and functional analysis of their role in auxin perception; transgenic tobacco plants have been generated expressing these proteins and demonstrating increased auxin sensitivity and altered root growth characteristics (MPI, GIF). Analysis of their cellular expression and trafficking will present a major step forward to understand their function. Anti-peptide antibodies with auxin agonist activity identify the auxin binding region of an auxin receptor; two monoclonal antibodies against the auxin receptor are excellent markers for ER, especially in mammalian cells (HRI). Development of an assay system for early auxin responses; functional analysis of auxin "agonist" antibodies; demonstration of increased auxin sensitivity in transgenic tobacco plants overexpressing an auxin binding protein (GIF). Mutant *cdc2* genes influence cell division in plants and provide perspectives to alter development; the availability of two *cdc2* genes and five cyclin genes allow us now to study the influence of plant hormones, in particular auxins and cytokinins, on 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 (RUG). Peptides have been synthesized that correspond in sequence to a number of G-protein sequences (ULeeds), and antisera raised against these peptides have been characterized and distributed to a number of participant laboratories (MPI, HRI, GIF).

WIDER CONSIDERATIONS:

Progress obtained from our subgroup (MPI, HRI, ISV, ULeeds, Danisco) has confirmed that an auxin-binding protein is involved in influencing cellular auxin sensitivity. This will allow - for the first time - to probe into the mechanisms of auxin action. The identification of proteins and genes involved plant signal transduction and cell cycle control provides the basis for the links between the signal, e.g. auxin, and cell division control.

COOPERATIVE ACTIVITIES:

Joint Meetings: sub-group meeting, MPI, Köln; general T-project meeting in Dourdan. Several visits have been organized between the different laboratories: visits by Napier (1 day) and Venis (2 days) to ISV lab for discussions; by Lovegrove to ULeeds for 1 month for work on G proteins. Anti-abp antibodies, seeds, materials exchanged between sub-group partners ISV, MPI, RUG and provided to other T-project labs - Klämbt (Bonn), Libbenga (Leiden), Terzi (Padova), Costantino (Rome), Van Lammeren (Wageningen), Hall (Aberystwyth). Transgenic tobacco plants provided to subgroup partner (ISV), exchange of materials with DANISCO/AS.

LIST OF JOINT PUBLICATIONS/PATENTS WITH TRANS-NATIONAL AUTHORSHIP:

BARBIER-BRYGOO, H., EPHRITIKINE, G., KLÄMBT, D., MAUREL, C., PALME, K., SCHELL, J., and GUERN, J. (1991) Perception of the auxin signal at the plasmalemma of tobacco protoplasts. *The Plant Journal* 1, 83-93.

HEMERLY, A., BERGOUNIOUX, C., VAN MONTAGU, M., INZÉ, D., and FERREIRA, P. (1992). Genes regulating the plant cell cycle: isolation of a mitotic-like cyclin from *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA*, in press.

MAUREL, C., BARBIER-BRYGOO, H., SPENA, A., TEMPE, J., and GUERN, J. (1991) Single *rol* genes from *Agrobacterium rhizogenes* TL-DNA alter some of the cellular responses to auxin in *Nicotiana tabacum*. *Plant Physiol.* 97, 212-216.

PALME, K., HESSE, T., CAMPOS, N., GARBERS, C., YANOFSKY, M.F., and SCHELL, J. (1992) The auxin-binding protein from *Arabidopsis thaliana* is located on chromosome 4. *The Plant Cell* 4, 193-201.

VENIS, M.A., THOMAS, M.W., BARBIER-BRYGOO, H., EPHRITIKHINE, G., and GUERN, J. (1990) Impermeant auxin analogues have auxin activities. *Planta* 182, 232-235.

VENIS, M.A., NAPIER, R.M., BARBIER-BRYGOO, H., MAUREL, C., PERROT-RECHENMANN, C. and GUERN, J. Antibodies to a peptide from the maize auxin-binding protein have auxin agonist activity. *Proc. Natl. Acad. Sci. USA* (in press).

OTHER PUBLICATIONS:

ANUNTALABHOCHAI, S., TERRYN, N., VAN MONTAGU, M., and INZÉ, D. (1991) Molecular characterization of an *Arabidopsis thaliana* cDNA encoding a small GTP-binding protein, Rha1. *Plant J.* 1, 167-174.

CAMPOS, N., FELDWISCH, J., ZETTL, R., BOLAND, W., SCHELL, J., AND PALME, K. (1991) Identification of auxin-binding proteins using an improved assay for photoaffinity labeling with 5-N_3-[7-H]-indole-3-acetic acid. *Technique* 3, 69-75.

CLARKSON, J., WHITE, I.R. AND MILLNER, P.A. (1991) Specific immune detection and partial purification of G-proteins from *A. thaliana*. *Biochem. Soc. Trans.* 20, 9S.

FELDWISCH, J., ZETTL, R., HESSE, F., SCHELL, J., and PALME, K. (1991) Novel auxin binding proteins are localised to the plasma membrane of maize coleoptile cells: Identification by photoaffinity labeling and purification of a 23 kDa polypeptide. *Proc. Natl. Acad. Sci. USA* 89, 475-479.

FELLE, H., PETERS, W., and PALME, K. (1991) The electrical response of maize to auxins. *Biochim. Biophys. Acta* 1064, 199-204.

FERREIRA, P.C.G., HEMERLY, A.S., VILLARROEL, R., VAN MONTAGU, M., and INZÉ, D. (1991). The *Arabidopsis* functional homolog of the $p34^{cdc2}$ protein kinase. *Plant Cell* 3, 531-540.

FINAN, P.M., WHITE, I.R., FINDLAY, J.B.C. AND MILLNER, P.A. (1991). Identification of nucleoside diphosphate kinase from pea microsomal membranes. *Biochem. Soc. Trans.* 20, 10S.

HESSE, T., and PALME, K. (1992) The search for phytohormone receptors: A role for auxin binding proteins? In: *Biochemical Mechanisms Involved in Growth Regulation*. Chiatante, D., Gallon, J., Smith, C., Zocchi, (eds.). Oxford University Press, in press.

INZÉ, D., FERREIRA, P., HEMERLY, A., and VAN MONTAGU, M. (1992). Control of cell division in plants. *Biochem. Soc. Trans.* 20, 80-84.

KÖRBER, H., STRIZHOV, N., STAIGER, D., FELDWISCH, J., OLSSON, O., SANDBERG, G., PALME, K., SCHELL, J., and KONCZ, C. (1991) T-DNA gene 5 of *Agrobacterium tumefaciens* modulates auxin response by autoregulated synthesis of a growth hormone antagonist in plants. *EMBO J.* 10, 3983-3991.

MOORE, I., FELDWISCH, J., CAMPOS, N., ZETTL, R., BRZOBOHATY, B., BAKO, L., SCHELL, J., and PALME, K. (1991) Auxin-binding proteins of *Zea mays* identified by photoaffinity labelling. *Biochem. Soc. Transact.* 20, 70-73.

NAPIER, R.M., FOWKE, L.C., HAWES, C., LEWIS, M. and PELHAM, H.R.B. Immunological evidence that plants use both HDEL and KDEL for targeting proteins to the endoplasmic reticulum. *J. Cell Sci.* (in press).

NAPIER, R.M. and VENIS, M.A. Epitope mapping reveals conserved regions of an auxin-binding protein. *Biochem J.* (in press).

NAPIER, R.M. and VENIS, M.A. The auxin receptor: structure and distribution. In *Transport and Receptor proteins of Plant Membranes*, eds D.T. Clarkson and D.T. Cooke Long Ashton Symposium vol. 12. Plenum, New York (in press).

PALME, K., HESSE, T., MOORE, I., CAMPOS, N., FELDWISCH, J., GARBERS, C., HESSE, F., and J. SCHELL (1991) Hormonal regulation of plant growth: The role of auxin perception. *Mech. Devel.* 33, 97-106.

PALME, K., and SCHELL, J. (1991) Auxin receptors take shape. *Current Biology* 4, 137-139.

PALME, K. (1992) Molecular analysis of plant signalling elements: The relevance of eucaryotic signal transduction models. *Int. Review Cytol.* 132, 223-286.

PALME, K. (1992) The relevance of eucaryotic signalling models to understand the perception of auxin. In *Control of Plant Gene Expression*. Verma, D.P.S. (ed.) Telford Press, in press.

PALME, K., DIEFENTHAL, T., VINGRON, M., SANDER, C., and SCHELL, J. (1992) Molecular cloning and structural analysis of genes from *Zea mays* (L.) coding for members of the *ras*-related *ypt* gene family. *Proc. Natl. Acad. Sci. USA* 89, 787-791.

PALME, K., FELDWISCH, J., PETERS, W.F., SCHELL, J., ZETTL, R., CAMPOS, N., and FELLE, H. (1992) Auxin-binding proteins are located in the ER and in the plasma membrane: Identification by photoaffinity-labelling and characterization. In: *Plant Growth Substances 1991*, C.M. Karssen (ed.), in press.

TERRYN,N., ANUNTALABHOCHAI, S., VAN MONTAGU, M., and INZÉ, D. (1992). Analysis of a *Nicotiana plumbaginifolia* cDNA encoding a novel small GTP-binding protein. *FEBS Lett.* 299, 287-290.

VENIS, M.A. and NAPIER, R.M. (1992). Plant hormone receptors: past, present and future. *Biochem. Soc. Trans.* **20**, 55-59.

WHITE, I.R., WISE, A., FINAN, P.M., CLARKSON, J. AND MILLNER, P.A. (1991) GTP-binding proteins in higher plant cells. *Proceedings of 12th Long Ashton Symposium*. In the press.

WISE, A. AND MILLNER, P.A. (1991) Evidence for the presence of GTP-binding proteins in tobacco leaf and maize hypocotyl plamalemma. *Biochem. Soc. Trans.* 20, 7S.

ZETTL, R., FELDWISCH, J., BOLAND, W., SCHELL, J., and PALME, K. (1992) Azido-[3,6-$^3H_2$]-*N*-naphthylphtalamic acid, a novel photo-activatable probe for auxin carrier proteins from higher plants: Identification of a 23 kDa protein from maize coleoptile plasma membranes. *Proc. Natl. Acad. Sci. USA* 89, 480-484.

TITLE: The *rol* genes as privileged tool to study plant morphogenesis

CONTRACT NUMBER: BIOT-CT90-0179 (SMA)

OFFICIAL STARTING DATE: March 1st 1991.

COORDINATOR:
P. Costantino, Dip. Gen. Biol. Mol., Università "La Sapienza", Roma, IT (DGBM).

PARTICIPANTS:
A. Spena, Max-Planck-Institut, Köln, DE (MPI);
J. Tempé, Institut Sciences Végétales CNRS, Gif-sur Yvette, FR (ISV);
H. Van Onckelen, Universitaire Instelling Antwerpen, Antwerp, BE (UIA);
D. Chriqui, Cytol. Embriol. Végétales, Université P. et M. Curie, Paris, FR (CEMV);
F. Souq, Louis Vuitton Moet Hennessy Recherche, Paris, FR (LVMH);
D. Mariotti, Istituto Radiobiol. Ecofisiol. Vegetali CNR, Monterotondo, IT (IREV).

OBJECTIVES SET FOR THE REPORTING PERIOD:
Analysis of: regulation of *rol* genes (DGBM, MPI) and other relevant Ri T-DNA genes (ISV); biochemical role of the *rolB* (DGBM, MPI) and *rolC* (MPI) gene products; interaction of *rol* genes with plant genes (DGBM, MPI); hormonal levels of different tissues and plants transgenic for *rol* constructs (UIA); morphological and developmental traits of *rol* transgenic plants (CEMV); effects of other T-DNA genes (ISV) . Exploitation as useful traits of the effects of *rol* and other T-DNA genes (MPI, LVMH, IREV).

MAJOR PROBLEMS ENCOUNTERED:
Impossibility to visualize the *rolB* gene product with specific anti-RolB antisera (DGBM, ISV). Impossibility to overexpress *rolA* in *Escherichia coli* (MPI). Difficulties in defining regeneration procols for rose (LVMH) and woody species (IREV).

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. DGBM has been concentrating on the regulation of *rolB*, whose promoter has been shown to be under hormonal, developmental and tissue-specific regulation. Detailed histological analysis of GUS gene fusion expression has pointed out a developmental regulation of the *rolB* promoter which has also been followed during zygotic embryogenesis (DGBM). The hormonal response of the *rolB* promoter has also been characterized - the gene has been shown to be auxin-inducible (DGBM, ISV) - and is currently being linked to the developmental control of expression of this gene. Very recent data point to an unexpected role of the TATA box in determining tissue specificity and hormonal and developmental regulation of *rolB* (DGBM). The role of *rolB* in triggering rooting is being studied at MPI, where an indoxyl-β-glucosidase activity has been assigned to RolB, and at DGBM, where a RolB-related auxin binding activity in normal plant cells has been identified. This auxin-binding activity is much enhanced in *rolB*-transformed cells (DGBM) where a greatly increased sensitivity to auxin has been measured (ISV). At MPI also *rolC* is being studied and a conjugated cytokinin hydrolase activity assigned to its gene product. The intracellular localization of the *rolB* and *rolC* gene products has been defined (MPI). Plant genetic mosaics for the expression of a cytokinin gene and plants expressing constitutively *rol* genes have been constructed (MPI) and analysed in collaboration with UIA and CEMV. ISV is mainly concerned with ORFs 13 and 14 of the Ri T-DNA, the characterization of their promoters and the identification of their gene products. In particular, ORF13 has been shown to be

controlled by a wound-inducible promoter and to encode for an as yet uncharacterized low molecular weight morphogenic substance (ISV). UIA is currently measuring hormone levels of plants transgenic for prom35S-ORF13 gene fusions. CEMV has characterized at the hystological and ultrastructural level the developmental and morphological modifications induced by the *rol* genes *A*, *B* and *C* in collaboration with MPI and UIA. At UIA various advanced microanalytical techniques are being utilized to measure hormone levels in even small districts of plant tissues transformed by the *rol* genes. These techniques have allowed to correlate alterations of endogenous hormones to specific developmental patterns of transgenic plants (UIA, MPI, CEMV). At the MPI-associated laboratory of LVMH, *Agrobacterium*-based transformation techniques have been developed for woody roses in order to exploit the developmental modifications induced by *rol* genes. Evaluation of the consequences of *rol* gene expression by a vast morphological and agronomical survey is currently underway at LVMH. The possible exploitation of the *rol*-induced morphological alterations in agronomical plants such as alfalfa and kiwi is the object of the research at the DGBM-associated laboratory IREV. *rolB*-transgenic kiwi plants show an improved rooting capability, while other interesting modifications are induced by *rol* genes in alfalfa (IREV).

HIGHLIGHTS/MILESTONES:

Specific regulatory sequences within the *rolB* promoter which interact with endogenous plant regulatory factors and confer tissue specificity, hormonal and developmental control have been identified (DGBM). A non -systemic, wound-inducible promoter (ORF13) has been characterized, which could be used to control expression of plant defense genes (ISV). The existence on the membranes of untransformed tobacco cells of an auxin-binding activity immunologically related to the gene product of the plant oncogene *rolB* has been pointed out (DGBM). The biochemical activities of the *rolB* and *rolC* gene products - capable of hydrolysing indole glycosides and, respectively, cytokinin-n-glucoside - have been defined, suggesting a primary role of hormone metabolism in the alterations induced by the *rol* genes (MPI). A novel, low molecular weight substance capable of interfering with morphogenesis, encoded by ORF13 has been evidenced (ISV).

WIDER CONSIDERATIONS:

The results obtained from our subgroup as a whole (DGBM, ISV, MPI, UIA, CEMV, LVMH, IREV) has 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 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 for the first time that plant physiology and development can be modified by enzymatic systems able to synthesize or hydrolyse plant hormone conjugates.

COOPERATIVE ACTIVITIES:

A post-doc from DGBM has spent two years at ISV. Students have been exchanged between CEMV and UIA. 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. **Joint meetings**: Several meetings between partners in the subgroup: three ISV-DGBM in Gif; four UIA-CEMV in Paris; four MPI-UIA in Köln and Antwerp; two general meetings of the subgroup in Paris (Nov.'90 and Jan.'92); one general T-Project meeting in Dourdan (Nov.'91).

LIST OF JOINT PUBLICATIONS WITH TRANS-NATIONAL AUTHORSHIP:

Spena, A., Prinsen, E., Fladung, M., Schulze, S.C. and Van Onckelen, H.: The indoleacetic-Lysine synthetase gene of *Pseudomonas syringae* subsp. *savastanoi* induces developmental alterations in transgenic tobacco and potato plants. Mol. Gen. Genet. 227, 205-212, 1991.

Estruch, J.J., Chriqui, D., Grossmann, K., Schell, J. and Spena, A.: The plant oncogene *rolC* is responsible for the release of cytokinin from glucoside conjugates. The EMBO Journal 10, 2889-2995, 1991.

Estruch, J.J., Prinsen, E, Van Onckelen, H., Schell, J. and Spena, A.: Viviparous leaves produced by somatic activation of an inactive cytokinin-synthesizing gene. SCIENCE 254, 1364-1367, 1991.

Spena, A., Estruch, J.J., Nacken, W., Prinsen, E., Van Onckelen, H. and Sommer, H.: Anther-specific expression of the *rolB* gene of *Agrobacterium rhizogenes* increses IAA content in anthers and alters anther development. T.A.G. In press.

Spena, A., Estruch, J.J., Aalen, R.D., Prinsen, E., Parets-Soler, A., Nacken, W., Sommer, H., Chriqui, D., Grossmann, K., Van Onckelen, H. and Schell, J.: Transgenic plants and transgenic plant mosaics for the expression of pathogen derived genes able to affect phytohormone activity. In: Plant Growth Substances 1991. Kluwer publ. In press.

Maurel, C., Barbier-Brygoo, H., Spena, A., Tempé, J. and Guern, J.: Single *rol* genes from the *Agrobacterium rhizogenes* TL-DNA modify the response to auxin of tobacco mesophyll protoplasts. Plant Physiol. 97, 212-216, 1991.

Prinsen, E., Berchetche, J., Chriqui, D. and Van Onckelen, H.: *Pisum sativum* epicotyls inoculated with *Agrobacterium rhizogenes* agropine strains harbouring various T-DNA fragments: morphology, histology and endogenous indole-3-acetic acid and indole-3-acetamide content. J. Plant Physiol. In press.

Mengoli, M., Chriqui, D. and Bagni, N.: Protein, free aminoacid and polyamine content during development of hairy root *Nicotiana tabacum* plants. J. Plant Physiol. In press.

Mengoli, M., Ghellia, A., Chriqui, D. and Bagni, N.: Growth kinetics, polyamine pattern and biosynthesis in hairy root lines of *Nicotiana tabacum* plants. J. Plant Physiol. In press.

Hansen, G., Larribe, M., Vaubert, D., Tempé, J., Bierma, B.J., Montoya, A.L., Chilton, M.D. and Brevet, J.: *Agrobacterium rhizogenes* pRi8196 T-DNA: mapping and DNA sequence of functions involved in mannopine synthesis and hairy root differentiation. Proc. Natl. Acad. Sci. U.S.A. 88, 7763-7767, 1991.

OTHER PUBLICATIONS:

Capone, I., Cardarelli, M., Mariotti, D., Pomponi, M., De Paolis, A. and Costantino, P.: Different promoter regions control level and tissue specificity of expression of *Agrobacterium rhizogenes rolB* gene in plants. Plant Mol. Biol. 16, 427-436, 1991.

Altamura, M.M., Archilletti, T., Capone, I. and Costantino, P.: Histological analysis of the expression of *Agrobacterium rhizogenes rolB*-GUS gene fusions in transgenic tobacco. New Phytol. 118, 69-78, 1991.

Costantino, P., Capone, I., Cardarelli, M., De Paolis, A. and Trovato, M.: *rolB*: a bacterial gene capable of controlling auxin response and morphogenesis in plant cells. In: Biochemical Mechanisms Involved in Plant Growth Regulation. C.J. Smith ed., Oxford University Press. In press.

Filippini, F., Trovato, M., Lo Schiavo, F., Terzi, M. and Costantino, P.: The plant oncogene *rolB* alters binding of auxin to plant cell membranes. Submitted for publication.

Chichiriccò, G., Costantino, P. and Spanò, L.: Expression of the *rolB* oncogene from *Agrobacterium rhizogenes* during zygotic embryogenesis in tobacco. Submitted for publication.

Beinsberger, S., Valcke, R., Deblaere, R., Clijsters, H., De Greef, J. and Van Onckelen, H.: Effects of the introduction of *Agrobacterium tumefaciens* T-DNA *ipt* gene in *Nicotiana tabacum* cv Petite havana SR1 plant cells. Plant Cell Physiol. 32, 489-496, 1991.

Heylen C., Vendrig J. and Van Onckelen, H.: The accumulation and metabolism of plant growth regulators during organogenesis of thin cell layers of *Nicotiana tabacum*. Physiol. Plant. 83, 578-584, 1991.

Rugini, E., Pellegrineschi, A., Mencuccini, M. and Mariotti, D.: Increase of rooting ability in the woody species of kiwi (*Actnidia deliciosa* A Chev.) by transformation with *Agrobacterium rhizogenes rol* genes. Plant Cell Rep. 10, 291-295, 1991.

Estruch, J.J., Parets-Soler, A., Schmülling, T. and Spena, A.: Cytosolic localization in transgenic plants of the *rolC* peptide from *Agrobacterium rhizogenes*. Plant Mol. Biol. 17, 547-550, 1991

Estruch, J.J., Schell, J. and Spena, A.: The protein encoded by the *rolB* plant oncogene hydrolyses indole glucosides. The EMBO Journal 10, 3125-3128, 1991

Spena, A., Estruch, J.J. and Schell, J.: On microbes and plants: new insights in phytohormonal research. Current Opinion in Biotechnology. In press.

Spena, A. and Schulze, S.C.: Chimaeras and transgenic plant mosaics: a new tool in plant biology. In: Advances in Molecular Genetics of Plant-Microbe Interactions. Hennecke, H. and Verma, D.P.S. eds., Kluwer Academic Publ. 1, 352-355, 1991.

Dessaux, Y., Petit, A. and Tempé, J.: Opines in the biology of *Agrobacterium*. In: Molecular Signals in Plant-Microbe Communications, pp 109-136. Verma, D.P.S. ed., CRC Press, Boca Raton, 1991

Tempé, J.: Introduction of foreign genetic information in plants. Acta Hort. In press.

Phelep, M., Petit, A., Martin, L., Duyhoux, E. and Tempé, J.: Transformation and regeneration of a nitrogen-fixing tree *Allocasuarina verticillata* Lam. Biotechnology 9, 461-466, 1991.

David, C. and Tempé, J.: Transformation of *Catharantus*. In: Biotechnology in Agriculture and Forestry. Bajaj, Y.P.S. ed. Springer Verlag, Berlin, 1992.

Van der Linden, A., Gadeyne, J., Van Onckelen, H., Van laere, A. and Decleir, W.: Involvement of cyclic nucleotides in light-induced resumption of development of *Artemia* embryos. J. Exp. Zool. 258, 312-321, 1991

Prinsen, E., Chauvaux, N., Schmidt, J., John, M., Wieneke, U., De Greef, J., Schell, J. and Van Onckelen, H.: Stimulation of indole-£-Acetic acid production in *Rhizobium* by flavonoids. FEBS Lett. 1, 53-55, 1991.

Marcussen, J., Seiden, P., Olsen, C. and Van Onckelen, H.: Monoclonal antibodies toward a stable cyclic AMP-C18 antigen. Anal. Biochem. 198, 324-329, 1991.

Regulation of the inductive phase of microspore embryogenesis.

Contract Number: BIOT 900160

Official starting date: 01.01.1990

Coordinator: Dr Paul Pechan, MPI, Köln, G.

Participants: Dr Andre van Lammeren, Agricultural University, Wageningen, NL.

Dr Antonio Martin, IAPV, Cordoba, ES.

Mr Andrew McQueen, Nickersons, Cambridge, UK.

Dr Hans Dons, CPRO-DLO, Wageningen, NL.

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 double 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.

The following main objectives were set for the first year of our study. (a) Creation of *Brassica* cDNA libraries and isolation of clones associated with induction of microspore embryogenesis (MPI). (b) Investigate the *B. napus* cytoskeleton (microtubules and microfilaments) under normal and stress conditions (Agricultural University). (c) Investigate the cytoskeletal targets of the phosphorylation cascade leading to induction of microspore embryogenesis (CPRO-DLO). (d) Cross low and high embryogenesis responsive *Brassica* varieties (IAPV). (e) Identify potentially embryogenic barley microspores (Nickersons Int. Co.).

Major problems

Nothing to report.

Results

A 32°C temperature treatment of 8 h duration induces the embryogenic process in freshly isolated late uni- to early binucleate microspores. Consequently, molecular and cellular comparisons, based on the developmental stage of microspores, the severity of stress treatment and duration of the treatment, can be made.

(a) Max Planck Institute (MPI)

Because of the limited amount of microspores available, new techniques had to be adapted and developed to generate cDNA libraries. The techniques were based on PCR technology. Approximately 5 µg of cDNA can be synthesized from as little as 10,000-20,000 cells. Genes associated with induction of microspore embryogenesis were enriched for by subtractive hybridization. 15 cDNA libraries have so far been created in this fashion. Screening of the libraries is in progress. A number of clones have been isolated. An additional investigative approach is based on protein comparisons during the inductive phase of microspore embryogenesis. Microspores were also successfully transformed by electroporation mediated delivery of DNA into microspores. This and other techniques will be used to ascertain in the future the function of genes associated with the induction of microspore embryogenesis.

(b) Agricultural University

Changes in the organization of the microtubular and the microfilamental cytoskeletons were investigated during the normal development of *Brassica* microspores. The visualization of cytoskeletal elements was successful with immunological, fluorescence and electron

microscopical techniques. Stage specific arrangements were detected. The results point to a specific function of the microtubules in inducing or maintaining polarity within the microspores. Changes in the organization of the microtubular and microfilamental cytoskeletons in microspores under induction conditions were monitored during the first 12 h of culture and compared with the *in vivo* development. Clear differences were noted as compared with the *in vivo* development. Additionally, we observed differences in the structure between microspores cultured under inductive (32°C) and non-inductive (25°C) conditions. The detection of mRNA coding for tubulin and actin in normal developing microspores has been started. For this purpose probes were developed in Wageningen and digoxigenated at the MPI.
(c) Institute of Plant Breeding and Plant Reproduction (CPRO-DLO)
Continued microspore culture at 32°C leads to the formation of embryos. In contrast to this continuous culture at 18°C resulted in continuation of the normal gametophytic development giving rise to about 80% of the cells becoming mature pollen. Consequently, a simple culture system has been developed allowing comparison of *in vitro* sporophytic and gametophytic microspore development. 35S Met-labelled proteins were separated on 2-D gels and analysed. Most proteins were present under both conditions, but a limited number appeared to be specific for the initial phase of embryo induction. Both under embryogenic and non-embryogenic conditions a large number of proteins were phosphorylated, but mainly quantitative differences were observed. Using Western blotting several isotypes were detected for a- and b-tubulin and also for actin. By combining the results of the Western blotting and the 32 Pi labelling, it was demonstrated that some of the isotypes of the cytoskeletal proteins were phosphorylated.
(d) Instituto de Agronomas y Proteccion Vegetal (IAPV)
Crosses among Topas and Hanna were carried out. F1, F2 and backcrosses generations of the two reciprocals have been obtained. We also tested the possibility of speeding up the flowering cycle of plants under starving conditions at high sowing density. We found that three generations could be obtained per year. Three rapid cycling varieties were crossed with Hanna, Topas and the low embryogenic response Topas line developing a diallel. The hybrid nature of the F1 was tested using electrophoresis (ADH, ACP, LAP, GPI and GPM systems). The RAPDs analysis of the different parentals will start in the near future, including the three plant clones brought from Köln. These, as the other lines, have been crosses to create SSD families for the study of the genetic system controlling somatic microsporogenesis and for relating this character with some ADN markers.
(e)Nickerson International Seed Company
The technology transfer unit is currently involved in research on anther/microspore culture in both mono- and dicotyledonous crop species including barley, maize and vegetable Brassicas. A system for the culture of isolated barley microspores (cv. Igri) has been developed whereby green plants may be recovered at a high frequency. Transfer of this system to MPI will facilitate a comparison of *Brassica napus* microspores and *Hordeum vulgare* microspores to identify common molecular features of microspore embryogenesis induction in di- and monocotyledonous species.

Highlights/Milestones

1. Rapid and simple methodology has been developed for creation and screening of cDNA libraries. A number of clones associated with induction of microspore embryogenesis have been isolated.
2. Procedures to visualize microtubules in sectioned microspores and microfilaments in whole microspores as well as for detection of mRNAs *in situ* have been established.
3. A system has been developed for the gametophytic and sporophytic development of microspores under *in vitro* conditions.
4. The first documentation of the phosphorylation of cytoskeletal proteins in plant cells.
5. Crosses between various tissue culture responsive and non-responsive *Brassica napus* varieties have been carried out.

Wider considerations

The *Brassica napus* microspore culture system proved to be a very good model to study plant embryogenesis. The microspore culture is unique in that it is both a vehicle for the study of plant cell proliferation and an important tool for the plant breeder. 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.

Cooperative activities

Throughout the year a number of meetings were held between the coordinating laboratory (MPI) and members of participating laboratories.

IAPV

Total of two meetings were held. The April meeting in Cordoba dealt with practical aspects of setting up the experimental system for carrying out crossing experiments between low and high embryogenic *B. napus* lines. Theoretical considerations for such experiments were also discussed. The second meeting was held in Köln. Dr D. Cabrera visited Köln for three days in June to continue discussions on the genetic aspects of microspore embryogenesis and to learn microspore *in vitro* culture.

Agricultural University

Total of four meetings were held (three in Wageningen, one in Köln) between January and December 1991. The meetings were held to inform the participants about the present state of the art in the various laboratories and to coordinate future research activities.

CPRO-DLO

Regular meetings were held with project partners at the Department of Plant Cytology and Morphology, Agricultural University, Wageningen. Moreover, coordinator Paul Pechan paid several visits to the institute. Dr F Corke from the John Innes Research Centre UK (Group Keith Roberts) visited CPRO-DLO on 13-14 February 1992 to improve 2-D gel electrophoresis of cell wall proteins.

Meeting of group 5 took place in Köln on November 11-12, 1991

Training in microspore culture was given by the MPI to the laboratories participating. Seeds of various inbred lines were provided by the MPI to the participating laboratories. More recently, exchange of antibodies and cDNA probes has also taken place.

Publications

J.G. Duijs, R.E. Voorrips, D.L. Visser and J.B.M. Custers. Microspore culture is successful in most crop types of *Brassica oleracea* L. Euphytica 1992 (in press).

J.B.M. Custers, Y. Nollen, J.H.G. Cordewener and R. Busink. Temperature control of sporophytic and gametophytic development in microspore culture of *Brassica napus* L. (In preparation.)

G. Hause, B. Hause and A.A.M. van Lammeren. Microtubular and actin-filament configurations during microspore and pollen development in *Brassica napus* L. cv. Topas. Can. J. Bot. 1992 (in press).

P. Pechan. Heat shock proteins and cell proliferation. FEBS 1991, 280:1-4.

TITLE: The molecular analysis of higher plant embryogenesis

CONTRACT NUMBER: BIOT-CT90-0177 (T-project 'Regeneration' Group 4)

OFFICIAL STARTING DATE: 01 April 1991

COORDINATOR: S.C. de Vries, AUW, Wageningen, NL

PARTICIPANTS: **01.** T. Hendriks and E.A. Meijer, AUW, Wageningen, NL
02. H.A. Verhoeven, CPRO, Wageningen, NL
03. M. Terzi and F. Cozzani, Univ. Padova, Padova, I
04. K. Roberts and T. Baldwin, JII, Norwich, UK
06. J.D. Mikkelsen and K.M. Kragh, Maribo, Kopenhagen, DK
07. S.C. Fry and R. Hetherington, Univ. Edin., Edinburgh, UK

OBJECTIVES SET FOR THE REPORTING PERIOD: 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. In the first year several objectives have been pursued by each of the participants.

01/02.: Small scale purification of 32 and 46 kD secreted glycoproteins shown to influence somatic embryogenesis, preparation of antibodies and cDNAs for these proteins, fluorescence activated flow sorting of single embryogenic cells, functional analysis of 32 and 46 kD proteins. **03.**: establishment of new complementable and non-complementable somatic cell mutants. **04.**: small and large scale purification of arabinogalactans (AGPs) from the culture medium, AGP distribution during somatic and zygotic embryogenesis. **06.**: large scale purification of 32 and 46 kD proteins, role of PR and stress proteins (chitinases and glucanases) in embryogenesis. **07.**: biochemical analysis of 32, 46 kD and other secreted proteins in relation to embryogenesis.

MAJOR PROBLEMS ENCOUNTERED: None

RESULTS: 01/02.: Both the 32 and 46 kD secreted glycoproteins have been purified to homogeneity and tested for their effects on impaired embryo development in the carrot line ts11. The 32 kD protein consistently showed rescue activity allowing the formation of globular and heart stage embryos at the non-permissive temperature. The effect of the 46 kD protein, resulting in an increase of the number of embryos obtained in ts11, could not be reproduced. Both specific antisera as well as partial cDNA clones for these proteins have been obtained. The 32 kD protein has been identified as an acidic endochitinase, both on the basis of sequence homology and catalytic properties. A 10 kD seceted lipid transfer protein, that marks the presence of embryogenic cells in suspension cultures, has been purified to homogeneity. The purified protein has been shown to be able to transfer fatty acids and phospholipids in vitro. A system to follow the fate of up to 10,000 individual immobilized carrot suspension cells over a 14 d period has been developed. This involved the simultanous testing of new cell-embedding compounds, media composition as well as the computer-controlled recording of phase-contrast or CSLM images on video tape or on WORM discs. To aid in discriminating between actively proliferating and inactive cells, the RNA-specific fluorochrome thiazol-orange was succesfully tested as a vital stain for immobilized cells during several days of culture. **03.**: Biological tests have been developed employing various cell lines to evaluate the role of secreted glycoproteins on somatic embryogenesis. The results show that embryogenic efficiency can be either increased or decreased upon addition of partially purified glycoproteins to either wild-type or variant cell lines. New variant lines obtained are unable to produce even the earliest embryo stages. All these new lines exhibit a greater resistance to auxin when compared with the wild-type lines. For many of these

new variant lines, the ability to produce somatic embryos can be acquired by preculture in the presence of high concentrations of auxin. In addition, it was shown that auxin induces its own binding sites and that this induction was different in embryogenic cells when compared to non-embryogenic cells. **04.**: An anti-AGP monoclonal antibody, JIM 8, has been used to map the complex appearance of its cognate oligosaccharide epitope during all stages of flower development in both Brassica and Arabidopsis. The same antibody labels a complex pattern of stele cells in Arabidopsis roots that includes the endodermis. The relationship of these patterns to those revealed by MAC 207 has been clarified. A secreted AGP has been immunoaffinity-purified to homogeneity in large amounts from carrot cell suspension medium and is being used for a.) deglycosylation prior to sequencing and antibody production, b.) identification of putative receptor molecules in the extracellular matrix and c.) high resolution EM studies and FTIR microspectroscopy. **06.**: A system for large scale culturing (100 L) of embryogenic cell suspensions of carrot in a rotating drum fermentor has been developed. The pattern of extracellular proteins from cultures grown in the fermentor and cultures grown in Erlenmeyer flasks are very similar and cultures grown in the fermentor retain their embryogenic potential. Large scale purification of an acidic 32 kD protein have been performed by batch and column anion exchange chromatography, gelfiltration, and hydrophobic interaction chromatography. This protein has chitinolytic activity. Antibodies raised against the basic sugar beet chitinase 4 crossreact with the acidic 32 kD protein. Partial amino acid sequences obtained from tryptic peptides of the 32 kD protein show similarity to sequences from the sugar beet chitinase 4 and other chitinases and indicate that the 32 kD protein is a chitinase with an N-terminal chitin binding domain. **07.**: The question has been addressed whether the secreted proteins that are active in somatic embryogenesis are involved in a process that results in small, adherent cell types, either by releasing oligosaccharides that are biologically active, or by causing direct physical changes in the cell walls. Conversely, cell wall enzymes that would loosen the cell wall by hydrolysis of wall polymers would be expected to antagonize the formation of small adherent cell types. One of these enzymes is xyloglucan endotransglycosilase (XET) that was found to be present in carrot culture media. The enzyme activity was shown to decrease during the first four days of somatic embryogenesis. Both the 32 and 46 kD as well as a previously purified 52/54 kd secreted glycoprotein did not have XET activity. The seceted protein exhibiting high XET activity is now being purified to homogeneity. None of the proteins purified sofar (32, 46 and 52/54 kD proteins) did release measurable amounts of oligosaccharides from preparations of random radiolabeled cell walls. These experiments are currently being repeated with cell walls labeled with specific sugars. On the basis of these findings it is likely that the secreted proteins active in somatic embryogenesis cause direct changes in the cell walls, rather then releasing biologically active oligosaccharides.

HIGHLIGHTS/MILESTONES: 01/02. Biochemical identification of the 32 kD secreted protein able to rescue ts11 somatic embryogenesis as an acidic endochitinase, establishment of regeneration system starting from single cells. **03.** Use of high levels of auxin shown to initiate embryogenic potential in otherwise non-embryogenic cell lines. **04.**: Pure AGP produced in large quantities and shape determined by electron microscopy. **06.**: Culturing of up to 100 L of embryogenic cell suspension cultures in a fermentor allowing large scale purifications of extracellular proteins involved in embryogenesis and AGPs. **07.**: Detection of high amounts of XET activity in conditioned media.

WIDER CONSIDERATIONS: The aim of this research project is twofold, to obtain and characterize molecular markers specific for different stages of the acquisition of embryogenic potential in plant cell suspension cultures, and to identify and characterize compounds that promote the formation of somatic embryos in plant cell suspension cultures. As a model system somatic embryogenesis in carrot is employed in which the focus of interest is on compounds secreted into the culture medium. This approach has

yielded non-destructive molecular markers for embryogenic potential and also purified compounds that promote somatic embryogenesis after addition to the culture medium. In addition, procedures have been developed to increase embryogenic potential in suspension cultures. Significant progress has been made in all areas of the project and most of the objectives set for the first year have either been achieved or have exceeded the expectations. This is strikingly illustrated by the very rapid establishment of a large scale culturing system in the laboratory of the industrial partner (**06**). The purification of not only glycoproteins but also of AGPs in the amounts necessary for meaningful experiments has been a major step forward and is of great benefit to all participants of the project. A characteristic feature of this project is the unusually high level of mutual interactions that have resulted in numerous joint and complementary experiments. It is clear that this is a direct result of the transnational collaborations that would be very difficult to maintain without the continued support of the EC.

COOPERATIVE ACTIVITIES: General activities: A meeting of Group 4 was held in Wageningen in May 1991, a general meeting as well as the second Group 4 meeting were held in Dourdan in November 1991. Specific activities: Working discussions have been held between participants **01** and **06** in May 1991 and in March 1992 and also between **04** and **06**. A technician of **01** has been to **04** for training in immunolocalization of proteins. 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.

LIST OF JOINT PUBLICATIONS/PATENTS WITH TRANSNATIONAL AUTHORSHIP:

De Jong, A.J., Cordewener, J.H.C., Lo Schiavo, F., Terzi, M., Vandekerckhove, J., van Kammen, A., de Vries, S.C. (1992) A carrot somatic embryo mutant is rescued by chitinase. Plant Cell (in press)

OTHER PUBLICATIONS/PATENTS:

Filippini, F., Terzi, M., Cozzani, F., Lo Schiavo, F. (1992) Modulation of auxin-binding proteins in cell suspensions. II. Isolation and initial characterization of carrot cell variants impaired in somatic embryogenesis. TAG (in press)

Fry, S.C., Smith, R.C., Renwick, K.F., Martin, D.J., Hodge, S.K., Metthews, K.J. (1992) Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity from plants. Biochem. J. (in press)

Knox, J.P., Linstead, P.J., Peart, J., Cooper, C., Roberts, K. (1991) Developmentally regulated epitopes of cell surface arabinogalactan proteins and their relation to root tissue pattern formation. Plant J. 1, 317-326

Lo Schiavo, F., Filippini, F., Cozzani, F., Vallone, D., Terzi, M. (1991) Modulation of auxin-binding proteins in cell suspensions. I. Differential responses of carrot embryo cultures. Plant Physiol. 97, 60-64

Pennell, R.I., Janniche, L., Kjellbom, P., Scofield, G.N., Peart, J.M., Roberts, K. (1991) Developmental regulation of a plasma membrane arabinogalactan protein epitope in oilseed rape flowers. Plant Cell 3, 1317-1326

Smith, R.C., Fry, S.C. (1991) Endotransglycosylation of xyloglucans in plant cell suspension cultures. Biochem. J. 279, 529-535

Sterk, P., Booij, H., Schellekens, G.A., van Kammen, A., de Vries, S.C. (1991) Expression pattern of the carrot EP2 gene encoding an extracellular lipid transfer protein. Plant Cell 3, 907-921

Van Engelen, F.A., De Vries, S.C. (1992) Extracellular proteins in plant embryogenesis. Trends in Genet. 8, 66-70

TITLE: Plant Growth Regulators: perception, interaction and response.

CONTRACT NUMBER: BIOT-CT90-0158

OFFICIAL STARTING DATE: 01.02.1991

COORDINATOR: Professor M A Hall, UCW Aberystwyth, UK.

PARTICIPANTS: Professor K R Libbenga, Univ. of Leiden
Professor D Klambt, Univ. of Bonn
Professor P Aducci, Univ. of Rome 'Tor Vergata'
Dr A Hoekema, Mogen International NV

OBJECTIVES SET FOR THE REPORTING PERIOD:
The objectives set involved progress in the following with the emphasis on items 1 to 3.

1. Isolation and characterisation of gene(s) for ABP.
2. Isolation and characterisation of gene(s) for EBP.
3. Isolation and characterisation of gene for FCBP.
4. Separation of components of transduction chains.
5. Development of protoplast systems to test functionality and testing of components.
6. Development of in vitro reconstitution systems to test functionality and testing of components.

MAJOR PROBLEMS ENCOUNTERED:
Difficulties in obtaining sufficient solubilized membrane-bound ABP in an active form from tobacco. Similar problems with putative transduction proteins integral to the plasmalemma. N-terminal blockage of protein preparations. Strategies are being developed to solve those problems by means of new methods for the separation of hydrophobic integral membrane proteins and a method to analyse C-terminal residues of polypeptides.

RESULTS:

In Leiden, several genes encoding ABP-like proteins have been isolated from rice and are currently under investigation. Tobacco protoplasts from a transgenic cell line have been used to investigate the role of membrane-bound factors involved in signal transduction using antibodies from Bonn and from Dr M A Venis. Anti-binding site antibodies blocked NAA-induced gene expression in the protoplasts. Promoter-GUS fusions have been used to investigate the promoters of auxin-induced genes and have led to the identification of a region important for auxin-induced and root tip specific expression.

In Bonn, studies have centred on attempts to detect ABP at the plasmamembrane. The use of two phase separation of membranes or additional free flow electrophoresis has demonstrated the association of ABP with plasmamembrane, perhaps via a disulphide link. Iodinated ABP binds to microsomes and plasmamembrane vesicles but not to those from the endomembrane system. Auxin has been shown to modulate the secretion of α-amylase by exocytosis.

In Rome, the FCBP has been successfully isolated and its

structural analysis is in progress at Mogen where the development of techniques for C-terminal analysis is likely to prove most useful. There is now preliminary evidence regarding the structure of an endogenous ligand suppressing FC-stimulated H^+ extrusion. Progress with reconstituted systems has shown that the response of such systems is specific to FC which does not alter the K_m of the H^+-ATPase but rather the V_{max}. There is evidence for the involvement of the phosphoinositide cycle in signal transduction vis a vis fusicoccin.

In Aberystwyth, a new electrophoretic method for the separation of hydrophobic proteins has been developed. Using this technique, samples of the EBP have been sent to Mogen and preliminary N-terminal sequences of subunits obtained. There is no homology with any known protein. Studies on protein kinases indicate that ethylene may modulate the phosphorylation of its own receptor which in turn appears to modulate the affinity of the receptor for ethylene.

There have been frequent meetings to discuss progress in the various laboratories and, as noted in other sections, considerable exchange of tools and personnel. In particular the progress made on sequencing the EBP and the FCBP could not have been achieved in anything like the timescale without the wholehearted cooperation of the partners involved.

HIGHLIGHTS/MILESTONES:

1. Partial sequence of subunits of EBP.
2. Progress with sequencing of FCBP.
3. Development of patch-clamp technique to investigate transduction mechanisms; characterisation of inward and outward directed K^+ channels in tobacco protoplasts.
4. Initial characterisation of involvement of protein kinase activity in transduction of the ethylene signal.
5. Detection of ABP at the plasmamembrane.
6. Demonstration that exocytosis by non-growing cells is mediated by auxin.
7. Possible involvement of phosphoinositide signalling pathway in transduction of the fusicoccin effect.

WIDER CONSIDERATIONS:

The progress being made in the elucidation of the perception and transduction pathways does render feasible the production of transgenic crop plants improved in a number of characteristics.

COOPERATIVE ACTIVITIES:

a) Within group: EBP, FCBP to Mogen; anti-EBP antibodies to Leiden; anti-ABP antibodies from Bonn to Leiden.
b) With other groups within T-project: Impermeable auxin-conjugates from M A Venis to Bonn; anti-ABP antibodies from M A Venis to Aberystwyth, Leiden and Bonn.
c) With other groups in EC programmes: Group of M van Montagu (Dr D v.d. Straeten) - screening (by Aberystwyth) of ethylene insensitive mutants for ethylene binding.

Dr N V J Harpham from Aberystwyth is spending a year in

Leiden (in collaboration with the Institute of Molecular Plant Sciences and Mogen International NV) for the purpose of cloning the EBP gene.

Visit by Dr E.J. v.d. Zaal to Aberystwyth to discuss protein purification.

- Two day meeting in Leiden immediately prior to beginning of contract (December 1990) involving all contractors and associated personnel as well as the group of H. Barbier-Brygoo.
- Meeting of whole T-group at Dourdan (November 1991).
- Meeting of N-group in Rome (May 2 to 5 1992) involving all contractors and associated personnel and also the groups of P. Constantino, M. Terzi and other interested parties.
- Meeting between the Leiden and Bonn participants and the group of Professor J Guern at Gif-sur-Yvette (Nov 15 1991).

In accordance with the original agreement there has been considerable interaction with other groups in the T-project particularly that coordinated by K Palme, both in the exchange of appropriate tools, cooperative activities and joint meetings.

JOINT PUBLICATIONS:

A M Mennes, A Maan & M A Hall (1991) In: Cell to Cell Signals in Plants and Animals: Progress Report (eds. V Neuhoff & J Friend), NATO/ASI Series H: Cell Biology, Vol. 51, pp. 301-314. Springer/Verlag.

H Barbier-Brygoo, G Ephritikhine, D Klambt, C Maurel, K Palme, J Schell & J Guern (1991) The Plant Journal 1, 83-93.

OTHER SELECTED PUBLICATIONS:

I O Sanders, A R Smith & M A Hall (1991) Planta 183, 209-217.

N V J Harpham, A W Berry, E M Knee, G Roveda-Hoyos, I Raskin, I O Sanders, A R Smith, C K Wood and Hall, M A (1991) Annals of Botany 68, 55-61.

I O Sanders, N V J Harpham, I Raskin, A R Smith and M A Hall (1991) Annals of Botany 68, 97-104.

D Klambt, B Knauth, I Dittman (1992) Physiol. Plant., submitted.

D. Klambt (1992) Planta, submitted.

P Aducci, A Ballio, M R Fullone & M Marra (1992) In: "Biochemical mechanisms involved in growth regulation", Smith, CJ, ed., Oxford University Press, in press.

P Aducci, A Ballio, V Fogliano, M R Fullone, M Marra & D Verzili (1992) In: "Transport and receptor proteins of plant membranes", Clarkson, D.T. & Cooke, D.T., eds., Plenum Publishing Corp., in press.

M Marra, A Ballio, M R Fullone & P Aducci (1992) Plant Physiology, in press.

E J van der Zaal, F N J Droog, C J M Boot, L A M Hensgens, J H C Hoge, R A Schilperoort & K R Libbenga (1991) Plant Mol. Biol. 16, 983-998.

A M Mennes, A Quint, J H Gribnau, C J M Boot, E J van der Zaal, A C Maan & K R Libbenga (1992) Plant Mol. Biol. 18, 109-117.

PARTICIPANT INDEX

KEYWORD INDEX

Contractors' names and addresses

UNIVERSITE CATHOLIQUE DE LOUVAIN
PLACE DE L'UNIVERSITE 1
B 1348 LOUVAIN-LA-NEUVE
Tel.: 3210473614
Telex: 3210474745
E-mail: AGROFYSA@BUCLLNII

EUROPEAN MOLECULAR BIOLOGY LABORATORY
DATA LIBRARY
MEYERHOFSTRASSE 1
D 6900 HEIDELBERG
Tel.: 4962213870
Telex: 496221387306
Fax: 461613 EMBL D

DR. M. ADOLPHE
DIRECTOR OF THE LABORATORY OF CELLULAR
LABORATORY OF CELLULAR PHARMACOLOGY
ECOLE PRATIQUE DES HAUTES ETUDES
RUE DE L'ECOLE DE MEDECINE 15
F 75006 PARIS
Tel.: 33143292869
Telex: 33143298546

DR. P. ADUCCI
ASSOCIATE PROFESSOR
DIPARTIMENTO DI BIOLOGIA
UNIVERSITY OF ROME 'TOR VERGATA'
VIA E. CARNEVALE
I 00173 ROMA
Tel.: 39679794343
Telex: 3962023500
Fax: 396626382

DR. M. AIGLE / DR. M. CROUZET
DR.
UNIVERSITE BORDEAUX II
RUE LEO SAIGNAT 146
F 33076 BORDEAUX
Tel.: 3356806800
Telex: 3356990380
Fax: UNIBXII 572237F
E-mail: AIGLE@FRDBXII

A.D.L. AKKERMANS
TBC
DEPARTMENT OF MICROBIOLOGY
AGRICULTURAL UNIVERSITY WAGENINGEN
HESSELINK VAN SUCHTELENWEG 4
NL 6703 CT WAGENINGEN
Tel.: 31837083486
Telex: 31837083829
Fax: 45854 LUWAG
E-mail: SCHRAAG@ACLWAU.NL

L. ALBERGHINA
PROFESSOR OF COMPARATIVE BIOCHEMISTRY
DEPT. OF GENERAL PHYSIOLOGY AND BIOCHE
DIV. OF COMPARATIVE BIOCHEMISTRY
UNIVERSITA DEGLI STUDI DI MILANO
VIA CELORIA 26
I 20133 MILANO
Tel.: 3922364218
Telex: 3922361070

DR. L. ANDERSSON
RESEARCHER
UPPSALA MEDICAL CENTRE
SWEDISH UNIVERSITY OF AGRICULTURAL SCI
BOX 7055
S 750 07 UPPSALA
Tel.: 4618671000
Telex: 4618673389
E-mail: ULTLA@BMC.UU.SE

DR. W. ANSORGE
DR.
EUROPEAN MOLECULAR BIOLOGY LABORATORY
MEYERHOFSTRASSE 1
POSTFACH 10.2209
D 6900 HEIDELBERG
Tel.: 496221387355
Telex: 496221387306
Fax: 461613EMBLD
E-mail: SCHWAGER@EMBL-HEIDELBERG.DE

DR. A.L. ARCHIBALD
SENIOR SCIENTIFIC OFFICER
DEPT. OF MOLECULAR GENETICS
EDINBURGH RESEARCH STATION
AFRC INSTITUTE OF ANIMAL PHYSIOLOGY AN
UK EH25 9PS ROSLIN, MIDLOTHIAN
Tel.: 44314402726
Telex: 44314400434
E-mail: ARCHIBALD@IAPE.AFRC.AC.UK

DR. P. AROSIO
RICERCATORE UNIVERSITARIO CONFERMATO
DIPARTIMENTO DI SCIENZE E TECNOLOGIE B
UNIVERSITA DEGLI STUDI DI MILANO
VIA OLGETTINA 60
I 20132 MILANO
Tel.: 39221702459 / 392217
Telex: 3922641198
Fax: 320484 UNIMII

PROF. H.N. ARST
PROFESSOR OF BACTERIOLOGY
ROYAL POSTGRADUATE MEDICAL SCHOOL
DUCANE ROAD 150
UK W12 ONN LONDON
Tel.: 44817403243
Telex: 44817492281
Fax: 926128 HHRPMS

DR. P.J. ARTYMIUK
ROYAL SOCIETY UNIVERSITY RESEARCH FELL
KREBS INSTITUTE FOR BIOMOLECULAR RESEA
DEPARTMENT OF MOLECULAR BIOLOGY AND BI
SHEFFIELD UNIVERSITY
WESTERN BANK
UK S10 2TN SHEFFIELD
Tel.: 44742768555423
Telex: 44742728697
Fax: 547216 UGSHEF G

DR. F. AZORIN
INVESTIGADOR CIENTIFICO CSIC
CENTRO DE INVESTIGACION Y DESARROLLO CSIC
JORDI GIRONA SALGADO 18-26
E 08034 BARCELONA
Tel.: 3432040600
Telex: 3432045904
Fax: 97977 IDEB

DR. P.H. BACH
HEAD OF THE SCHOOL
SCHOOL OF SCIENCE
POLYTECHNIC OF EAST LONDON
ROMFORD ROAD
UK E15 4LZ LONDON
Tel.: 44818493496
Telex: 44815193740

DR. A. BALLESTEROS
CAREER INVESTIGATOR
LABORATORY OF BIOCATALYSIS
INSTITUTE OF CATALYSIS
C.S.I.C.
SERRANO 119
E 28006 MADRID
Tel.: 34126194002412
Telex: 3415642431

PROF. M. BALLS
FRAME (FUND FOR THE REPLACEMENT OF ANI
MEDICAL EXPERIMENTS)
STONEY STREET 34
EASTGATE HOUSE
UK NG1 1NB NOTTINGHAM
Tel.: 44602584740
Telex: 44602503570

DR. J.V. BANNISTER
PROFESSOR OF BIOLOGICAL CHEMISTRY
CRANFIELD INSTITUTE OF TECHNOLOGY
UK MK43 OAL CRANFIELD, BEDFORDSHIRE
Tel.: 44234752738
Telex: 444750907

DR. H. BARBIER-BRYGOO
CHARGEE DE RECHERCHE
GROUPE DE PHYSIOLOGIE CELLULAIRE
INSTITUT DES SCIENCES VEGETALES
CNRS
AVENUE DE LA TERRASSE 1
BATIMENT 22
F 91198 GIF-SUR-YVETTE
Tel.: 33169823868
Telex: 33169823768
Fax: 601137F

DR. M. BASAGLIA
HELIGENETICS S.P.A.
VIA PROVINCIALE 62A
I 45030 GAIBA (ROVIGO)
Tel.: 39425709709
Telex: 39425709650

DR. A. BELAYEW
SENIOR RESEARCH ASSOCIATE
LABORATOIRE DE BIOLOGIE MOLECULAIRE ET GENIE GENETIQUE
UNIVERSITE DE LIEGE
INSTITUT DE CHIMIE B6
B 4000 SART TILMAN
Tel.: 3241563311
Telex: 3241562968
E-mail: U218101@BLEULG11

DR. G.J. BELSHAM
SENIOR SCIENTIFIC OFFICER
PIRBRIGHT LABORATORY
INSTITUTE FOR ANIMAL HEALTH
ASH ROAD, PIRBRIGHT
UK GU24 ONF WOKING, SURREY
Tel.: 44483232441
Telex: 44483232448
Fax: 859137 AVRI G

DR. J.-P. BELTRAN PORTER
INVESTIGADOR CIENTIFICO
INST. DE AGROQUIMICA Y TECNOLOGIA DE ALIMENTOS
C.S.I.C.
JAIME ROIG 11
E 46010 VALENCIA
Tel.: 3463690800
Telex: 64 197 AYTVE
Fax: 3463930001
E-mail: BIOVEG@IATA.CSIC.ES

PROF. J. BENNEDSEN
HEAD OF DEPARTMENT
STATENS SERUMINSTITUT
ARTILLERIVEJ 5
DK 2300 S COPENHAGEN
Tel.: 453195281727O1
Telex: 4531952869
E-mail: TELECOM GOLD 10075:DBI0706

DR. K. BERGEMANN
STAFF SCIENTIST
ABT. BIOTECHNISCHE PRODUKTION
DR. KARL THOMAE GMBH
BIRKENDORFER STRASSE 65
D 7950 BIBERACH
Tel.: 497351544836
Telex: 497351542167

DR. J.E. BERINGER
HEAD OF BOTANY DEPARTMENT
BOTANY DEPARTMENT
SCHOOL OF BIOLOGICAL SCIENCES
UNIVERSITY OF BRISTOL
WOODLAND ROAD
UK BS8 1UG BRISTOL
Tel.: 44272303759
Telex: 44272732657
Fax: 445938 BSUNIV G

DR. M. BEVAN
SENIOR SCIENTIFIC OFFICER
AFRC INSTITUTE OF PLANT SCIENCE RESEAR
CAMBRIDGE LABORATORY
JOHN INNES CENTRE
COLNEY
UK NR4 7UJ NORWICH
Tel.: 4460352571
Telex: 44603502270
Fax: 975122 JIINOR G

DR. M.E. BIANCHI
ASSOCIATE PROFESSOR
DIPARTIMENTO DI GENETICA E MICROBIOLOG
UNIVERSITA DEGLI STUDI DI PAVIA
SANT'EPIFANIO 14
I 27100 PAVIA
Tel.: 39382303016
Telex: 3938232234

PROF. D. BITTER-SUERMANN
FULL PROFESSOR
INSTITUT FUER MEDIZINISCHE MIKROBIOLOG
MEDIZINISCHE HOCHSCHULE HANNOVER
KONSTANTY-GUTSCHOW-STRASSE 8
D 3000 HANNOVER
Tel.: 495115324369
Telex: 495115324366

DR. D.N. BLACK
GROUP LEADER
INSTITUTE FOR ANIMAL HEALTH
PIRBRIGHT LABORATORY
ASH ROAD
UK GU24 ONF WOKING, SURREY
Tel.: 44232441
Telex: 44232448
Fax: 859137 AVRI G
E-mail: BLACK@UK.AC.AFRC.AVRI

PROF. C. BLANCO
PROFESSOR
CNRS -URA LSG - LABORATOIRE DE GENETIQ PHYSIOLOGIE MICROBIENNE
UNIVERSITE DE RENNES
CAMPUS DE BEAULIEU
UFR SVE AVENUE DU GENERAL LECLERC
F 35042 RENNES O42
Tel.: 3399286140
Telex: 3399286700

DR. A. BI.EASBY
SEQNET MANAGER
DARESBURY LABORATORY
SCIENCE AND ENGINEERING RESEARCH COUNC
DARESBURY
UK WA4 4AD WARRINGTON
Tel.: 44925603348
Telex: 44925603100
E-mail: AJB@DLVH.DARESBURY.AC.UK

DR. H. BLOECKER
HEAD OF RESEARCH GROUP
DNA-SYNTHESE
GBF - GESELLSCHAFT FUER BIOTECHNOLOGIS
FORSCHUNG MBH
MASCHERODER WEG 1
D 3300 BRAUNSCHWEIG
Tel.: 495316181220
Telex: 495316181292
Fax: 952667 GEBIO D
E-mail: BLOECKER@VENUS.GBF-BRAUNSCHWEIG.DBP.DE

PROF. K. BOCK
PROF./HEAD OF DEPT.
CARLSBERG LABORATORY
GAMLE CARLSBERG VEJ 10
DK 2500 VALBY-COPENHAGEN
Tel.: 453122 10225221
Telex: 4531211366
E-mail: CARLKEM@NEUVM1

DR. L. BOE
ASSISTANT PROFESSOR
DEPARTMENT OF MICROBIOLOGY
TECHNICAL UNIVERSITY OF DENMARK
BUILDING 221
DK 2800 LYNGBY
Tel.: 45428840663506
Telex: 4545932809
Fax: 37529 DTHDIADK

DR. P. BOISTARD
DIRECTOR OF THE LABORATORY
LABORATOIRE DE BIOLOGIE MOLECULAIRE DE
RELATIONS PLANTES-MICROORGANISMES
CNRS-INRA
BP 27
F 31326 CASTANET TOLOSAN CEDEX
Tel.: 3361285046
Telex: 3361285061
Fax: 520009
E-mail: COLLEC@TOULOUSE.INRA.FR

DR. M. BOLOTIN
INSTITUT DE GENETIQUE ET MICROBIOLOGIE
URA 1354 CNRS
UNIVERSITE DE PARIS-SUD
BATIMENT 400
F 91405 ORSAY CEDEX
Tel.: 33169416206
Telex: 33169417296

DR. U. BONAS
STAFF SCIENTIST
INSTITUT FUER GENBIOLOGISCHE FORSCHUNG
IHNESTRASSE 63
D 1000 BERLIN 33
Tel.: 493083000783
Telex: 493083000736

DR. C.Y. BOQUIEN
JUNIOR SCIENTIST
LABORATOIRE DE GENIE DES PROCEDES BIOTECHNOLOGIQUES AGRO-ALIMENTAIRES
F 78850 THIVERVAL GRIGNON
Tel.: 33130815353
Telex: 33130815457
Fax: 697388

DR. J. BOS
SENIOR LECTURER
DEPARTMENT OF DERMATOLOGY
UNIVERSITEIT VAN AMSTERDAM
ACADEMISCH MEDISCH CENTRUM
MEIBERGDREEF 9
NL 1105 AZ AMSTERDAM
Tel.: 31205662587
Telex: 31205664440

DR. A.A. BOSMA
ASSISTENT-PROFESSOR (UNIVERSITAIR HOOF
DEPARTMENT OF FUNCTIONAL MORPHOLOGY
FACULTY OF VETERINARY MEDICINE
RIJKS UNIVERSITEIT UTRECHT
P.O. BOX 80.157
NL 3508 TD UTRECHT
Tel.: 3130534336
Telex: 3130516863

PROF. V. BOTTAZZI
FULL PROFESSOR
ISTITUTO DI MICROBIOLOGIA
FACOLTA DI AGRARIA
UNIVERSITA DEL SACRO CUORE
EMILIA PARMENSE 84
I 29100 PIACENZA
Tel.: 3952368275
Telex: 3952365205
E-mail: MICROB@IPCUCSC.BITNET

DR. C. BOUCHER
DIRECTEUR DE RECHERCHE
LABORATOIRE DE BIOLOGIE MOLECULAIRE DE
RELATIONS PLANTES-MICROORGANISMES
CENTRE DE RECHERCHES INRA
BP 27
F 31326 CASTANET-TOLOSAN CEDEX
Tel.: 3361285028
Telex: 3361285280
Fax: 520009 F

DR. A. BRADBURY
HEAD OF PROJECT
SEZIONE BIOTECHNOLOGIE
SOCIETA ITALIANA PER LA RICERCA SCIENT
GIOVANNI PAISIELLO 47/C
I 00198 ROMA
Tel.: 3968442224
Telex: 3968442579

DR. B. BRAEU
SCIENTIST
HOECHST AG
BRUENINGSTRASSE 50
POSTFACH 80 03 20
D 6230 FRANKFURT/MAIN 80
Tel.: 49693056447
Telex: 4969331320
Fax: 41234-OHO D

DR. G.M. BRESSAN
TBC
INSTITUTE OF HISTOLOGY AND EMBRYOLOGY
UNIVERSITY OF PADOVA
VIA TRIESTE 25 75
I 35100 PADOVA
Tel.: 39498286613
Telex: 39498072213

DR. S.W.J. BRIGHT
RESEARCH MANAGER
JEALOTT'S HILL RESEARCH STATION
ICI SEEDS
WAREFIELD
UK RGI2 6EY BRACKNELL
Tel.: 44344424701/4821
Telex: 44344434996
Fax: 847556 ICIPPJG

DR. S. BRON
ASSISTANT PROFESSOR
DEPARTMENT OF GENETICS
UNIVERSITY OF GRONINGEN
KERKLAAN 30
NL 9751 NN HAREN
Tel.: 3150632105
Telex: 3150635205
E-mail: BRON@HGRRUG52

PROF. G.G. BROWNLEE
PROFESSOR OF CHEMICAL PATHOLOGY
CHEMICAL PATHOLOGY UNIT
SIR WILLIAM DUNN SCHOOL OF PATHOLOGY
UNIVERSITY OF OXFORD
SOUTH PARKS ROAD
UK OX1 3RE OXFORD
Tel.: 44865275559
Telex: 44865275556

DR. A. BRUNEL
DIRECTION DU DEVELOPPEMENT
SEPIA INTERNATIONAL
ECLOSERIE MARINE DE GRAVELINES
VOIE DES ENROCHEMENTS
F 59820 GRAVELINES
Tel.: 3328655151
Telex: 33130579745
Fax: 6990734

DR. Y. BRYGOO
DIRECTEUR DE RECHERCHES
CRYPTOGAMIE, INRA
UNIVERSITE PARIS-SUD
BAT. 400
F 91405 ORSAY
Tel.: A3316169417006
Telex: 3316169417296
Fax: 602166 F
E-mail: LAFAY@FRCITI51.BITNET

PROF. O. BUCHARDT
PROFESSOR
H.C. OERSTED INSTITUTE
UNIVERSITY OF COPENHAGEN
UNIVERSITETSPARKEN 5
DK 2100 COPENHAGEN
Tel.: 4531353133
Telex: 4531350766

IR. A.F. BUECKMANN
SENIOR SCIENTIST
DEPARTMENT OF ENZYME TECHNOLOGY
GBF
MASCHERODER WEG 1
D 3300 BRAUNSCHWEIG
Tel.: 495316181307
Telex: 495316181302
E-mail: ABU@VENUS.GBF-BRAUNSCHWEIG.DBP.DE

DR. J.-M. BUHLER
LABORATOIRE D'INGENIERIE DES PROTEINES
CENTRE D'ETUDES NUCLEAIRES DE SACLAY
COMMISARIAT A L'ENERGIE ATOMIQUE
F 91191 GIF-SUR-YVETTE CEDEX
Tel.: 33169086418
Telex: 33169084712

PROF. M. BUIATTI
FULL PROFESSOR OF GENETICS
DIPARTIMENTO DI BIOLOGIE ANIMALE E GENETICA
UNIVERSIAT DEGLI STUDI DI FIRENZE
VIA ROMANA 17
I 50125 FIRENZE
Tel.: 3955229453
Telex: 3955222565

PROF. H. BUJARD
PROFESSOR
ZENTRUM FUER MOLEKULARE BIOLOGIE (ZMBH
UNIVERSITAET HEIDELBERG
IM NEUENHEIMER FELD 282
D 6900 HEIDELBERG
Tel.: 496221566800
Telex: 496221565507

DR. J. BULLERDIEK
HEAD OF LABORATORY
CENTER FOR HUMAN GENETICS
UNIVERSITY OF BREMEN
LEOBENER STRASSE ZHG
D 2800 BREMEN 33
Tel.: 494212183589
Telex: 494212184039

DR. M. CABOCHE
DIRECTEUR DE RECHERCHES
LABORATOIRE DE BIOLOGIE CELLULAIRE
INST. NATIONAL DE LA RECHERCHE AGRONOM
ROUTE DE SAINT CYR
F 78026 VERSAILLES
Tel.: 33130833017
Telex: 33130833111

PROF. J. CABRAL
ASSOCIATE PROFESSOR
SECTION OF BIOTECHNOLOGY
DEPT. OF CHEMICAL ENGINEERING
INSTITUTO SUPERIOR TECNICO
AVENIDA ROVISCO PAIS
P 1000 LISBOA 1096
Tel.: 3511802045/1233
Telex: 35118480072
Fax: 63423 ISTUTL P

DR. C.-M. CALBERG-BACQ
CHEF DE TRAVAUX
DEPARTEMENT DE MICROBIOLOGIE

UNITE DE VIROLOGIE FONDAMENTALE ET IMM
UNIVERSITE DE LIEGE
INSTITUT DE PATHOLOGIE B23
SART-TILMAN
B 4000 LIEGE
Tel.: 3241562451
Telex: 3241562433

PROF. J.A. CALLOW
PROFESSOR
UNIVERSITY OF BIRMINGHAM
P.O. BOX 363
UK B15 2TT BIRMINGHAM
Tel.: 01044214145559
Telex: 0214145925
Fax: SPAPHY G 338938

DR. CHR. CAMBILLAU
CHARGE DE RECHERCHES
LABORATOIRE DE CRISTALLOGRAPHIE ET CRISTALLISATION DES MACROMOLECULES BIOLOGIQUES / CNRS
BD PIERRE DRAMARD
FAC. DE MEDICINE NORD
F 13326 MARSEILLE 15
Tel.: 3391698908
Telex: 3391698913

DR. G.N. CAMERON
HEAD OF DATA LIBRARY
DATA LIBRARY
EUROPEAN MOLECULAR BIOLOGY LABORATORY
MEYERHOFSTRASSE 1
D 6900 HEIDELBERG
Tel.: 496221387257
Telex: 496221387306
Fax: 461613 EMBL D
E-mail: CAMERON@EMBL-HEIDELBERG.DE

DR. J.M. CARAZO
CHIEF OF BIOCOMPUTING UNIT. SCIENTIST
UNIDAD DE BIOCOMPUTACION
CENTRO NACIONAL DE BIOTECNOLOGIA
UNIVERSIDAD AUTONOMA DE MADRID
E 28049 MADRID
Tel.: 3413978430
Telex: 3413974799
E-mail: CARAZO@EMDCC11.BITNET

DR. M. CARILLO
INVESTIGADOR CIENTIFICO DEL CSIC
INSTITUTO DE ACUICULTURA DE TORRE DE L
C.S.I.C.
RIBERA DE CABANES
E 12595 TORRE DE LA SAL
Tel.: 3464310325
Telex: 3464310350
Fax: 42182

DR. R.J. CARR
ASSISTANT HEAD OF GROUP
CENTRE FOR APPLIED MICROBIOLOGY & RESE
PORTON DOWN
UK SP4 OJG SALISBURY, WILTSHIRE
Tel.: 44980610391
Telex: 44980611320

PROF. M.J. CARRONDO
ASSOCIATE PROFESSOR/DIRECTOR OF THE LA
INSTITUTO DE BIOLOGIA EXPERIMENTAL E TECNOLOGICA (IBET)
APARTADO 12
P 2780 OEIRAS
Tel.: 35114427787
Telex: 35114421161

DR. J.I. CASAL
IMMUNOLOGIA Y GENETICA APLICADA S.A.
MOLECULAR BIOLOGY UNIT (INGENASA)
HERMANOS GARCIA NOBLEJAS 41
E 28037 MADRID
Tel.: 3412680501 / 3412680
Telex: 3414087598

PROF. A. CATTANEO
FULL PROFESSOR
ISTITUTO SISTEMI INTELLIGENTI
ISAS
STRADA COSTIERA 11
I 34014 TRIESTE
Tel.: 394022491
Telex: 39402249249
Fax: 460269 SISSA
E-mail: CATTANEO@ITSSISSA

PROF. E. CERNIA
CTB PRESIDENT
CONSORZIO PER IL TRASFERIMENTO DELLE BIOTECHNOLOGIE
VIA SARDEGNA 38
I 00187 ROMA
Tel.: 396485451
Telex: 3964885614

DR. F. CERVONE
ASSOCIATE PROFESSOR
LABORATORIO DI FISIOLOGIA VEGETALE
DIPT. DI BIOLOGIA VEGETALE
UNIVERSITA DI ROMA 'LA SAPIENZA'
PIAZZA ALDO MORO 5
I 00185 ROMA
Tel.: 39649912641
Telex: 396463865

PROF. G. CESARENI
PROFESSOR OF MOLECULAR GENETICS
LABORATORIO DI GENETICA MOLECOLARE
DIPARTIMENTO DI BIOLOGIA II
UNIVERSITA DI ROMA TORVERGATA
VIA CARNEVALE
LA ROMANINA
I 00173 ROMA
Tel.: 39679794315
Telex: 3962023500
Fax: 626382 FIUNTVI
E-mail: CESARENI@VAXBTV.INFN.IT

DR. J. CHAMBAZ
MAITRE DE CONFERENCES DES UNIVERSITES
LAB. DE BIOCHIMIE
FAC. MEDECINE SAINT-ANTOINE
UNIVERSITE PIERRE ET MARIE CURIE
CHALIGNY 27
F 75012 PARIS CEDEX
Tel.: 33140011341
Telex: 33143453687

DR. G. CHARALAMBOS
RESEARCH SCIENTIST
DEVELOPMENT & TECHNOLOGY TRANSFER
OPTIMUM LTD. RESEARCH
ARISTOTELOUS STREET 177
GR 11251 ATHENS
Tel.: 3018670234
Telex: 3018677747

MR. M. CHAUVET
SECRETARY GENERAL, BRG
BUREAU DES RESSOURCES GENETIQUES (BRG)
RUE CUVIER 57
F 75231 PARIS CEDEX 05
Tel.: 33147071575
Telex: 33145357015
Fax: FFSPN 260921 F

DR. D.J. CHISWELL
HEAD OF R&D, GENERAL MANAGER AND CHIEF
CAMBRIDGE ANTIBODY TECHNOLOGY LIMITED
THE DALY RESEARCH LABORATORIES
BABRAHAM HALL
UK CB2 4AT CAMBRIDGE
Tel.: 44223837526
Telex: 44223837524

DR. M.-C. CHOPIN
DIRECTEUR DE RECHERCHE
LABORATOIRE DE GENETIQUE MICROBIENNE
INRA
DOMAINE DE VILVERT
F 78352 JOUY EN JOSAS CEDEX
Tel.: 33134652530
Telex: 33134652273
E-mail: ACHOPIN@BIOTECJOUY.INRA.FR

DR. D. CHOURROUT
CHARGE DE RECHERCHES, INRA
DIRECTEUR D
LABORATOIRE DE GENETIQUE DES POISSONS INRA
DOMAINE DE VILVERT
F 78352 JOUY-EN-JOSAS
Tel.: 33134652391
Telex: 33134652390
Fax: INRACRZ695431F

PROF. D. CHRIQUI
PROFESSOR
UNIVERSITY PIERRE AND MARIE CURIE
PLACE JUSSIEU 4
BAT. N2
F 75252 PARIS
Tel.: 33144274583
Telex: 33144273866

DR. C. CHRISTIANSEN
MANAGING DIRECTOR
MOLECULAR BIOTECHNOLOGY SECTION
BIOTECHNOLOGICAL INSTITUTE
BUILDING 227
LUNDTOFTEVEJ 100
P.O. BOX 199
DK 2800 LYNGBY
Tel.: 45458766699
Telex: 45459328888

DR. G. CILIBERTO
PROJECT LEADER
ISTITUTO DI RICERCHE DI BIOLOGIA MOLEC
MERCK, SHARP AND DOHME RESEARCH
VIA PONTINA
KM 30,600
I 00040 POMEZIA (ROMA)
Tel.: 3963125530

DR. D. CLAUS
HEAD OF THE INFORMATION CENTRE
INFORMATION CENTRE FOR EUROPEAN CULTUR
COLLECTIONS (ICECC)
MASCHERODER WEG 1B
D 3300 BRAUNSCHWEIG
Tel.: 49531618715/22
Telex: 49531618718
E-mail: TELECOM GOLD 10075:DBI0274

DR. A. CLOAREC
DIRECTOR OF THE BIOLOGY DEPARTMENT
DEPT. OF BIOLOGY
LABORATORIES UPSA
DANTON 128
F 92500 RUEIL-MALMAISON
Tel.: 33147490500
Telex: 33147516879
Fax: 203269

PROF. M. CLYNES
PROFESSOR OF BIOTECHNOLOGY/DIRECTOR NA
NATIONAL CELL AND TISSUE CULTURE CENTR
SCHOOL OF BIOLOGICAL SCIENCES
DUBLIN CITY UNIVERSITY
GLASNEVIN
IRL 9 DUBLIN
Tel.: 35317045720
Telex: 35317045484
Fax: 353130690

DR. D. COCCO
PROFESSOR OF BIOCHEMISTRY
UNIVERSITY OF CAGLIARI
VIA DELLA PINETA 77
I 09125 CAGLIARI
Tel.: 3970300728

DR. E.S. COEN
SENIOR SCIENTIFIC OFFICER
JOHN INNES INSTITUTE
COLNEY LANE
UK NR4 7UH NORWICH
Tel.: 4460352571
Telex: 4460356844
Fax: 975122 JII NOR G

DR. T.M. COGAN
HEAD, DAIRY MICROBIOLOGY DEPARTMENT.
TEAGASC
MOOREPARK
FERMOY
IRL CO. CORK
Tel.: 3532531422 EXT. 227
Telex: 3532532563
Fax: 75020 IE

DR. M.A. COLLINS
LECTURER
DEPARTMENT OF FOOD MICROBIOLOGY
THE QUEEN'S UNIVERSITY OF BELFAST
NEWFORGE LANE
UK BT9 5PX BELFAST
Tel.: 44232661166 EXT. 231
Telex: 44232669551
Fax: QUBADM 74487

DR. M.D. COLLINS
HEAD, MOLECULAR BIOLOGY SECTION
DEPARTMENT OF MICROBIOLOGY
READING LABORATORY
AFRC INSTITUTE OF FOOD RESEARCH
SHINFIELD
UK RG2 9AT READING
Tel.: 447348831032225
Telex: 44734884763

PROF. L. COLOMBO
FULL PROFESSOR OF COMPARATIVE
ENDOCRIN
DEPARTMENT OF BIOLOGY
LABORATORY OF COMPARATIVE
ENDOCRINOLOG
UNIVERSITY OF PADOVA
VIA TRIESTE 75
I 35121 PADOVA
Tel.: 390498286114
Telex: 39498286100
E-mail: BIOCOL@UNIPAD.UNIPD.IT

PROF. C. COLSON
LECTURER
LABORATOIRE DE GENETIQUE
MICROBIENNE (
UNIVERSITE CATHOLIQUE DE LOUVAIN
PLACE CROIX DU SUD 4
B 1348 LOUVAIN-LA-NEUVE
Tel.: 3210473483
Telex: 3210473109
Fax: 59037 UCL-B
E-mail: COLSON@BUCLLN11

DR. H.S. CONRADT
TBC
DEPARTMENT OF GENETICS
PROJECT GROUP "PROTEIN GLYCOSYLATION"
GBF
MASCHERODER WEG 1
D 3300 BRAUNSCHWEIG
Tel.: 495316181212/219
Telex: 495316181202
Fax: 952668 GEBIO D

DR. H.J. COOKE
ACTING HEAD OF STRUCTURE STUDIES DIVIS
MRC HUMAN GENETICS UNIT
WESTERN GENERAL HOSPITAL
CREWE ROAD
UK EH4 2XU EDINBURGH
Tel.: 44313322471
Telex: 44313432620

DR. B.J.C. CORNELISSEN
GROUPLEADER
MOGEN INTERNATIONAL NV
EINSTEINWEG 97
NL 2333 CB LEIDEN
Tel.: 3171258282
Telex: 3171221471

DR. R. CORTESE
ISTITUTO DI RICERCHE DI BIOLOGIA MOLEC
(IRBM)
MERCK, SHARP AND DOHME RESEARCH
VIA PONTINA
KM 30,600
I 00040 POMEZIA (ROMA)
Tel.: 3969124582
Telex: 3969125530

PROF. P. COSTANTINO
PROFESSOR OF MOLECULAR BIOLOGY/
GROUP L
DIP. GENETICA E BIOLOGIA MOLECOLARE
UNIVERSITA DI ROMA "LA SAPIENZA"
PIAZZALE ALDO MORO 5
I 00185 ROMA
Tel.: 3964455344
Telex: 3964440812

DR. G. COUPLAND
SENIOR SCIENTIFIC OFFICER
INSTITUTE OF PLANT SCIENCE RESEARCH,
CAMBRIDGE LABORATORY
COLNEY LANE
UK NR4 7UH NORWICH
Tel.: 4460352571
Telex: 4460356844
Fax: 975122

PROF. P. COZZONE
DIRECTOR OF CRMBM
CENTRE DE RESONANCE MAGNETIQUE
BIOLOGI
ET MEDICALE (CRMBM), UNITE CNRS 1186
FACULTE DE MEDICINE DE MARSEILLE
BOULEVARD JEAN MOULIN 27
F 13005 MARSEILLE
Tel.: 33912556529
Telex: 3391256539

PROF. M. CRESTI
FULL PROFESSOR BOTANY
DIPARTIMENTO DI BIOLOGIA AMBIENTALE
UNIVERSITA DEGLI STUDI DI SIENA
VIA P.A. MATTIOLI 4
I 53100 SIENA
Tel.: 49577298854
Telex: 49577298860
Fax: 572459 UNIV SI I
E-mail: VAXTIEZZI@SIVAX.CINECA.IT

DR. R.R. CROY
SENIOR EXPERIMENTAL OFFICER
PLANT MOLECULAR BIOLOGY GROUP
DEPT. OF BIOLOGICAL SCIENCES
UNIVERSITY OF DURHAM
SOUTH ROAD
UK DH1 3LE DURHAM
Tel.: 44913742433
Telex: 44913743741
Fax: 537 351 DURLIB G
E-mail: CROY@UK.AC.DURHAM

PROF. E. CUNDLIFFE
PROFESSOR OF BIOCHEMISTRY
DEPARTMENT OF BIOCHEMISTRY
UNIVERSITY OF LEICESTER
UNIVERSITY ROAD
UK LE1 7RH LEICESTER
Tel.: 44533523451
Telex: 44533523369
Fax: 342318 LEIBIO G

DR. P.J. DALE
DEPUTY HEAD OF BRASSICA AND
OILSEEDS R
INSTITUTE OF PLANT SCIENCE RESEARCH
CAMBRIDGE LABORATORY
JOHN INNES CENTRE FOR PLANT SCIENCE RE
COLNEY LANE
UK NR4 7UJ NORWICH
Tel.: 446035271
Telex: 44603502270
Fax: 975122 JILNOR G

DR. K. DALSGAARD
DEPT. CHEMISTRY/IMMUNOLOGY SECTION
STATE VETERINARY INSTITUTE FOR VIRUS R
LINDHOLM
DK 4771 KALVEHAVE
Tel.: 4555814523
Telex: 4555811766
Fax: 40306 VIRUS DK

PROF. C. DALY / DR. G. FITZGERALD
PROFESSOR AND LECTURER
FOOD MICROBIOLOGY DEPARTMENT AND
THE NATIONAL FOOD BIOTECHNOLOGY
CENTRE
UNIVERSITY COLLEGE
WESTERN ROAD
IRL CORK
Tel.: 35321276871 EXT. 284
Telex: 35321276318
Fax: 76050 UNIC EI

DR. J.L. DANGL
GROUP LEADER
MAX-DELBRUECK-LABORATORIUM IN DER
MPG
CARL-VON-LINNE -WEG 10
D 5000 KOELN 30
Tel.: 492215062430
Telex: 492215062613

DR. M.J. DANIELS
HEAD OF LABORATORY
THE SAINSBURY LABORATORY
NORWICH RESEARCH PARK
COLNEY
UK NR4 7UH NORWICH
Tel.: 4460352571
Telex: 44603568 44
Fax: 975122JIINORG

DR. R. DANKERT
DIRECTOR RZ RESEARCH BV
RZ RESEARCH BV
ROPTAWEG 4
NL 9123 ZR METSLAWIER
Tel.: 3151921442
Telex: 3151921774
Fax: 46050 ZPCLNL

DR. L. DAVIDSE
ROYAL SLUIS
P.O. BOX 22
NL 1600 AA ENKHUIZEN
Telex: 31228014404

DR. W. DAVIES
RESEARCHER
NORWEGIAN COLLEGE OF VETERINARY
MEDICI
ULLEVAALSVEIEN 72
P.O.BOX 8146
N 0033 OSLO
Tel.: 472964500
Telex: 472600985
E-mail: WILLIAM@BIOMED.UIO.NO

DR. C. DAVIES
RESEARCH AND ENGINEERING DIVISION
UNILEVER RESEARCH
COLWORTH HOUSE
UK MK44 11Q SHARNBROOK, BEDFORD
Tel.: 44234222559
Telex: 44234222552
Fax: 82229

DR. I.C. DE ALMEIDA
PRINCIPAL INVESTIGATOR
DEPARTAMENTO DE VIROLOGIA
LABORATORIO NACIONAL DE INVESTIGACAO
VETERINARIA
ESTRADA DE BENFICA 701
P 1500 LISBOA
Tel.: 3517162075
Telex: 35117160039
Fax: 18248VETERI P

DR. J.A.M.P. DE ALMEIDA
PROFESSOR AUXILIAR
INSTITUTO SUPERIOR DE AGRONOMIA
TAPADA DA AJUDA
P 1399 LISBOA CODEX
Tel.: 35113638161
Telex: 35114428766
Fax: 44700 ISATEL

PROF. P. DE BAETSELIER
PROFESSOR
IMB/VUB - INSTITUUT VOOR MOLECULAIRE B
VRIJE UNIVERSITEIT BRUSSEL
PAARDENSTRAAT 65
B 1640 SINT-GENESIUS-RODE
Tel.: 3223590358
Telex: 3223590390
Fax: 61091 VUBCO B

PROF. J.A.M. DE BONT
HEAD OF DEPARTMENT
DIVISON OF INDUSTRIAL MICROBIOLOGY
AGRICULTURAL UNIVERSITY WAGENINGEN
BOMENWEG 2
NL 6703 HD WAGENINGEN
Tel.: 31837084749
Telex: 318370847..

MR. J. DE BRABANDERE
PROGRAM MANAGER
BELGIAN COORDINATED COLLECTIONS OF MICROORGANISMS
SCIENCE POLICY OFFICE OF BELGIUM
RUE DE LA SCIENCE 8
B 1040 BRUSSELS
Tel.: 3222383411520..
Telex: 3222305912
Fax: 24501 PROSCI B

DR. C. DE CASTELLARNAU
CHIEF OF THE ATHEROTHROMBOSIS UNIT
ATHERO THROMBOSIS UNIT
INSTITUT RICERCA BIOMEDICA, DEPT. OF L
FUNDACIO' D'INVESTIGACIO' ST.PAU (FISP
AVDA SANT ANTONI M. CLARET 167
E 08025 BARCELONA
Tel.: 3433473133 EXT. 409
Telex: 3432552331

PROF. G. DE HAAS
PROFESSOR IN BIOCHEMISTRY
DEPARTMENT OF BIOCHEMISTRY
UNIVERSITY OF UTRECHT
PADUALAAN 8
CBLE / TRANSITORIUM III / "DE UITHOF"
NL 3584 CH UTRECHT
Tel.: 3130533186/3186
Telex: 3130522478

DR. V. DE LORENZO
COLABORADOR CIENTIFICO / CIB-STAFF MEM
CENTRO DE INVESTIGACIONES BIOLOGICAS
C.S.I.C.
VELAZQUES 144
E 28006 MADRID
Tel.: 3415854243
Telex: 3415627518

DR. L. DE RIDDER
DIRECTOR OF DEPARTMENT OF
HISTOLOGY, R
LABORATORIUM VOOR HISTOLOGIE
RIJKSUNIVERSITEIT GENT
LOUIS PASTEURLAAN 2
B 9000 GENT
Tel.: 3291240224 ext. 351
Telex: 3291240499

DR. E. DE SIMONE
STAFF SCIENTIST
CEINGE-CENTRO DI INGEGNERIA
GENETICA S
C/O DIPARTIMENTO DI BIOCHIMICA
E BIOTECHNOLOGIE MEDICHI
VIA SERGIO PANSINI 5
I 80131 NAPOLI
Tel.: 39817463073
Telex: 39817463074

PROF. W.M. DE VOS
RESEARCH GROUP LEADER NIZO,
PROFESSOR
NIZO
KERNHEMSEWEG 2
P.O. BOX 20
NL 6710 BA EDE
Tel.: 31838059558
Telex: 31838050400
E-mail: NIZO@CAOS.CAOS.KUN.NL

DR. S.C. DE VRIES
SENIOR LECTURER
DEPARTMENT OF MOLECULAR BIOLOGY
AGRICULTURAL UNIVERSITY WAGENINGEN
DREIJENLAAN 3
NL 6703 HA WAGENINGEN
Tel.: 31837084325
Telex: 31837083584

PROF. R. DE WACHTER
PROFESSOR
ONDERZOEKSGROEP MOLEKULAIRE
BIOLOGIE
DEPARTEMENT BIOCHEMIE
UNIVERSITAIRE INSTELLING ANTWERPEN
B 2610 ANTWERPEN
Tel.: 3238202319
Telex: 3238202248
Fax: 33646
E-mail: DEWACHTER@CCV.UIA.AC.BE

DR. P.J.G.M. DE WIT
SENIOR LECTURER
DEPT. OF PHYTOPATHOLOGY
AGRICULTURAL UNIVERSITY WAGENINGEN
BINNENHAVEN 9
NL 6709 PD WAGENINGEN
Tel.: 31837083130
Telex: 8341283412
Fax: 45854

DR. C. DEAN
SENIOR SCIENTIFIC OFFICER
INSTITUTE OF PLANT SCIENCE RESEARCH,
CAMBRIDGE LABORATORY
COLNEY LANE
UK NR4 7UH NORWICH
Tel.: 4460352571
Telex: 4460356844
Fax: 975122

DR. E. DEJANA
CHIEF OF THE LABORATORY OF
VASCULAR BI
VASCULAR BIOLOGY LABORATORY
ISTITUTO DI RICERCHE FARMACOLOGICHE
"MARIO NEGRI"
VIA ERITREA 62
I 20157 MILANO
Tel.: 39239014480
Telex: 3923546277
Fax: 331268 NEGRI I

PROF. F. DEL REY
ASSOCIATE PROFESSOR OF MICROBIOLOGY
INSTITUTO DE MICROBIOLOGIA BIOQUIMICA
FACULTAD DE BIOLOGIA
UNIVERSIDAD DE SALAMANCA
PLAZA DE LA MERCED
E 37008 SALAMANCA
Tel.: 3423294461
Telex: 3423294513

PROF. S. DELROT
CO DIRECTOR OF UA CNRS 574
UNITE ASSOCIEE CNRS 574
UNIVERSITE DE POITIERS
RUE DU FAUBOURG SAINT-CYPRIEN 25
F 86000 POITIERS
Tel.: 334941186
Telex: 3349559374

DR. M. DELSENY
DIRECTEUR DE RECHERCHE CNRS
LABORATOIRE DE PHYSIOLOGIE ET BIOLOGIE
MOLECULAIRE VEGETALE (URA CNRS 565)
UNIVERSITE DE PERPIGNAN
AVENUE DE VILLENEUVE
F 66025 PERPIGNAN CEDEX
Tel.: 3368668848
Telex: 3368668499

DR. PH. DESSEN
DIRECTEUR DE RECHERCHE - CNRS -
LABORATOIRE DE BIOCHIMIE
ECOLE POLYTECHNIQUE
F 91128 PALAISEAU
Tel.: 33169334884
Telex: 33169333013
E-mail: DESSEN@FRCTI51.BITNET

DR. K.M. DEVINE
UNIVERSITY LECTURER
DEPARTMENT OF GENETICS
TRINITY COLLEGE
UNIVERSITY OF DUBLIN
IRL 2 DUBLIN
Tel.: 3531772941 EXT. 1872
Telex: 3531798558
Fax: 93782 TCDEI
E-mail: KDEVINE@VAX1.TCD.IE

DR. D. DI LORENZO
CNR RESEARCHER
ISTITUTO DI CHIMICA E PROPEDEUTICA BIO
UNIVERSITY OF BRESCIA
PIAZZALE SPEDALI CIVILI 1
OSPEDALE CIVILE
I 25100 BRESCIA
Tel.: 393039951554
Telex: 3930307251

PROF. H.G. DICKINSON
PROFESSOR AND HEAD OF SCHOOL OF PLANT
DEPARTMENT OF PLANT SCIENCES
UNIVERSITY OF OXFORD
SOUTH PARKS ROAD
UK OX1 3RB OXFORD
Tel.: 44865275800
Telex: 44865275074

DR. B. DIDERICHSEN
HEAD OF DEPARTMENT
NOVO NORDISK
NOVO ALLE
DK 2880 BAGSVAERD
Tel.: 45444900333401
Telex: 4544490070
Fax: 37173

DR. J. DIEZI
TBC
INSTITUT DE PHARMACOLOGIE ET DE TOXICO
UNIVERSITE DE LAUSANNE
RUE DE BUGNON 27
CH 1005 LAUSANNE
Tel.: 41213132700
Telex: 41213132775

PROF. L. DIJKHUIZEN
SENIOR LECTURER IN MICROBIOLOGY
DEPARTMENT OF MICROBIOLOGY
UNIVERSITY OF GRONINGEN
KERKLAAN 30
NL 9751 NN HAREN
Tel.: 3150632153
Telex: 3150635205
Fax: 53410 RUGRO

PROF. B.W. DIJKSTRA
UNIVERSITAIR HOOFDDOCENT (ASSOC. PROFE
LABORATORY OF CHEMICAL PHYSICS
UNIVERSITY OF GRONINGEN
NIJENBORGH 16
NL 9747 AG GRONINGEN
Tel.: 3150634381
Telex: 3150634200
Fax: 53935
E-mail: BAUKE@HGRRUG52

PROF. G. DODSON
PROFESSOR
DEPARTMENT OF CHEMISTRY
UNIVERSITY OF YORK
UK YOI 5DD HESLINGTON, YORK
Tel.: 44904430000
Telex: 44904410519

DR. R. DOELZ
BIOCOMPUTING
BIOZENTRUM DER UNIVERSITAET BASEL
KLINGELBERGSTRASSE 70
CH 4056 BASEL
Tel.: 41612672247
Telex: 41612616760
E-mail: DOELZ@URZ.UNIBAS.CH

DR. H. DOMDEY
LABORATORIUM FUER MOLEKULARE BIOLOGIE
GENZENTRUM DER LMU MUENCHEN
AM KLOPFERSPITZ 18A
D 8033 MARTINSRIED
Tel.: 498985783942
Telex: 498985783795
Fax: 521740MPIB

PROF. E. DOMINGO
PROFESOR DE INVESTIGACION
CENTRO DE BIOLOGIA MOLECULAR
UNIVERSIDAD AUTONOMA DE MADRID
CSIC
CANTO BLANCO
E 28049 MADRID
Tel.: 3413975070
Telex: 3413974799
Fax: 42182

PROF. A. DOMINGUEZ
PROFESOR TITULAR DE MICROBIOLOGIA
DEPTO. DE MICROBIOLOGIA, GENETICA,
MEDICINA PREVENTIVA Y SALUD PUBLICA
FAC. DE BIOLOGIA / UNIVERSIDAD DE SALA
PLAZA DE LA MERCED S/N
E 37071 SALAMANCA
Tel.: 3423294461
Telex: 3423294513
Fax: 26828 EDUCI

DR. J. DOMMES
PROJECT LEADER
EUROGENTEC SA
ALLEE DU AOUT, B6
CAMPUS DU SART-TILMAN
B 4000 LIEGE
Tel.: 3241563310
Telex: 3241562968

PROF. P. DONINI
FULL PROFESSOR
DIPARTIMENTO DI BIOLOGIA
CELLULARE E D
SEZ. DI SCIENZE MICROBIOLOGICHE
UNIVERSITA' DI ROMA "LA SAPIENZA"
VIA DEGLI APULI 1
I 00185 ROMA
Tel.: 396493232
Telex: 39649910789 / +39649

DR. H. DONS
CENTRE FOR PLANT BREEDING AND
REPRODUCTIVE RESEARCH
DROEVENDAALSESTEEG 1
POSTBUS 16
NL 6700 AA WAGENINGEN
Tel.: 31837077000
Telex: 31837018094

DR. D. DOWLING
PROJECT MANAGER
DEPT. OF FOOD MICROBIOLOGY
BIOTECHNOLOGY CENTRE
UNIVERSITY COLLEGE
IRL 30 CORK
Tel.: 35321272097
Telex: 35321275934
Fax: 76050 UNIC EI
E-mail: STDM 8008

DR. A. DOYLE
HEAD OF ECACC GROUP
ECACC - EUROPEAN COLLECTION OF
ANIMAL CELL CULTURES
PHLS CENTRE
PORTON DOWN
UK SP4 OJG SALISBURY
Tel.: 44980610391
Telex: 44980611315
E-mail: TELECOM GOLD 10075:DBI0008

PROF. L. DUBERTRET
DIRECTEUR DE L'UNITE INSERM 312
LAB. DE PHARMACOLOGIE CUTANEE
UNITE INSERM 312
AV. DU MARECHAL DE LATTRE DE
TASSIGNY 51
HOPITAL HENRI MONDOR
F 94010 CRETEIL
Tel.: 33149813606
Telex: 33142070704

PROF. J.A. DUINE
PROFESSOR IN APPLIED ENZYMOLOGY
BIOTECHNOLOGY
FACULTY OF CHEMICAL TECHNOLOGY
DELFT UNIVERSITY OF TECHNOLOGY
JULIANALAAN 67
NL 2628 BC DELFT
Tel.: 31157891115051
Telex: 3115782355
Fax: 38151 BUTUD NL

PROF. B. DUJON
PROFESSOR
DEPARTEMENT BIOLOGIE MOLECULAIRE
INSTITUT PASTEUR
RUE DU DOCTEUR ROUX 25
F 75724 PARIS 15
Tel.: 33145688482
Telex: 33145688521
Fax: 250609F
E-mail: DUJON@PASTEUR

PROF. C. DUMAS
PROFESSOR
RECONNAISSANCE CELLULAIRE ET
AMELIORAT
LA INRA 879
INSTITUT DE LA RECHERCHE
AGRONOMIQUE
BOULEVARD DU 11 NOVEMBRE 1918 43
F 69622 VILLEURBANNE CEDEX
Tel.: 3372448302
Telex: 3372448501
Fax: 810310

PROF. L.K. DUNICAN
PROFESSOR OF MICROBIOLOGY;
HEAD OF THE
UNIVERSITY COLLEGE
IRL GALWAY
Tel.: 3539124411
Telex: 3539125700
Fax: 50023 UCG E.I.

DR. J.M. DUNWELL
PROJECT LEADER
JEALOTTS HILL RESEARCH STATION
ICI SEEDS
UK RG12 6EY BRACKNELL
Tel.: 44344414823
Telex: 44344414818
Fax: 847556 ICIPPJG

PROF. G. DURAND
PROFESSEUR
DEP. DE GENIE BIOCHIMIQUE ET ALIMENTAI
INSTITUT NATIONAL DES SCIENCES
APPLIQUEES (INSA)
AVENUE DE RANGUEIL
F 31077 TOULOUSE
Tel.: 3361559720
Telex: 3361559652

DR. D. DWYER
PROJECT LEADER
DEPARTMENT OF MICROBIOLOGY
GBF
MASCHERODER WEG 1
D 3300 BRAUNSCHWEIG
Tel.: 495316181400
Telex: 495316181411
Fax: 952667 GEBIOD

DR. M.R. EGMOND
RESEARCH MANAGER
UNILEVER RESEARCH LABORATORIUM
OLIVIER VAN NOORTLAAN 120
NL 3133 AT VLAARDINGEN
Tel.: 31104605039/5037
Telex: 31104605383
Fax: 23261

DR. S.D. EHRLICH
DIRECTEUR DE RECHERCHE,
HEAD OF THE LA
LABORATOIRE DE GENETIQUE
MICROBIENNE
INRA
DOMAINE DE VILVERT
F 78350 JOUY EN JOSAS
Tel.: 33134652511
Telex: 33134652521
E-mail: EHRLICH@BIOTECJOUY.INRA.FR

DR. V. ELFERINK
RESEARCH COORDINATOR / PROJECT
MANAGER
ANDENO B.V.
GRUBBENVORSTERWEG 8
NL 5928 NX VENLO
Tel.: 31778995558992
Telex: 3177899300
Fax: 58310 AVE NL

PROF. K.-D. ENTIAN / DR. M. BRENDEL
INSTITUT FUER MIKROBIOLOGIE
J.W. GOETHE-UNIVERSITAET FRANKFURT/M.
THEODOR-STERN-KAI 7
HAUS 75A
D 6900 FRANKFURT
Tel.: 496963016468
Telex: 496963017639

DR. E. FALSEN
CCUG CULTURE COLLECTION
DEPARTMENT OF CLINICAL BACTERIOLOGY
UNIVERSITY OF GOTEBORG
GULDHEDSG. 10
S 413 46 GOTEBORG
Tel.: 4631604625
Telex: 4631825484
E-mail: TELECOM GOLD 10075:DBI0070 /
TIXCC@SEG

PROF. H. FELDMANN
UNIVERSITAETS PROFESSOR
INSTITUT FUER PHYSIOLOGISCHE CHEMIE
LUDWIG-MAXIMILIANS-UNIVERSITAET
SCHILLERSTRASSE 44
D 8000 MUENCHEN 2
Tel.: 49895996451
Telex: 49895996316
Fax: 529860

PROF. I. FERRERO
PROFESSOR OF GENETICS
INSTITUTE OF GENETICS
UNIVERSITY OF PARMA
VIALE DELLE SCIENZE
I 43100 PARMA
Tel.: 39521580601
Telex: 39521580665

PROF. A.R. FERSHT
DIRECTOR
MRC UNIT FOR PROTEIN FUNCTION AND
DESI
DEPT. OF CHEMISTRY
UNIVERSITY OF CAMBRIDGE
LENSFIELD ROAD
UK CB2 IEW CAMBRIDGE
Tel.: 44223336341
Telex: 44336362

PROF. DR. W. FIERS
LABORATORY OF MOLECULAR BIOLOGY
RIJKS UNIVERSITEIT GENT
K.L. LEDEGANCKSTRAAT 35
B 9000 GENT
Tel.: 3291645131
Telex: 3291645348
Fax: 11995 GENGEN

DR. J. FIGUEIREDO MARQUES
SENIOR RESEARCHER/DIRECTOR OF THE
LAB.
LABORATORIO DE MICROBIOLOGIA
INSTITUTO DE BIOLOGIA EXPERIMENTAL
E TECNOLOGICA
APARTADO 12
P 2780 OEIRAS
Tel.: 35114427787
Telex: 35114421161

DR. R. FLEER
SENIOR SCIENTIST. RESEARCH GROUP
UGENE
CENTRE DE RECHERCHE DE VITRY ET
ALFORT
INSTITUT DE BIOTECHNOLOGIE (IBV)
RHONE POULENC SANTE
QUAI JULES GUESDE 13
F 94403 VITRY SUR SEINE
Tel.: 33145737890
Telex: 33145737796

PROF. M. FONSECA
ASSISTANT PROFESSOR
INSTITUTO SUPERIOR TECNICO
AVENIDA ROVISCO PAIS
P 1000 LISBOA 1096
Tel.: 3511802045 EXT. 1682
Telex: 35118480072
Fax: 63423 ISTUTL P

DR. B.G. FORDE
ROTHAMSTED EXPERIMENTAL STATION
BIOCHEMISTRY AND PHYSIOLOGY DEPT.
THE LAWES AGRICULTURAL TRUST
UK AL5 2JQ HARPENDEN
Tel.: 44582763133
Telex: 44582760981

DR. F. FOURY
UNITE DE BIOCHIMIE PHYSIOLOGIQUE
FYSA
UNIVERSITE CATHOLIQUE DE LOUVAIN
PLACE CROIX DU SUD 1
BTE 8
B 1348 LOUVAIN LA NEUVE
Tel.: 3210473618
Telex: 3210473872

DR. F.C. FRANKLIN
LECTURER
SCHOOL OF BIOLOGICAL SCIENCES
UNIVERSITY OF BIRMINGHAM
UK B15 2TT BIRMINGHAM
Tel.: 44214145910
Telex: 44214145925

DR. M. FREDHOLM
SENIOR SCIENTIST
DEPARTMENT OF ANIMAL SCIENCE AND ANIMA
DIVISION OF ANIMAL GENETICS
THE ROYAL VETERINARY AND AGRICULTURAL
BUELOWSVEJ 13
DK 1870 FREDERIKSBERG C
Tel.: 4535283074
Telex: 4535283042

DR. H. FREDRICKSON
PROJECT LEADER
ABTEILUNG MIKROBIOLOGIE
GBF
MASCHERODER WEG 1
D 3300 BRAUNSCHWEIG
Tel.: 495316181419
Telex: 495316181411
Fax: 952667 GEBIO D

PROF. J. FRISVAD
DEPT. OF BIOTECHNOLOGY
DANMARK TEKNISCHE HOEJSKOLE
BUILDING 221
DK 2800 LYNGBY
Tel.: 4545931222
Telex: 4542884922
Fax: 37529 DTHDIA DK

DR. R. FRITZ
DIRECTOR
DIMDI - DEUTSCHES INSTITUT FUER MEDIZI
DOKUMENTATION UND INFORMATION
WEISSHAUSSTRASSE 27
P.O. BOX 420580
D 5000 KOELN 41
Tel.: 4922147241211
Telex: 49221411429
Fax: 8881364

PROF. L. FRONTALI
PROFESSOR
DEPARTMENT OF CELL AND DEVELOPMENTAL BIOLOGY
UNIVERSITY OF ROMA "LA SAPIENZA"
PIAZZALE ALDO MORO 5
I 00185 ROMA
Tel.: 3964453950
Telex: 39649912351

DR. S.C. FRY
READER
DEPT. OF BOTANY
UNIVERSITY OF EDINBURGH
THE KING'S BUILDINGS
MAYFIELD ROAD
UK EH9 3JH EDINBURGH
Tel.: 44316671081330
Telex: 44316683870
Fax: 727442 UNIVED G

DR. J.C. FRY
SENIOR LECTURER
SCHOOL OF PURE AND APPLIED BIOLOGY
COLLEGE OF CARDIFF
UNIVERSITY OF WALES
P.O. BOX 915
UK CF1 3TL CARDIFF
Tel.: 442228740005878
Telex: 442228743O5
Fax: 498635

DR. S.C. FRY
READER
DEPARTMENT OF BOTANY
UNIVERSITY OF EDINBURGH
MAYFIELD ROAD
THE KING'S BUILDINGS
UK EH9 3JH EDINBURGH
Tel.: 44316671081330
Telex: 44316683870
Fax: 727442 UNIVED G

PROF. DR. H. FUKUHARA
INSTITUT CURIE, SECTION DE BIOLOGIE
CENTRE UNIVERSITAIRE, BATIMENT 110
RUE GEORGES CLEMENCEAU 15
F 91405 ORSAY
Tel.: 33169863063
Telex: 33169861703
Fax: CURIE 270479F
E-mail: FUKUHARA@FRCITI51

PROF. H. FUKUHARA
HEAD OF LABORATORY
LABORATOIRE DE GENETIQUE MOLECULAIRE
INSTITUT PASTEUR
RUE GEORGES CLEMENCEAU 15
F 91405 ORSAY
Tel.: 33169863063
Telex: 33169869429
Fax: FACORS 692 116 F
E-mail: FUKUHARA@FRCITI51

PROF. N.E. FUSENIG
PROFESSOR, HEAD OF DIVISION
INSTITUTE OF BIOCHEMISTRY
DEUTSCHES KREBSFORSCHUNGS-ZENTRUM - DK
IM NEUENHEIMER FELD 280
D 6900 HEIDELBERG
Tel.: 4962214841507
Telex: 496221474457
Fax: 461 562 DKFZ D

PROF. C. GAILLARDIN
PROFESSOR
LABORATOIRE DE GENETIQUE DES MICRO-ORG
INAPG - CENTRE DE BIOTECHNOLOGIE AGRO-
INRA
F 78850 THIVERVAL-GRIGNON
Tel.: 33130544510
Telex: 33130545690
Fax: 697388

DR. F. GALIBERT
HEAD OF LABORATORY
LABORATOIRE RECOMBINAISON GENETIQUE
HOPITAL ST LOUIS / CENTRE HAYEM
CNRS
VELLEFEAUX 1
HOPITAL ST LOUIS
F 75010 PARIS
Tel.: 33142021605
Telex: 33142411470
Fax: IRM2L2309F

DR. P. GALLOIS
MAITRE DE CONFERENCES
LABORATOIRE DE PHYSIOLOGIE VEGETALE
UNIVERSITE DE PERPIGNAN
AVENUE DE VILLENEUVE
F 66025 PERPIGNAN
Tel.: 3368668848
Telex: 3368668499

PROF. F. GANNON
ASSOCIATE PROFESSOR AND DIRECTOR, NATI
DEPARTMENT OF MICROBIOLOGY
UNIVERSITY COLLEGE GALWAY
UNIVERSITY ROAD
IRL GALWAY
Tel.: 3539124411
Telex: 3539166570
Fax: 55023 EI

DR. M-I. GARCIA LUQUE
ASSISTANT PROFESSOR
C.S.I.C.
VELAZQUEZ 44
E 28006 MADRID
Tel.: 3412611800
Telex: 3412627518

DR. R.M. GASKELL
RESEARCH FELLOW
DEPARTMENT OF VETERINARY PATHOLOGY
AND
MEDICAL MICROBIOLOGY
UNIVERSITY OF LIVERPOOL
VETERINARY FIELD STATION LEAHURST
UK L64 7TE NESTON, WIRRAL
Tel.: 44517946019
Telex: 517946005
Fax: 62095 UNILPL G

PROF. M.J. GASSON
HEAD OF DEPARTMENT
AFRC INSTITUTE OF FOOD RESEARCH
NORWICH RESEARCH PARK
COLNEY
UK NR4 7UA NORWICH
Tel.: 4460356122 EXT. 248
Telex: 44603507723
Fax: 975453

DR. A. GEIS
BUNDESANSTALT FUER MILCHFORSCHUNG
KIEL
HERMANN-WEIGMANN-STRASSE 1
D 2300 KIEL
Tel.: 49431609352
Telex: 49431609222

PROF. H. GELDERMANN
UNIV.- PROF.
INSTITUET FUER TIERHALTUNG AND
TIERZUE
FACHGEBIET TIERZUECHTUNG 470
UNIVERSITAET HOHENHEIM
GARBENSTRASSE 17
D 7000 STUTTGART 70
Tel.: 497114592474
Telex: 497114593290

DR. P. GIACOMONI
LABORATOIRE DE RECHERCHE DE L'OREAL
AVENUE EUGENE SCHUELLER 1
BP 22
F 93601 AULNAY SOUS BOIS
Tel.: 33148689710
Telex: 33148699889

DR. A.L.J. GIELKENS
HEAD DEPARTMENT MOLECULAR BIOLOGY
DEPARTMENT OF MOLECULAR BIOLOGY
CENTRAL VETERINARY INSTITUTE
HOUTRIBWEG 39
P.O. BOX 365
NL 8200 AJ LELYSTAD
Tel.: 31320076611/76604
Telex: 31320042804
Fax: 47907

DR. J. GIRAUDAT
CHARGE DE RECHERCHE, GROUP LEADER
INSTITUT DES SCIENCES VEGETALES
CENTRE NATIONAL DE LA RECHERCHE
SCIENT
AVENUE DE LA TERRASSE
F 91198 GIF-SUR-YVETTE CEDEX
Tel.: 33169823696
Telex: 33169823695
Fax: 601137F

DR. G. GIULIANO
STAFF SCIENTIST
ENEA - COMITATO NAZIONALE PER LO SVILU
RICERCA DELLA ENERGIA NUCLEARE E
DELLE ENERGIE ALTERNATIVE.
STRADA PROVINCIALE ANGUILLARESE
P.O. BOX 2400
I 00100 AD ROMA
Tel.: 39630486343
Telex: 39630484995

PROF. N.N. GLANSDORFF /DR. A. PIERARD
RESEARCH INSTITUTE OF THE CERIA - COOV
AVENUE E. GRYSON 1
B 1070 BRUXELLES
Tel.: 3225267275 / 3225267
Telex: 3225267042
E-mail: R1310@BBRFE01

DR. T. GODEFROY-COLBURN
DIRECTEUR DE RECHERCHE 2E CLASSE,
CNRS
INSTITUT DE BIOLOGIE MOLECULAIRE DES P
RUE DU GENERAL ZIMMER 12
F 67084 STRASBOURG
Tel.: 3388417255
Telex: 3388614442

DR. J.L. GORDON
DIRECTOR OF RESEARCH
RESEARCH DIVISION
BRITISH BIO-TECHNOLOGY LIMITED
BROOK HOUSE
WATLINGTON ROAD
COWLEY
UK OX4 5LY OXFORD
Tel.: 44865748747 EXT. 226
Telex: 44865717598
Fax: 838083 BIOTEC G

PROF. P.H. GOUYON
PROFESSEUR
LABORATOIRE D'EVOLUTION ET
SYSTEMATIQUE DES VEGETAUX
UNIVERSITE DE PARIS SUD
BATIMENT 362
F 91405 ORSAY
Tel.: 33169417222
Telex: 33164461992

DR. M.A. GRANDBASTIEN
STAFF SCIENTIST (CR1)
LABORATOIRE DE BIOLOGIE CELLULAIRE
INRA
CENTRE DE VERSAILLES
F 78026 VERSAILLES
Tel.: 33130833024
Telex: 33130833099
Fax: 695269

PROF. P. GRAY
PROFESSOR (FULL)
DEPARTMENT OF COMPUTING SCIENCE
UNIVERSITY OF ABERDEEN
KINGS COLLEGE
UK AB9 2UB ABERDEEN
Tel.: 44224272292
Telex: 44224274322
E-mail: PGRAY@CSD.ABDN.AC.UK

DR. J.B. GRIFFITHS
DEPUTY HEAD DIVISION OF BIOLOGICS
DIVISION OF BIOLOGICS
PUBLIC HEALTH LABORATORY SERVICE
CAMR
PORTON DOWN
UK SP4 OJG SALISBURY, WILTSHIRE
Tel.: 449806103912340
Telex: 44980611310
Fax: 47683 PH CAMR G
E-mail: TELECOM GOLD 10075:DBI0222

DR. R. GRIFFITHS
LECTURER IN BIOCHEMISTRY
DEPARTMENT OF BIOCHEMISTRY AND
MICROBI
UNIVERSITY OF ST. ANDREWS
NORTH STREET
UK KY16 9AL ST ANDREWS
Tel.: 4433476161
Telex: 4433474674

PROF. P.A.D. GRIMONT
CHIEF OF UNIT
UNITE DES ENTEROBACTERIES
INSTITUT PASTEUR
RUE DU DOCTEUR ROUX 28
F 75724 PARIS CEDEX 15
Tel.: 33145688340
Telex: 33145688837
Fax: 250609 F

PROF. DR. L. GRIVELL
SECTION FOR MOLECULAR BIOLOGY
DEPT. OF MOLECULAR CELL BIOLOGY
UNIVERSITY OF AMSTERDAM
KRUISLAAN 318
NL 1098 SM AMSTERDAM
Tel.: 31205257924
Telex: 31206685086
E-mail: UOO193HASARA5

DR. M.A.M. GROENEN
BIOTECHNICAL RESEARCHER
DEPARTMENT OF ANIMAL BREEDING
AGRICULTURAL UNIVERSITY WAGENINGEN
MARIJKEWEG 40
P.O. BOX 338
NL 6700 AH WAGENINGEN
Tel.: 31837083747
Telex: 31837083962
Fax: 45015 NL
E-mail: GROENEN@ZOA.LUW.NL

PROF. F. GRUMMT
PROFESSOR
UNIVERSITAET WUERZBURG
ROENTGENRING 11
D 8700 WURZBURG
Tel.: 4993131648

DR. J. GUARDIOLA
SENIOR RESEARCHER AND GROUP LEADER
ISTITUTO INTERNAZIONALE DI GENETICA E
CNR
VIA GUGLIELMO MARCONI 10
I 80125 NAPOLI
Tel.: 39815935370
Telex: 39815936123
E-mail: NA0402@ICNUCEVM

DR. W.H. GUENZBURG
SENIOR SCIENTIST AND PROJECT LEADER
INSTITUT FUER MOLEKULARE VIROLOGIE
GSF - FORSCHUNGSZENTRUM FUER UMWELT
UND GESUNDHEIT
INGOLSTAEDTER LANDSTRASSE 1
D 8042 NEUHERBERG
Tel.: 498931873126
Telex: 498931873329
Fax: 523125 STRAL D
E-mail: WOLFI@DMOGSFII
Teletex: 898947

PROF. J. GUERN
C.N.R.S.
SCE DE PHYSIOLOGIE CELLULAIRE
VEGETALE
AVE DE LA TERRASSE
B.P. N° 1
F 91190 GIF-SUR-YVETTE
Tel.: +33.1.69823868
Telex: +33.1.69823768

PROF. R. GUERRERO
DIRECTOR GRUPO INVESTIGACION
DEPARTAMENTO DE MICROBIOLOGIA
UNIVERSIDAD DE BARCELONA
AV. DIAGONAL 645
E 08028 BARCELONA
Tel.: 3434021484
Telex: 3434110592
E-mail: D3MIJMC1@EBOUBO11

DR. M. GUILLON
RESEARCH AND DEVELOPMENT MANAGER
CALLIOPE S.A.
RUE DU CHAPEAU ROUGE 7
F 34325 BEZIERS
Tel.: 3367281678
Telex: 3367288902
Fax: 480947 F

DR. CHR. GUILLOUZO
DIRECTOR OF RESEARCH
INSERM U 49
HOSPITAL DE PONTCHAILLOU
F 35033 RENNES
Tel.: 331699543737
Telex: 33145856856

PROF. M.L. GULLINO
PROFESSEUR
UNIVERSITA DEGLI STUDI DI TORINO
VIA GIURIA 15
I 10126 TORINO
Tel.: 39116505236
Telex: 39116502139

DR. L GUSTAVSSON
PROFESSOR
DEPARTMENT OF ANIMAL BREEDING AND GENE
SWEDISH UNIVERSITY OF AGRICULTURAL SCI
BOX 7023
S 750 07 UPPSALA
Tel.: 4618671000
Telex: 4618302965

DR. J. HACHE
SCIENTIFIC DIRECTOR
BERTIN AND CIE
RUE PIERRE CURIE 59
Z.I. DES GATINES / BP N° 3
F 78373 PLAISIR CEDEX
Tel.: 33134818500
Telex: 33130540414
Fax: 69623 F

DR. C. HADFIELD
RESEARCH GROUP LEADER
LEICESTER BIOCENTRE
UNIVERSITY OF LEICESTER
UNIVERSITY ROAD
UK LE1 7RH LEICESTER
Tel.: 44533522269
Telex: 44533551219

PROF. M.A. HALL
PROFESSOR OF BOTANY
DEPARTMENT OF BIOLOGICAL SCIENCES
THE UNIVERSITY COLLEGE OF WALES
PENGLAIS
UK SY23 3DA ABERYSTWYTH
Tel.: 0970622313
Telex: 0970617172
Fax: 35I8IABYUCWG
E-mail: MZH UK.AC.ABERYSTWYTH

DR. W.D. HAMILTON
GROUP LEADER OF APPLIED PLANT TECHNOLO
AGRICULTURAL GENETICS COMPANY LIMITED
SCIENCE PARK MILTON ROAD 154
UK CB4 4GG CAMBRIDGE
Tel.: 44223420882
Telex: 44223420801
Fax: 817568AGGENEG

PROF. W.P. HAMMES
DIRECTOR OF THE INSTITUTE
HOHENHEIM UNIVERSITY
GARBENSTRASSE 25
D 7000 STUTTGART 70
Tel.: 497114592305
Telex: 497114593443

DR. E.B. HANSEN
HEAD OF DEPARTMENT, PH.D.
CHR. HANSEN'S LABORATORIUM DANMARK A/S
BOEGE ALLE 10
DK 2970 HOERSHOLM
Tel.: 4545767676 EXT. 4027
Telex: 4545765455
Fax: 19184 HANSN DK

PROF. C. HARDISSON
CATEDRATICO DE UNIVERSIDAD
DEPARTAMENTO DE BIOLOGIA FUNCIONAL
AREA MICROBIOLOGIA
UNIVERSIDAD DE OVIEDO
JULIAN CLAVERIA S/N
E 33006 OVIEDO
Tel.: 3485103557
Telex: 3485232255
Fax: 84322

DR. R. HARPER
RESEARCH SCIENTIST
CENTRE FOR SCIENTIFIC COMPUTING
FINNISH STATE COMPUTER CENTRE
P.O. BOX 40
SF 02101 ESPOO
Tel.: 35804572076
Telex: 35804572302
E-mail: HARPER@CSC.FI

DR. H. HAUSER
GROUP LEADER
GESELLSCHAFT FUER BIOTECHNOLOGISCHE FORSCHUNG MBH
MASCHERODER WEG 1
D 3300 BRAUNSCHWEIG
Tel.: 495316181250
Telex: 495316181202515
Fax: 952668 GEBIO D

PROF. D.L. HAWKSWORTH
DIRECTOR CMI
CAB INTERNATIONAL MYCOLOGICAL INSTITUT
(IMI)
FERRY LANE
UK W9 3AF KEW
Tel.: 44819404086
Telex: 44813321171
Fax: 9312102252 MIG
E-mail: DIALCOM 84:CAU009

DR. Y. HENRY
CRI CNRS
LABORATOIRE DE GENETIQUE VEGETALE
UNIVERSITE PARIS SUD
BT 360
F 91405 ORSAY
Tel.: 33169417960
Telex: 33169853715
Fax: 692166 F

DR. P.E. HERNANDEZ CRUZ
ASSISTANT PROFESSOR
DPTO. NUTRICION Y BROMATOLOGIA III
FACULTAD DE VETERINARIA
UNIVERSIDAD COMPLUTENSE
AVDA. PUERTA DE HIERRO S/N
E 28040 MADRID
Tel.: 3413943747
Telex: 3413943883
Fax: 41805 UCVET

DR. J. HIGGINGS
CRANFIELD BIOTECHNOLOGY LTD.
UK BEDFORD
Tel.: 44234750665
Telex: 44234750907
Fax: 825072 CITECH G

PROF. C.W. HILBERS
HEAD OF DEPARTMENT, PROFESSOR OF PHYSI
NIJMEGEN SON RESEARCH CENTRE (NSR CENT
LABORATORY FOR BIOPHYSICAL CHEMISTRY
FAC. NATUURWETENSCHAPPEN / K.U. NIJMEG
TOERNOOIVELD
NL 6525 ED NIJMEGEN
Tel.: 3180612160
Telex: 3180652112

DR. F. HILGER / DR. D. PORTETELLE
LABORATOIRE DE MICROBIOLOGIE
FACULTE DES SCIENCES AGRONOMIQUES
AVENUE MARECHAL JUIN 6
B 5800 GEMBLOUX
Tel.: 3281612355
Telex: 3281614544
Fax: 59482FSAGXT

DR. J. HILLE
UNIVERSITAIR HOOFDDOCENT
DEPT. OF GENETICS
FACULTY BIOLOGY
VRIJE UNIVERSITEIT AMSTERDAM
DE BOELELAAN 1087
NL 1081 HV AMSTERDAM
Tel.: 31205485753
Telex: 3120442556
Fax: 11329 DPVVU
E-mail: HILLE@BIO.VU.NL

PROF. H.-J. HINZ
PROFESSOR
INSTITUT FUER PHYSIKALISCHE CHEMIE
ABTEILUNG BIOPHYSIKALISCHE CHEMIE
UNIVERSITAET MUENSTER
SCHLOSSPLATZ 4/7
D 4400 MUENSTER
Tel.: 49251833427
Telex: 49251839163

DR. D.A. HODGSON
LECTURER IN MICROBIOLOGY
DEPARTMENT OF BIOLOGICAL SCIENCES
UNIVERSITY OF WARWICK
UK CV4 7AL COVENTRY
Tel.: 44203523559
Telex: 44203523701
Fax: 317472 UNIREG

DR. M.G. HOEFLE
ASSOCIATE SCIENTIST
ABTEILUNG MIKROBIOLOGIE
GBF
MASCHERODER WEG 1
D 3000 BRAUNSCHWEIG
Tel.: 495316181419
Telex: 495316181411
Fax: 952667 GEBIO D

DR. A. HOEKEMA
GROUP LEADER
MOGEN INTERNATIONAL NV
EINSTEINWEG 97
NL 2333 CB LEIDEN
Tel.: 3171258282
Telex: 3171221471

DR. F. HOEKSTRA
ASSOCIATE PROFESSOR
DEPARTMENT OF PLANT PHYSIOLOGY
AGRICULTURAL UNIVERSITY WAGENINGEN
ARBORETUMLAAN 4
NL 6703 BD WAGENINGEN
Tel.: 31837082454
Telex: 31837084740
Fax: NL45015
E-mail: HOEKSTRA@RCL.WAU.NL

PROF. W. HOL
PROFESSOR OF BIOSTRUCTURAL CHEMISTRY
LABORATORY OF CHEMICAL PHYSICS
UNIVERSITY OF GRONINGEN
NIJENBORGH 16
NL 9747 AG GRONINGEN
Tel.: 3150634383
Telex: 3150634800
Fax: 53935 CHRUG NL

PROF. C.P. HOLLENBERG
PROFESSOR FUER MIKROBIOLOGIE
HEINRICH-HEINE-UNIVERSITAET
UNIVERSITAETSSTRASSE 1
D 4000 DUESSELDORF
Tel.: 492113114720
Telex: 49211342229
Fax: 8587348 UNI D
E-mail: HOLLENB@DDORUD8I

DR. E. HONIKMAN-LEBAN
PHD BIOLOGY RESEARCH INGENEER
DEPARTMENT OF CHEMICAL PROTECTION AND
PHOTOBIOLOGICAL RESEARCH IN VITRO
UNIT OF CELL CULTURES / L'OREAL RESEAR
AVENUE EUGENE SCHUELLER 01
F 93600 AULNAY SOUS BOIS
Tel.: 33148689224
Telex: 33148689004

DR. P.J.J HOOYKAAS
UNIVERSITAIR HOOFDDOCENT
CLUSIUS LABORATORY
DEPT. PLANT MOLECULAR BIOLOGY
LEIDEN UNIVERSITY
WASSENAARSEWEG 64
NL 2333 AL LEIDEN
Tel.: 3171274933
Telex: 3171274999

PROF. D.A. HOPWOOD
PROFESSOR AND HEAD OF GENETICS
DEPARTM
JOHN INNES INSTITUTE
COLNEY LANE
UK NR4 7UH NORWICH
Tel.: 4460352571
Telex: 4460356844
Fax: 975122 G

DR. J. HUBER
ACTING DIRECTOR
INSTITUTE FOR BIOLOGICAL CONTROL
BBA
HEINRICHSTRASSE 243
D 6100 DARMSTADT
Tel.: 49615144061
Telex: 496151422502

DR. J.H.J. HUIS IN 'T VELD
HEAD DEPARTMENT OF MICROBIOLOGY
TNO NUTRITION AND FOOD RESEARCH
UTRECHTSEWEG 48
NL 3704 HE ZEIST
Tel.: 31340444730
Telex: 31340454186
Fax: 40022 CIVO NL

DR. R. HULL
SENIOR PRINCIPAL SCIENTIFIC OFFICER (U
JOHN INNES INSTITUTE
JOHN INNES CENTRE
NORWICH RESEARCH PARK
COLNEY LANE
UK NR4 7UH NORWICH
Tel.: 4460352571
Telex: 4460356844
Fax: 975122JIINORG
E-mail: HULL@UK.AC.AFRC.JII

DR. M. HUMPHREYS
AFRC INSTITUTE OF GRASSLAND AND
ENVIRONMENTAL RESEARCH
WPBS
PLAS GOGERDDAN
UK SY23 3EB ABERYSTWYTH, DYFED
Tel.: 44970828255
Telex: 44970828357

DR. J.C. HUTTON
BDA READER
DEPARTMENT OF CLINICAL BIOCHEMISTRY
ADDENBROOKE'S HOSPITAL
UNIVERSITY OF CAMBRIDGE
HILLS ROAD
UK CB2 2QR CAMBRIDGE -
Tel.: 44223336790
Telex: 44223216862
Fax: -
E-mail: JCH@UK.AC.CAM.PHX
Teletex: -

DR. M. HUYSMANS
SENIOR ENGINEER
SSI/R.D.
BIM
KWIKSTRAAT 4
B 3078 EVERBERG
Tel.: 3227595925
Telex: 3227594783
Fax: 63518
E-mail: MARTINA@SUNBIM.BE

DR. M. IACCARINO
INTERNATIONAL INSTITUTE OF GENETICS AN
BIOPHYSICS, CNR
VIA MARCONI 12
I 80125 NAPOLI
Tel.: 39815932428
Telex: 39815936123
E-mail: NA0402@ICNUCEVM.BITNET

DR. D. INZE / M. VAN MONTAGU
GROUP LEADER
LABORATORIUM VOOR GENETICA
RIJKSUNIVERSITEIT GENT
K.L. LEDEGANCKSTRAAT 35
B 9000 GENT
Tel.: 3291645170
Telex: 3291645349
E-mail: JECOP@GENGENP.RUG.AC.BE

DR. E. JACOBSEN
DEPARTMENT OF PLANT BREEDING
AGRICULTURAL UNIVERSITY WAGENINGEN
LAWICKSE ALLEE 166
P.O.BOX 386
NL 6700 AJ WAGENINGEN
Tel.: 31837082859
Telex: 31837083457

PROF. C. JACQ
GENETIQUE MOLECULAIRE
ECOLE NORMALE SUPERIEURE
RUE D'ULM 46
F 75230 PARIS
Tel.: 33143258737
Telex: 33143298172

DR. M. JACQUET
UNIVERSITE DE PARIS-SUD
BATIMENT 400
F 91405 ORSAY CEDEX
Tel.: 33169417963
Telex: 33169417296
Fax: 602166FACOR
E-mail: UBEX001@FROS

DR. J.-C. JAUNIAUX
DR.
LABORATOIRE DE MICROBIOLOGIE
UNIVERSITE LIBRE DE BRUXELLES
BD DU TRIOMPHE
CP 244
B 1050 BRUXELLES
Tel.: 322640015
Telex: 3226463148

DR. S.M. JENSEN
SCIENTIFIC OFFICER
ECOLOGICAL DIVISION
MINISTRY OF THE ENVIRONMENT
THE NATIONAL FOREST AND NATURE AGENCY
SLOTSMARKEN 13
DK 2970 HORSHOLM
Tel.: 45765376
Telex: 45765477

DR. A. JESTIN
CHARGE DE RECHERCHES
LABORATOIRE CENTRAL DE RECHERCHES AVICOLE ET PORCINE
CENTRE NATIONAL ETUDES VETERINAIRES ET
B.P. 9
F 22440 PLOUFRAGAN
Tel.: 3396941090
Telex: 3396786861
Fax: 950743

PROF. A. JIMENEZ
RESEARCH PROFESSOR
CENTRO DE BIOLOGIA MOLECULAR
C.S.I.C.
UNIVERSIDAD AUTONOMA CANTOBLANCO
E 28049 MADRID
Tel.: 3413975070
Telex: 3413974799
Fax: 27810 EDUCI E

DR. R. JIMENEZ DIAZ
COLABORADOR CIENTIFICO ,
INSTITUTO DE LA GRASA Y SUS DERIVADOS
I. DE BIOTECNOLOGIA DE ALIMENTOS
PADRE GARCIA TEJERO 4
E 41012 SEVILLA
Tel.: 3454691054
Telex: 3454691262
Fax: 72497 IGSE E

DR. M. JOHN
SCIENTIST / GROUP LEADER
MAX-PLANCK-INSTITUT FUER ZUECHTUNGSFOR
CARL-VON-LINNE-WEG 10
D 5000 KOELN 30
Tel.: 492215062235
Telex: 492215062213
Fax: 498881028 MPIZ D

PROF. L.M. JOHNSON
PROFESSOR OF MOLECULAR BIOPHYSICS
LABORATORY OF MOLECULAR BIOPHYSICS
DEPARTMENT OF BIOCHEMISTRY
REX RICHARDS BUILDING
SOUTH PARKS ROAD
UK 0X1 3QU OXFORD
Tel.: 44865275397/5365
Telex: 44865510454
Fax: 83147

DR. M. JOIGNY
DIRECTEUR
INSTITUT DU TABAC DE BERGERAC
SEITA
DOMAINE DE LA TOUR
F 24100 BERGERAC
Tel.: 3353636600
Telex: 3353636608
Fax: 570 220 SEITABE

DR. J. JONES
SENIOR SCIENTIST
THE SAINSBURY LABORATORY
JOHN INNES CENTRE
COLNEY LANE
UK NR4 7UH NORWICH
Tel.: 4460352571
Telex: 44603250024
Fax: 975122 JIINORG
E-mail: JONES@JII.AFRC.AC.UK

DR. J. JOSEPHSEN
ASSOCIATE PROFESSORS
ROYAL VETERINARY AND AGRICULTURAL UNIV
BUELOWSVEJ 13
DK 1870 FREDERIKSBERG C
Tel.: 4535283215
Telex: 4535282079

DR. A. JOYEAUX
DIRECTOR R AND D
LABORATOIRE DE SAINT SIMON
SOCIETE EQUIPHARM
F 15130 SAINT SIMON
Tel.: 3371471195
Telex: 3371471248
Fax: 306022

PROF. G.-G. JUNG
PROFESSOR OF ORGANIC CHEMISTRY AND BIO
INSTITUT FUER ORGANISCHE CHEMIE
EBERHARD-KARLS UNIVERSITAET
AUF DER MORGENSTELLE 18
D 7400 TUEBINGEN
Tel.: 497071296237
Telex: 497071296925

PROF. G. KALANTZOPOULOS
PROFESSOR GREEK COORDINATED COLLECTION OF MICROO
AGRICULTURAL UNIVERSITY OF ATHENS
BOTANIKOS
GR 118 55 ATHENS
Tel.: 3013465333
Telex: 3013469160
Fax: 225018 AGSA
E-mail: TELECOM GOLD 10075:DBI0256

DR. E.F. KALETA
RESEARCH SCIENTIST
INSTITUT FUER GEFLUEGELKRANKHEITEN
JUSTUS-LIEBIG UNIVERSITAET
FRANKFURTER STRASSE 87
D 6300 GIESSEN
Tel.: 496417024865
Telex: 496417024911

DR. J. KALLERHOFF
RESEARCH PROGRAM COORDINATOR FOR PLANT
LABORATOIRE DE BIOLOGIE CELLULAIRE ET MOLECULAIRE BIOSEM S.A.
AVENUE DES LANDAIS 24
CAMPUS UNIVERSITAIRE
F 63170 AUBIERE
Tel.: 3373427970
Telex: 3373275736

PROF. P.J. KALTSIKES
DEPT. OF PLANT BREEDING AND BIOMETRY
ATHENS AGRICULTURAL UNIVERSITY
IERA ODOS 75
GR 11855 ATHENS
Tel.: 3013471504
Telex: 3013460885

PROF. R. KAPTEIN
PROFESSOR
DEPARTMENT OF ORGANIC CHEMISTRY
UNIVERSITY OF UTRECHT
PADUALAAN 8
NL 3584 CH UTRECHT
Tel.: 3130533787/2652
Telex: 3130540980

DR. A. KARAGOUNI
LECTURER
INSTITUTE OF GENERAL BOTANY
UNIVERSITY OF ATHENS
PANEPISTIMIOUPOLIS
GR 15784 ATHENS
Tel.: 3017243345
Telex: 3017231634

DR. A. KARP
UG7 (PRINCIPLE SCIENTIFIC OFFICER)
DEPARTMENT OF AGICULTURAL SCIENCES
UNIVERSITY OF BRISTOL
AFRC INSTITUTE OF ARABLE CROPS
RESEARC
LONG ASHTON RESEARCH STATION
UK BS18 9AF BRISTOL
Tel.: 44275392181
Telex: 44275394007

DR. C. KARSSEN
PROFESSOR
DEPARTMENT OF PLANT PHYSIOLOGY
AGRICULTURAL UNIVERSITY WAGENINGEN
ARBORETUMLAAN 4
NL 6703 BD WAGENINGEN
Tel.: 33837083645
Telex: 33837084761

DR. T. KAVANAGH
LECTURER IN MOLECULAR GENETICS
TRINITY COLLEGE
UNIVERSITY OF DUBLIN
COLLEGE GREEN
IRL 2 DUBLIN
Tel.: 3531772941 EXT.1908
Telex: 3531798558
Fax: 93782 TCD EI
E-mail: KAVANAGH@VAX1.TCD.IE

DR. G.M. KEIL
DR.RER.NAT., SENIOR SCIENTIST, LEADER
INSTITUTE FOR MICROBIOLOGY
FEDERAL RESEARCH CENTRE FOR
VIRUS DISEASES OF ANIMALS
PAUL-EHRLICH-STRASSE 28
D 7400 TUEBINGEN
Tel.: 497071603323
Telex: 497071603201
Fax: 17707131

DR. G.D. KEIZER
HEAD OF RESEARCH, DEVELOPMENT AND
PROD
HBT HOLLAND BIOTECHNOLOGY BV
NIELS BOHRWEG 13
NL 2333 CA LEIDEN
Tel.: 3171219521
Telex: 3171211507
Fax: 39350

DR. F.J. KENNEDY
CAPTEC
COMPUTER APPLIED TECHNIQUES LTD
ST JAMES' TERRACE 3
IRL MALAHIDE, CO DUBLIN
Tel.: 3531450921
Telex: 3531450136
E-mail: FRED@CAPTEC.IE

PROF. K. KERSTERS
ASSOCIATE PROFESSOR
LABORATORIUM VOOR MICROBIOLOGIE
UNIVERSITEIT GENT
K.L. LEDEGANCKSTRAAT 35
B 9000 GENT
Tel.: 3291645105
Telex: 3291645346
E-mail: GELS@LMGMICRO.RUG.AC.BE

PROF. K. KERSTERS
ASSOCIATE PROFESSOR
LABORATORIUM VOOR MICROBIOLOGIE
FACULTY OF SCIENCES
RIJKSUNIVERSITEIT GENT
K.L. LEDEGANCKSTRAAT 35
B 9000 GENT
Tel.: 3291645105
Telex: 3291645346
Fax: 9312133486 GK G
E-mail: KERSTERS@LMGMICRO.RUG.AC.BE

PROF. H. KESSLER
TECHNICAL UNIVERSITY MUNICH
LICHTENBERGSTRASSE 4
D 8046 GARCHING
Tel.: 498932093300
Telex: 498932093210

MS. B. KIRSOP
EXECUTIVE DIRECTOR
MICROBIAL STRAIN DATA NETWORK
(MSDN)
HUNTINGDON ROAD 307
UK CB3 OJX CAMBRIDGE
Tel.: 44223276622
Telex: 44223277605
Fax: 9312133976 MS G
E-mail: TELECOM GOLD 10075:DBI0001/DBI0005

PROF. S. KJELLEBERG
PROFESSOR
DEPARTMENT OF GENERAL AND MARINE
MICROBIOLOGY
UNIVERSITY OF GOTEBORG
CARL SKOTTSBERGS GATAN 22
S 41319 GOTEBORG
Tel.: 4631418700
Telex: 4631826790

DR. D. KLAMBT
PROFESSOR
BOTANISCHES INSTITUT
UNIVERSITAET BONN
MECKENHEIMER ALLEE 170
D 5300 BONN 1
Tel.: 49228732680
Telex: 49228735545

DR. J. KNOP
HEAD, DEPARTMENT OF DERMATOLOGY, ASSIS
DERMATOLOGIE KLINIK
KLINIKUM DES JOHANNES GUTENBERG-UNIVER
LANGENBECKSTRASSE 1
POSTFACH 3960
D 6500 MAINZ
Tel.: 496131177112
Telex: 496131176614
Fax: 4187402 UK MZ

DR. M. KOKKINIDIS
PROF UNIV CRETE; GROUP LEADER AT IMBB
DEPARTMENT OF PROTEIN STRUCTURE AND FUNCTION
I.M.B.B.
P.O. BOX 1527
GR 71110 HERAKLION
Tel.: 3081210031
Telex: 3081239735
Fax: 26282896 IMBB GR
E-mail: KOKKINIDIS@GRIMBB.BITNET

DR. F.N. KOLISIS
RESEARCHER
THE NATIONAL HELLENIC RESEARCH FOUNDAT
VAS.CONSTANTINOU AVENUE 48
GR 116 35 ATHENS
Tel.: 3017239965
Telex: 3017212729
Fax: 224064

DR. C. KONCZ
MAX-PLANCK-INSTITUT FUER ZUECHTUNGSFOR
CARL-VON-LINNE-WEG 10
D 5000 KOELN 30
Tel.: 492215062350
Telex: 492215062413
Fax: 8 881 028 MPIZ D

DR. A. KONDOROSI
DIRECTEUR DE RECHERCHE, DIRECTOR OF IS
INSTITUT DES SCIENCES VEGETALES CNRS
AVENUE DE LA TERRASSE
F 91198 GIF-SUR-YVETTE CEDEX
Tel.: 33169823696
Telex: 33169823695
E-mail: ADAM—K@FRCTI51.BITNET

PROF. W.N. KONINGS
PROFESSOR OF MICROBIOLOGY; CHAIRMAN OF
DEPARTMENT OF MICROBIOLOGY
UNIVERSITY OF GRONINGEN
KERKLAAN 30
NL 9751 NN HAREN
Tel.: 3150632152
Telex: 3150635205

DR. A. KOOL
MANAGER BIOTECHNOLOGY
ZAADUNIE B.V.
WESTEINDE 62
P.O. BOX 26
NL 1600 AA ENKHUIZEN
Tel.: 31228011411
Telex: 31228012818
Fax: 57053 SGNL

DR. M. KOORNNEEF
SENIOR LECTURER
DEPARTMENT OF GENETICS
AGRICULTURAL UNIVERSITY WAGENINGEN
DREIJENLAAN 2
NL 6703 HA WAGENINGEN
Tel.: 31837082150
Telex: 31837084731

PROF. U. KOSZINOWSKI
PROFESSOR OF VIROLOGY, MEDICAL DIRECTO
INSTITUTE OF MICROBIOLOGY
DEPT. OF VIROLOGY
UNIVERSITY OF ULM
ALBERT-EINSTEIN-ALLEE 11
D 7900 ULM
Tel.: 497315023340
Telex: 497315023337

PROF. M. KREIS
PROFESSOR
INSTITUT DE RECHERCHE SUR LES PLANTES
BIOLOGIE MOLECULAIRE VEGETALE
UNIVERSITE DE PARIS-SUD
BATIMENT 430, RDC
F 91405 ORSAY
Tel.: 33169417239
Telex: 33169853715
Fax: 692 166 F
E-mail: BMV@FRUPS51

PROF. TH. KRIEG
UNIVERSITATSPROFESSOR
KLINIK UND POLIKLINIK FUER DERMATOLOGI
UND VENEROLOGIE DER
UNIVERSITAET ZU KOELN
JOSEPH-STELZMANN STRASSE 9
D 5000 KOELN 41
Tel.: 492214784500
Telex: 492214784549

DR. R. KRON-MORELLI
AGRIFUTUR SRL.
VIA CAMPAGNOLE 8
I 25020 ALFIANELLO (BRESCIA)
Tel.: 39309305697
Telex: 39309305048

DR. R.M. KROPPENSTEDT
DIRECTOR
DSM-DEUTSCHE SAMMLUNG VON MIKROORGANIS
UND ZELLKULTUREN GMBH
MASCHERODER WEG 1 B
D 3300 BRAUNSCHWEIG
Tel.: 4953161870
Telex: 495316187l8
Fax: 531104 DSM
E-mail: TELECOM GOLD 10075:DBI0225

DR. V. KRUTOVSKIKH
INTERNATIONAL AGENCY FOR RESEARCH ON C
COURS ALBERT THOMAS 150
F 69372 LYON 08
Tel.: 3372738496
Telex: 3372738575
Fax: 380 023

DR. H.E. KURZWELLY
DIMDI
DEUTSCHES INSTITUT FUER MEDIZINISCHE DOKUMENTATION UND INFORMATION
POSTFACH 420580
D 5000 KOELN 41
Tel.: 492214724252
Telex: 49221411429
Fax: 8881364

DR. W.E. LANCASHIRE
BIOTECHNOLOGY MANAGER, RESEARCH AND DE
RESEARCH AND DEVELOPMENT DEPARTMENT
WHITBREAD AND COMPANY PLC
WHITBREAD TECHNICAL CENTRE
PARK STREET
UK LU1 3ET LUTON
Tel.: 44582424200
Telex: 44582416750
Fax: 824204

DR. L. LANFRANCONE
DIRECTOR OF THE RESEARCH
LABORATORIA DI BIOLOGIA MOLECULARE
ISTITUTO CLINICA MEDICA I
UNIVERSITA DEGLI STUDI
VIA BRUNAMONTI 51
I 06100 PERUGIA
Tel.: 39755783255
Telex: 39755783444

PROF. C.M. LAPIERE
PROFESSOR - HEAD OF DEPARTMENT
LABORATORY OF EXPERIMENTAL DERMATOLOGY
CENTRE HOSPITALO-UNIVERSITAIRE
UNIVERSITY OF LIEGE
TOUR DE PATHOLOGIE B23
B 4000 SART-TILMAN
Tel.: 3241562456
Telex: 3241307234
Fax: UNIVLG 41397

DR. D.J. LEAK
LECTURER
CENTRE FOR BIOTECHNOLOGY
THE IMPERIAL COLLEGE OF SCIENCE, TECHNOLOGY AND MEDICINE
EXHIBITION ROAD
UK SW7 2AZ LONDON
Tel.: 44715895111 EXT. 709
Telex: 44712258942

DR. M. LEBRUN
INGENIEUR DE RECHERCHE
SERVICE DE BIOLOGIE MOLECULAIRE ET CELLULAIRE VEGETALE
RHONE-POULENC AGROCHIMIE
RUE PIERRE BAIZET 14
BP 9163
F 69263 LYON CEDEX 09
Tel.: 3372292848
Telex: 3372292297
Fax: 310098F RHONE

DR. C. LECOMTE
PROJECT LEADER
EUROGENTEC S.A.
RUE BOIS ST JEAN 14
B 4102 SERAING
Tel.: 3241654393
Telex: 3241562968
E-mail: U218101@BLEULG11

DR. A.M. LEDEBOER
TBC
UNILEVER RESEARCH LABORATORIUM
OLIVIER VAN NOORTLAAN 120
NL 3133 AT VLAARDINGEN
Tel.: 31104606933 EXT. 554
Telex: 31104605383
Fax: 23261

DR. J. LEEMANS
PLANT GENETIC SYSTEMS N.V.
J. PLATTEAUSTRAAT 22
B 9000 GENT
Tel.: 3291358498
Telex: 3291240694

PROF. M.-D. LEGOY
PROFESSOR
LABORATOIRE DE TECHNOLOGIE ENZYMATIQUE
UNIVERSITE TECHNOLOGIE DE COMPIEGNE
BP 649
F 60206 COMPIEGNE
Tel.: 3344200037
Telex: 3344203910
Fax: 150110

PROF. J. LEHMANN
PROF. OF CELL CULTURE TECHNOLOGY
TECHNISCHE FAKULTAET
AG ZELLKULTURTECHNIK
UNIVERSITAET BIELEFELD
UNIVERSITAETSSTRASSE 25
D 4800 BIELEFELD
Tel.: 495211066319
Telex: 495211066328

DR. J.-P. LEPOITTEVIN
CHARGE DE RECHERCHE AU C.N.R.S.
LABORATOIRE DE DERMATOCHIMIE
CLINIQUE DERMATOLOGIQUE
C.H.R. DE STRASBOURG
F 67091 STRASBOURG
Tel.: 3388350664
Telex: 3388161032

DR. K.R. LIBBENGA
FULL PROFESSOR
DEPARTMENT OF PLANT MOLECULAR BIOLOGY
BOTANICAL LABORATORY
LEIDEN UNIVERSITY
NONNENSTEEG 3
NL 2311 VJ LEIDEN
Tel.: 3171275064
Telex: 3171274900
Fax: 39427 BURUL NL

DR. K. LINDSEY
LEADER PLANT MOLECULAR BIOLOGY RESEARC
LEICESTER BIOCENTRE
UNIVERSITY OF LEICESTER
UNIVERSITY ROAD
UK LEI 7RH LEICESTER
Tel.: 44533522263
Telex: 44533551219
Fax: 342318 LEIBIO G

PROF. H.J. LIPPS
PROFESSOR DOKTOR, STIFTUNGSPROFESSOR
MEDIZINISCH-NATURWISSENSCHAFTLICHES FORSCHUNGSZENTRUM
UNIVERSITAET TUEBINGEN
OB DEM HIMMELREICH 7
D 7400 TUEBINGEN
Tel.: 497071295025
Fax: 49707187815

DR. M. LOMBARD
D.V.M. DIRECTOR OF FMDV PRODUCTION
RHONE MERIEUX DIRECTOR FA
RUE MARCEL MERIEUX 254
B.P. 7009
F 69342 LYON 07
Tel.: 3372723380
Telex: 3372723480

DR. H. LORZ / DR.P.T.H. BROWN
PROFESSOR AND HEAD OF DEPARTMENT
INSTITUT FUER ALLGEMEINE BOTANIK
UNIVERSITAET HAMBURG
OHNHORSTSTRASSE 18
D 2000 HAMBURG 52
Tel.: 49408222410
Telex: 49408222229

DR. G. LUCCHINI
DIPARTIMENTO DI GENETICA
E DI BIOLOGIA MOLECOLARE
UNIVERSITY OF MILANO
VIA CELORIA 26
I 20133 MILANO
Tel.: 3922663498
Telex: 3922664551
E-mail: GENETIC@IMIUCCA

DR. H. LUCIUS
Z.I.M.B. - ZENTRALINSTITUT FUER MOLEKU
ROBERT-ROESSLE-STRASSE 10
D O-1115 BERLIN BUCH
Tel.: 3729462362
Telex: 3729494161

PROF. A. MAGGI
PROFESSOR
INSTITUTE OF PHARMACOLOGICAL SCIENCES
LAB. DI BIOTECNOLOGIE APPLICATE ALLA F
BALZARETTI 9
I 20133 MILANO
Tel.: 392204881375
Telex: 39229404961

DR. F. MALPARTIDA
INVESTIGADOR CIENTIFICO DEL CSIC
BIOTECHNOLOGY DEPARTMENT
CENTRO NACIONAL DE BIOTECNOLOGIA.MICRO
CSIC
SERRANO 115, BI
E 28006 MADRID
Tel.: 3413975452
Telex: 3413150075
Fax: CSIC E-42182
E-mail: FMALPART@EMDCNB51

DR. G. MARIGO
MAITRE CONFERENCES
UNIVERSITE PAUL SABATIER/CNRS
ROUTE DE NARBONNE 118
F 31062 TOULOUSE CEDEX
Tel.: 3361556753
Telex: 3361556210

DR. D. MARIOTTI
SENIOR STAFF SCIENTIST/GROUP LEADER
ISTITUTO DI RADIOBIOCHIMICA ED ECOFISIOLOGIA VEGETALI
CONSIGLIO NAZIONALE DELLE RICERCHE
VIA SALARIA Km 29.3
I 00016 MONTEROTONDO SCALO (ROMA
Tel.: 39690020542
Telex: 3969005849

PROF. E. MARRE
FULL PROFESSOR
DEPARTIMENTO DI BIOLOGIA
VIA CELORIA 26
I 20133 MILANO
Tel.: 39226604390
Telex: 39226604407

DR. V.M. MARSHALL
HEAD OF CENTRE FOR SCIENCES OF FOOD AN
OXFORD POLYTECHNIC
GIPSYLANE
HEADINGTON
UK OX3 OBP OXFORD
Tel.: 44865819248
Telex: 44865819073
Fax: G83L47VIA

DR. P. MARTEL
RESEARCH RESPONSIBLE
LABORATOIRE DE NUTRITION ET SECURITE ALIMENTAIRE
INRA
DOMAINE DE VILVERT
F 78352 JOUY-EN-JOSAS CEDEX
Tel.: 33134652254
Telex: 33134652311
Fax: INRACRZ 695 431 F
E-mail: CHRONOPOSTE

DR. G.J.M. MARTENS
STAFF ASSISTANT PROFESSOR
DEPARTMENT OF ANIMAL PHYSIOLOGY
UNIVERSITY OF NIJMEGEN
TOERNOOIVELD
NL 6525 ED NIJMEGEN
Tel.: 3180652601
Telex: 3180553450
Fax: 48228 WINA NL

PROF. J. MARTIAL
PROFESSOR
LABORATOIRE DE BIOLOGIE MOLECULAIRE
ET
GENIE GENETIQUE / INSTITUT DE CHIMIE
UNIVERSITE DE LIEGE
CAMPUS SART TILMAN B6
B 4000 LIEGE
Tel.: 3241563311
Telex: 3241562968
Fax: 41397 UNIVLG

PROF. J.F. MARTIN
PROFESSOR OF MICROBIOLOGY
DIVISION OF MICROBIOLOGY
DEPT. OF ECOLOGY, GENETICS AND MICROBI
UNIVERSITY OF LEON
CAMPUS DE VEGAZANA, S/N
E 24071 LEON
Tel.: 3487291505
Telex: 3487291506
Fax: 89892 EDUCI E

DR. C. MARTIN
SENIOR SCIENTIFIC OFFICER
JOHN INNES INSTITUTE
COLNEY LANE
UK NR4 7UH NORWICH
Tel.: 4460352571 EXT. 279
Telex: 4460356844
Fax: 975122

DR. A. MARTIN
SENIOR RESEARCHER
C.S.I.C.
E 14080 CORDOBA
Tel.: 3457218509
Telex: 3457218563

DR. J. M. MARTINEZ ZAPATER
RESEARCHER A
DEPARTAMENTO DE PROTECCION VEGETAL
CENTRO DE INVESTIGACION Y TECNOLOGIA
INSTITUTO NACIONAL DES INVESTIGACIONES
CARRETERA DE LA CORUNA KM 7
E 28040 MADRID
Tel.: 3413572294209
Telex: 3413572293

PROF. A. MARTINI
FULL PROFESSOR, CURATOR OF THE
DBVPG C
COLLEZIONE DEI LIEVITI INDUSTRIALI DBV
DIPART. BIOLOGIA VEGETALE
UNIVERSITA' DI PERUGIA
BORGO 20 GIUGNO 74
I 06100 PERUGIA
Tel.: 397530458
Telex: 397558562999
Fax: UNIPG I 662078

DR. R. MASTERSON
SENIOR SCIENTIST
ABT. GENETISCHE GRUNDLAGEN DER
PFLANZE
MAX-PLANCK-INSTITUT FUER
ZUECHTUNGSFOR
CARL-VON-LINNE-WEG 10
D 5000 KOELN 30
Tel.: 492215062245
Telex: 492215062213
Fax: 8 881 028 MPIZ D

PROF. P.L. MATEO ALARCON
DEPARTMENT OF PHYSICAL CHEMISTRY
UNIVERSITY OF GRANADA
SEVERO OCHOA S/N
E 18071 GRANADA
Tel.: 3458272879
Telex: 3458274258
Fax: 345878435

DR. G. MATTHIJSSENS
GENERAL MANAGER
CORVAS INTERNATIONAL N.V.
JOZEF PLATEAUSTRAAT 22
B 9000 GENT
Tel.: 3291358406
Telex: 3291255005

DR. S.G. MAYHEW
STATUTORY LECTURER
DEPARTMENT OF BIOCHEMISTRY
UNIVERSITY COLLEGE DUBLIN
BELFIELD
IRL 4 DUBLIN
Tel.: 35317061572
E-mail: MAYHEWS@IRLEARN.BITNET

DR. D.J. MCCONNELL
PROF.
NATIONAL CENTRE OF PHARMACEUTICAL
BIOT
DEPARTMENT OF GENETICS
TRINITY COLLEGE
IRL DUBLIN 2
Tel.: 3531772941 EXT. 2008
Telex: 3531798558
Fax: 93782 TCD EI
E-mail: DMCCNNLL@VAXXI.TCD.IE

DR. W. MCDOWELL
GROUP LEADER GLYCOBIOLOGY
MRC COLLABORATIVE CENTRE
BURTONHOLE LANE 1
MILL HILL
UK NW7 1AD LONDON
Tel.: 44819063811
Telex: 44819061395
Fax: 9413459

DR. A.P. MCQUEEN
NICKERSON INTERNATIONAL SEED COMPANY
CAMBRIDGE SCIENCE PARK
MILTON ROAD 200
UK CB4 4GZ CAMBRIDGE
Tel.: 44223423933
Telex: 44223420925

DR. P.A. MEACOCK
DEPUTY DIRECTOR AND RESEARCH GROUP
LEA
UNIVERSITY OF LEICESTER
UNIVERSITY ROAD
UK LE1 7RH LEICESTER
Tel.: 44533522268
Telex: 44533551219
Fax: 342318 LEIBIO G

DR. R.H. MELOEN
LABORATORY FOR MOLECULAR
IMMUNOLOGY
CENTRAAL DIERGENEESKUNDIG INSTITUUT
EDELHERTWEG 15
NL 8219 PH LELYSTAD
Tel.: 31320073911
Telex: 31320073473

PROF. A. MENDES FAIA
PROFESSORA ASSOCIADA
UNIVERSIDADE DE TRAS-OS-MONTES
E ALTO DOURO
APARTADO 202
P 5001 VILA REAL CODEX
Tel.: 3515925031/9
Telex: 3515974480
Fax: 24436

DR. U. MENGE
GROUP LEADER
DEPARTMENT OF ENZYME TECHNOLOGY
GROUP FOR SCREENING AND DESIGN OF
TECH
GBF
MASCHERODER WEG 1
D 3300 BRAUNSCHWEIG
Tel.: 495316181310
Telex: 495316181302

DR. C. MEREDITH
SCIENTIFIC OFFICER,
IMMUNOTOXICOLOGY D
DEPT. OF IMMUNOTOXICOLOGY
BRITISH INDUSTRIAL BIOLOGICAL
RESEARCH ASSOCIATION (BIBRA)
WOODMANSTERNE ROAD
UK SM5 4DS CARSHALTON, SURREY
Tel.: 4416434411
Telex: 4416617029
Fax: 23438

DR. M. MERGEAY
HEAD OF LABORATORY
LABORATORY OF GENETICS &
BIOTECHNOLOGY
VLAAMSE INSTELLING VOOR
TECHNOL'›GISCH
ONDERZOEK (SCK-CEN/VITO)
BOERETANG 200
B 2400 MOL
Tel.: 3214333111 EXT. 5112
Telex: 3214320372
Fax: 31922
E-mail: MERGEAY@BMLSCK11

DR. H.W. MEWES
PROJECT LEADER MIPS DATENBANK FUR
PROT
MIPS AM MAX PLANCK INSTITUT FUER BIOCH
AM KLOPFERSPITZ 18
D 8033 MARTINSRIED
Tel.: 498985782657
Telex: 498985782655
E-mail: MEWES@MIPS

DR. P. MEYER
LEADER OF AN INDEPENDENT WORKING
GROUP
MAX-DELBRUEK-LABORATORIUM IN DER
MPG
CARL-VON-LINNE-WEG 10
D 5000 KOELN 30
Tel.: 492215062490
Telex: 492215062319

DR. G. MIGNOT
HEAD OF CELL CULTURE LABORATORY
CELL CULTURE LABORATORY
TM INNOVATION
AVENUE DES TROPIQUES 3
B.P. 100
F 91943 LES ULIS
Tel.: 33169077871
Telex: 33169071903
Fax: 602729

DR. J. MIKKELSEN
DANISCO A/S
LANGEBROGADE 1
P.O. BOX 17
DK 1001 COPENHAGEN
Tel.: 45319541002210
Telex: 4531953022
Fax: 31430 DANSUK DK

DR. P. MILLNER
LECTURER
DEPARTMENT OF BIOCHEMISTRY
UNIVERSITY OF LEEDS
UK LS2 9JT LEEDS
Tel.: 44532333149
Telex: 44532336017
Fax: 44532556473

DR. O. MISSET
HEAD DEPARTMENT PROTEIN CHEMISTRY &
EN
ROYAL GIST BROCADES N.V.
WATERINGSEWEG 1
NL 2611 XT DELFT
Tel.: 3115799111/2659
Telex: 3115793890
Fax: 38103 GB NLI NL R&D

DR. W. MOENS
SENIOR RESEARCH OFFICER
INSTITUT D'HYGIENE ET D'EPIDEMIOLOGIE
RUE JULIETTE WYTSMAN 14
B 1050 BRUXELLES
Tel.: 3226425512
Telex: 3226425519
Fax: 21034 IHE BRU

PROF. S. MOLIN
PROFESSOR
DEPARTMENT OF MICROBIOLOGY
THE TECHNICAL UNIVERSITY OF DENMARK
BUILDING 221
DK 2800 LYNGBY
Tel.: 4542882222
Telex: 45459328O9
Fax: 37529DTHDIADK

DR. J.-P. MORIN
CHARGE DE RECHERCHE INSERM
INSERM - UNITE 295
FACULTE MEDICINE-PHARMACIE
UNIVERSITE DE ROUEN
AVENUE DE L'UNIVERSITE
B.P. 47
F 76800 ST.ETIENNE DU ROUVRAY
Tel.: 3335655274 EXT. 21
Telex: 3335661278

DR. I. MOURA
ASSOCIATE PROFESSOR
CENTRO DE TECNOLOGIA QUIMICA E BIOLOGICA (CTQB)
RUA DA QUINTA GRANDE 6
APARTADO 127
P 2780 OEIRAS
Tel.: 35114428616346
Telex: 35114428766

DR. F. MULHOLLAND
HEAD, DEPARTMENT OF BIOTECHNOLOGY AND
READING LABORATORY
AFRC INSTITUTE OF FOOD RESEARCH
UK RG2 9AT SHINFIELD
Tel.: 44734883103228
Telex: 44734884763
Fax: 9312102022 LBG

DR. B. MULLIGAN
LECTURER
DEPARTMENT OF BOTANY
UNIVERSITY OF NOTTINGHAM
UK NG7 2RD NOTTINGHAM
Tel.: 44602484848 EXT. 346
Telex: 44602420825

DR. F. NALIN
TBC
LYVEN
ROUTE DE PARIS
F 14630 CAGNY
Tel.: 3331234532
Telex: 3331234572
Fax: 170305

DR. I.F. NES
LABORATORY OF MICROBIAL GENE TECHNOLOG
NLVF
AGRICULTURAL RESEARCH COUNCIL
P.O. BOX 51
N 1432 AAS
Tel.: 479949471/63
Telex: 479941465
E-mail: BIOIFN@NLH10000.NLH.NO

DR. H.J. NEWBURY
UNIVERSITY LECTURER
SCHOOL OF BIOLOGICAL SCIENCES
UK B15 2TT BIRMINGHAM
Tel.: 44214145581
Telex: 44214145925
Fax: SPAPHY G 338938

DR. H.O. NIELSEN
SECTION MANAGER
LABORATORY FOR TECHNICAL OPTICS
HJORTEKAERSVEJ 99
DK 2800 LYNGBY
Tel.: 4542888333
Telex: 4545930353
Fax: 37529 DTHDIADK

DR. N. NOLARD-TINTIGNER
SENIOR RESEARCH OFFICER
INSTITUT D'HYGIENE ET EPIDEMIOLOGIE (I
RUE JULIETTE WYTSMAN 14
B 1050 BRUXELLES
Tel.: 3226425517
Telex: 3226425519
Fax: 21034 IHE BRU

PROF. C. NOMBELA
FULL PROFESSOR AND HEAD OF DEPARTMENT
DEPARTAMENTO DE MICROBIOLOGIA II
FACULTAD DE FARMACIA
UNIVERSIDAD COMPLUTENSE DE MADRID
CIUDAD UNIVERSITARIA
E 28040 MADRID
Tel.: 3413941743
Telex: 3415440667
Fax: 41806 UCFAR

DR. J.H. NOORDIK
DIRECTOR/PROJECT MANAGER CAOS/CAMM CEN
CAOS/CAMM CENTER
UNIVERSITY OF NIJMEGEN
TOERNOOIVELD
NL 6525 ED NIJMEGEN
Tel.: 3180653386
Telex: 3180553450
E-mail: NOORDIK@CAOS.CAOS.KUN.NL

PROF. M.P. NUTI
PROFESSOR, AGRIC.MICROBIOLOGY - HEAD O
DIPARTIMENTO DI BIOTECNOLOGIE AGRARIE
UNIVERSITA DEGLI STUDI DI PADOVA
VIA GRADENIGO 6
I 35125 PADOVA
Tel.: 39498071442
Telex: 39498070517
Fax: 430176 UNPADU

DR. F. O' GARA
DIRECTOR - BIOTECHNOLOGY CENTRE.
MICROBIOLOGY DEPARTMENT
BIOTECHNOLOGY CENTRE
UNIVERSITY COLLEGE
IRL CORK
Tel.: 353212768712351
Telex: 35321275948
Fax: 76050 UNIC EI

$$DR. M. O'CONNELL
LECTURER
DUBLIN CITY UNIVERSITY
GLASNEVIN
IRL 9 DUBLIN
Tel.: 3531370077318
Telex: 3531360830
Fax: 30167

DR. F. O'GARA
DIRECTOR -NATIONAL FOOD BIOTECHNOLOGY
NATIONAL FOOD BIOTECHNOLOGY CENTRE
DEPT. OF FOOD MICROBIOLOGY
UNIVERSITY COLLEGE CORK
IRL CORK
Tel.: 35321276871 EXT. 264
Telex: 35321275934
Fax: 76050 UNICCEI
E-mail: STDM 8008

DR. D.J. OCKENDON
PROJECT LEADER
AFRC INSTITUTE OF HORTICULTURAL
RESEAR
WELLESBOURNE
UK CV35 9EF WARWICK
Tel.: 44789470382
Telex: 44789470552
E-mail: kING@UK.AC.AFRC.NVRS

PROF. P. OLESEN
RESEARCH PLANNING MANAGER,
PROFESSOR
BIOTECHNOLOGY
MARIBO SEED
LANGEBROGADE
P.O. BOX 17
DK 1001 COPENHAGEN
Tel.: 4531954100 EXT. 2210
Telex: 4531953022
Fax: 31169 MARIBO DK

DR. P. OLESEN
RESEARCH PLANNING MANAGER,
PROFESSOR
DANISCO A/S
LANGEBROGADE 1
P.O. BOX 17
DK 1001 COPENHAGEN K
Tel.: 4531954100
Telex: 4531953022
Fax: 31430 DANSUK

DR. P. OLESEN
RESEARCH PLANNING MANAGER,
PROFESSOR
DANISCO A/S
LANGEBROGADE 1
P.O. BOX 17
DK 1001 COPENHAGEN K
Tel.: 45319541002210
Telex: 4531953022
Fax: 31430 DANSUK DK

DR. P. OLESEN
RESEARCH PLANNING MANAGER,
PROFESSOR
DANISCO A/S
LANGEBROGADE P.O. BOX 17 1
DK 1001 COPENHAGEN K
Tel.: 45319541002210
Telex: 4531953022
Fax: 31430 DANSUK DK

DR. J. OLIVARES
PROFESOR DE INVESTIGACION
DEPT. DE MICROBIOLOGIA
ESTACION EXPERIMENTAL DEL ZAIDIN,
PROFESOR ALBAREDA 1
E 18006 GRANADA
Tel.: 3458121011258
Telex: 3458129600

DR. R. OLIVER
LECTURER
SCHOOL OF BIOLOGICAL SCIENCES
UNIVERSITY OF EAST ANGLIA
UK NR4 7TJ NORWICH
Tel.: 4460259492
Telex: 4460358553
Fax: 975B4

DR. R. OLIVER
LECTURER
SCHOOL OF BIOLOGICAL SCIENCES
UNIVERSITY OF EAST ANGLIA
UK NR4 7TJ NORWICH
Tel.: 4460356161/2889
Telex: 4460358553
Fax: 975334

PROF. S.G. OLIVER
PROFESSOR OF BIOTECHNOLOGY
MANCHESTER BIOTECHNOLOGY CENTRE
UNIVERSITY OF MANCHESTER
INSTITUTE OF SCIENCE AND TECHNOLOGY
SACKVILLE STREET
P.O. BOX 88
UK M60 1QD MANCHESTER
Tel.: 44612004222
Telex: 44612360409
Fax: 666094 UMIST G
E-mail: S.G.OLIVER@UK.AC.UMIST

PROF. F. OLLEVIER
FULL PROFESSOR
ZOOLOGICAL INSTITUTE
LABORATORY FOR ECOLOGY AND
AQUACULTURE
KATHOLIEKE UNIVERSITEIT LEUVEN
NAAMSESTRAAT 22
B 3000 LEUVEN
Tel.: 3216283966
Telex: 3216283902
E-mail: FGDCB01@BLEKUL11

DR. L. OLLIVIER
DIRECTEUR DE LA STATION DE
GENETIQUE Q
INRA STATION DE GENETIQUE
QUANTITATIVE
DOMAINE DE VILVERT CRJ
F 78350 JOUY-EN-JOSAS
Tel.: 33134652190
Telex: 33134652210
Fax: INRACRZ 695431
E-mail: VGENLOL@FRINRA11

DR. F.R. OPPERDOES
HEAD RESEARCH UNIT FOR TROPICAL
DISEAS
INTERNATIONAL INSTITUTE OF CELLULAR
AN
MOLECULAR PATHOLOGY
(ICP)
AVENUE HIPPOCRATE 74
B 1200 BRUSSELS
Tel.: 3227647439
Telex: 3227626853
Fax: 23722 UCLWOLB
E-mail: OPPERD@TROP.UCL.AC.BE

DR. M. PAGES TORRENS
DEPTO DE GENETICA MOLECULAR.
CENTRO DE INVESTIGACION Y DESARROLLO
C.S.I.C.
JORDI GIRONA 18
E 08034 BARCELONA
Tel.: 772046851
Telex: 772045904
Fax: 97977

DR. K. PALME
SENIOR SCIENTIST
MAX-PLANCK-INSTITUT FUER
ZUECHTUNGSFOR
CARL-VON-LINNE-WEG 10
D 5000 KOELN 30
Tel.: 4902215062385
Telex: 492215062213
Fax: 8 881 028 MPIZ D
E-mail: PALME@VAX.MPIZ-OELN.MPG.DBP.DE

PROF. F. PALTAUF
DEPARTMENT HEAD
DEPARTMENT OF BIOCHEMISTRY
AND FOOD CHEMISTRY
GRAZ UNIVERSITY OF TECHNOLOGY
SCHLOEGELGASSE 09
A 8010 A GRAZ
Tel.: 43316830642
Telex: 43316827685

DR. G.S. PANAYI
TBC
RHEUMATIC DISEASE UNIT
GUY'S AND ST.THOMAS'S HOSPITALS
UNITED MEDICAL AND DENTAL SCHOOLS
ST. THOMAS STREET
UK SE1 9RT LONDON
Tel.: 4419554394
Telex: 4414075134

DR. N.J. PANOPOULOS
ASSOCIATE RESEARCH SCIENTIST, PROF UNI
INSTITUTE OF MOLECULAR BIOLOGY AND
BIO
FOUNDATION FOR RESEARCH AND
TECHNOLOGY
P.O.BOX 1527
GR 711 10 HERAKLION
Tel.: 3081210091
Telex: 3081239735
Fax: 262896 IMBB GR
E-mail: SECRETARIAT@GRIMBB

DR. B. PARODI
MEDICAL DOCTOR, LABORATORY ASSISTANT
SERVIZIO TECNOLOGIE BIOMEDICHE
ISTITUTO NAZIONALE PER LA RICERCA
SUL CANCRO
VIALE BENEDETTO XV 10
I 16132 GENOVA
Tel.: 39103534515
Telex: 3910352888
Fax: 286353 ISTEX I
E-mail: TELECOM GOLD 10075:DB10503

PROF. P.P. PASTORET
PROFESSOR
VIROLOGY-IMMUNOLOGY
FACULTY OF VETERINARY MEDICINE
UNIVERSITY OF LIEGE
INSTITUT DE CHIMIE - B6
LOCAL R80
B 4000 SART TILMAN-LIEGE
Tel.: 3241563469
Telex: 3241563580

DR. J. PASZKOWSKI
WISSENSCHAFTLICHER ADJUNKT
INSTITUT FUER PFLANZENWISSENSCHAFTEN
EIDGENOESSISCHE TECHNISCHE
HOCHSCHULE
UNIVERSITAETSTRASSE 2
CH 8092 ZUERICH
Tel.: 4112564438
Telex: 4112529613

DR. D. PAUL
HEAD OF DEPARTMENT OF CELL BIOLOGY
DEPT. OF CELL BIOLOGY
FRAUNHOFER INSTITUTE FOR TOXICOLOGY
AND AEROSOL
NIKOLAI-FUCHS-STRASSE 1
D 3000 HANNOVER 61
Tel.: 4951153500263
Telex: 495115350155

PROF. H. PAULSEN
PROFESSOR
INSTITUT FUER ORGANISCHE CHEMIE
UNIVERSITAET HAMBURG
MARTIN-LUTHER-KING-PLATZ 6
D 2000 HAMBURG 13
Tel.: 494041232820
Telex: 494041233834
Fax: 214732 UNIHHD
E-mail: BUSCH@RZ.INFORMATIK.UNI-
HAMBURG.DBP.DE

DR. E. PAULSSEN
PH.D. STUDENT
THE BIOTECHNOLOGY CENTRE OF OSLO
BLINDERN
P.O. BOX 1125
N 0317 OSLO 3
Tel.: 472454093
Telex: 472454058
E-mail: L—PAULSSEN—E@USE.UIO.UNINETT

DR. F.J. PAZ-ARES RODRIGUEZ
COLABORADOR CIENTIFICO DEL C.S.I.C.
UNIDAD ESTRUCTURAL DE INGENIERIA
GENET
LABORATORIO DE BIOLOGIA MOLECULAR
CENTRO DE INVESTIGACIONES BIOLOGICAS
VELAZQUEZ 144
E 28006 MADRID
Tel.: 3412611800 EXT. 206
Telex: 3412627518

DR. P. PECHAN
MAX PLANCK INSTITUT FUER
ZUECHTUNGSFOR
CARL-VON-LINNE-WEG 10
D 5000 KOELN 30
Tel.: 492215062350
Telex: 492215062413
Fax: 8 881 028 MPIZ D
E-mail: PECHAN@VAX.MPIZ-
KOELN.MPG.DBP.DE

PROF. F.S. PEDERSEN
ASSOCIATE PROFESSOR
INSTITUT FOR MOLEKYLAER BIOLOGIE
UNIVERSITY OF AARHUS
C.F. MOELLERS ALLE 130
DK 8000 AARHUS
Tel.: 4586125177
Telex: 4586196226
Fax: 64767 AAUSCI DK

DR. J.E. PELSENEER-COREMANS
CHARGE DE COURS
LABORATOIRE DE PARASITOLOGIE
UNIVERSITE LIBRE DE BRUXELLES
ROUTE DE LENNIK 808
B 1070 BRUXELLES
Tel.: 3225556251
Telex: 3225366160

DR. M. PENALVA
COLABORADOR CIENTIFICO DEL C.S.I.C.
LAB. DE GENETICA MOLECULAR DE ASPERGIL
UNIDAD ESTRUCTURAL DE CITOGENETICA
CENTRO DE INVESTIGACIONES BIOLOGICAS
VELAZQUEZ 144
E 28006 MADRID
Tel.: 3415854358
Telex: 3412627518

DR. A. PEREIRA
MOLECULAR BIOLOGY DEPARTMENT
ONDERZOEK INSTITUUT
KEIJENBERGSEWEG 6
NL 6700 AA WAGENINGEN
Tel.: 31837091911
Telex: 31837014435

PROF. W. PFALLER
UNIVERSITAETSPROFESSOR
INSTITUT FUER PHYSIOLOGIE UND BALNEOLO
UNIVERSITAET INNSBRUCK
FRITZ-PREGLSTRASSE 3
A 6010 INNSBRUCK
Tel.: 435125072221
Telex: 43512581740
Fax: UNITE A 533808
E-mail: C50701@AINUNI01

PROF. P. PHILIPPSEN
PROFESSOR
INSTITUT FUER MIKROBIOLOGIE UND MOLEKULARBIOLOGIE
JUSTUS-LIEBIG-UNIVERSITAET
FRANKFURTER-STRASSE 107
D 6300 GIESSEN
Tel.: 496417025934
Telex: 496417027390

DR. W. PIEPERSBERG
PROFESSOR OF MICROBIOLOGY, C4
CHEMISCHE MIKROBIOLOGIE, FB 9
GESAMTHOCHSCHULE WUPPERTAL
BERGISCHE UNIVERSITAET
GAUSS-STRASSE 20
D 5600 WUPPERTAL
Tel.: 492024392521
Telex: 492024392698
Fax: 8592262 BUGHW

DR. L. PIJNACKER
SCIENTIST
CENTRE BIOLOGICAL SCIENCES
DEPARTMENT OF GENETICS
UNIVERSITY OF GRONINGEN
KERKLAAN 30
NL 9751 NN HAREN
Tel.: 3150632132
Telex: 3150632348

DR. L. PILSTROM
SENIOR LECTURER
DEPARTMENT OF IMMUNOLOGY
BIOMEDICAL CENTRE
UPPSALA UNIVERSITY
BOX 582
S 75123 UPPSALA
Tel.: 4618174218
Telex: 4618157579

DR. D. PIOLI
PRINCIPAL RESEARCH SCIENTIST
BIOTECHNOLOGY DEPARTMENT
ICI PHARMACEUTICALS
ALDERLEY PARK
UK SK10 4TG MACCLESFIELD
Tel.: 44625512986
Telex: 44625514463
Fax: 669095/669388

PROF. R.J. PLANTA
PROF.DR.
BIOCHEMISCH LABORATORIUM
VRIJE UNIVERSITEIT
DE BOELELAAN 1083
NL 1081 HV AMSTERDAM
Tel.: 31205485606
Telex: 3120461479

PROF. R. PLAPP
PROFESSOR
UNIVERSITAET KAISERSLAUTERN
ERWIN SCHROEDINGER STRASSE 23
POSTFACH 3049
D 6750 KAISERSLAUTERN
Tel.: 496312052353
Telex: 496312052998
Fax: 45627 UNIKL D
E-mail: RPLAPPRK.UNI-KL.DE

DR. F.M. POHL
DR.
FAKULTAET FUER BIOLOGIE
UNIVERSITAET KONSTANZ
POSTFACH 5560
D 7750 KONSTANZ
Tel.: 497531882134
Telex: 497531882966
Fax: 0733359

DR. R.D. POSSEE
SENIOR SCIENTIFIC OFFICER
INSTITUTE OF VIROLOGY AND ENVIRONMENTAL MICROBIOLOGY
NATURAL ENVIRONMENT RESEARCH COUNCIL
MANSFIELD ROAD
UK OX1 3SR OXFORD
Tel.: 44865512361
Telex: 4486559962
Fax: 83147
E-mail: POSSEE@VAX.OX.AC.UK

DR. P.W. POSTMA
SENIOR LECTURER IN BIOCHEMISTRY
E.C. SLATER INSTITUTE FOR BIOCHEMICAL RESEARCH, FACULTY OF CHEMISTRY
UNIVERSITY OF AMSTERDAM
PLANTAGE MUIDERGRACHT 12
NL 1018 TV AMSTERDAM
Tel.: 31205255126
Telex: 31205255124
E-mail: A311POST@HASARA11

DR. J.E. PRENOSIL
DOZENT
TECHNISCH-CHEMISCHES LABORATORIUM
(TCL
EIDGENOESSISCHE TECHNISCHE
HOCHSCHULE
ZUERICH (ETH-Z)
UNIVERSITAETSTRASSE 6
CH 8092 ZUERICH
Tel.: 4112563080
Telex: 4112521827

DR. J.R. PRIGENT
RESEARCH MANAGER
TEXEL (RHONE POULENC GROUP)
Z.A. DE BUXIERES
B.P. 10
F 86220 DANGE SAINT ROMAIN
Tel.: 3349937100
Telex: 3349937110
Fax: 791061 F

PROF. A. PUEHLER
HEAD OF THE DEPARTMENT
LEHRSTUHL FUER GENETIK
FAKULTAET FUER BIOLOGIE
UNIVERSITAET BIELEFELD
UNIVERSITAETSSTRASSE 25
POSTFACH 8640
D 4800 BIELEFELD 1
Tel.: 495211065607
Telex: 495211065626
Fax: 932362 UNIBI
E-mail: PUEHLER@BIOGENEL.UNI-
BIELEFELD.DE

DR. G. RAGNOTTI
DEPT. OF BIOMEDICAL SCIENCES AND BIOTE
UNIT OF GENERAL PATHOLOGY AND
IMMUNOLO
UNIVERSITY OF BRESCIA
VIA VALSABBINA 19
I 25123 BRESCIA
Tel.: 39303996310
Telex: 39303701157

DR. J.L. RAMOS
INVESTIGADOR CIENTIFICO
ESTACION EXPERIMENTAL DEL ZAIDIN
C.S.I.C.
PROFESOR ALBAREDA 1
E 18008 GRANADA
Tel.: 3458121011
Telex: 3458129600
Fax: 78798 EEZG E
E-mail: ZAIDIN@IAA.ES

DR. O.F. RASMUSSEN
SENIOR SCIENTIST
BIOTECHNOLOGICAL INSTITUTE
LUNDTOFTEVEJ 100
BUILDING 227
DK 2800 LYNGBY
Tel.: 4542876699
Telex: 4545932888

DR. C.J. RAWLINGS
ADVANCED RESEARCH FELLOW
BIOMEDICAL COMPUTING UNIT
IMPERIAL CANCER RESEARCH FUND
LABORATO
LINCOLN'S INN FIELDS 44
P.O. BOX 123
UK WC2A 3PX LONDON
Tel.: 44712693389
Telex: 44714301787
E-mail: C-RAWLINGS@ICRF.AC.UK

DR. L. RECHAUSSAT
DIRECTOR
CERDIC - CENTRE EUROPEEN DE
RECHERCHES DOCUMENTAIRES SUR LES
IMMUN
ROUTE DES CRETES 2229
C/O CICA
F 06560 VALBONNE
Tel.: 3392942288
Telex: 3392942294
E-mail: TELECOM GOLD 10075:DBI0098

DR. B. REISS
GROUP LEADER
MAX-PLANCK-INSTITUT FUER
ZUECHTUNGSFOR
CARL-VON-LINNE-WEG 10
D 5000 KOELN 30
Tel.: 492215062220
Telex: 492215062213

DR. M. RENARD
RAPESEED BREEDER
STATION D'AMELIORATION DES PLANTES
INRA
RUE DE L'UNIVERSITE 147
F 75341 PARIS 07
Tel.: 3399285126
Telex: 3399285120

DR. A. RENARD
SCIENTIFIC DIRECTOR
SOCIETE EUROPEENNE DE BIOTECHNOLOGIE
S
EUROGENTEC
CAMPUS SART TILMAN B6
B 4000 LIEGE
Tel.: 3241563379
Telex: 3241562968

DR. A. REY-SENELONGE
SENIOR SCIENTIST
LABORATOIRE IFFA
RHONE MERIEUX
RUE MARCEL MERIEUX 254
B.P. 7009
F 69342 LYON 07
Tel.: 3372723380
Telex: 3372723480
Fax: 375470 IFFA LYON

DR. D.J. RHODES
TEAM LEADER
JEALOTT'S HILL RESEARCH STATION
BIOTECHNOLOGY SECTION
ICI AGROCHEMICALS
UK RG12 6EY BRACKNELL
Tel.: 44344414201
Telex: 44344414996
Fax: 847556 icip8j9

DR. G. RIBA
DIRECTEUR DE RECHERCHES
STATION DE RECHERCHES DE LUTTE BIOLOGI
INRA
LA MINIERE - INRA
F 78285 GUYANCOURT
Tel.: 33130833635
Telex: 33130438097
Fax: 698450 F

DR. M. RIEGER
BIOTECHNOLOGISCHE UND MOLEKULARBIOLOGI
FORSCHUNG
ANGELHOFWEG 39
D 6916 WILHELMSFELD
Tel.: 4962208724
Telex: 4962208724

PROF. P. RITZENTHALER
CENTRE DE RECHERCHE DE BIOCHIMIE ET DE
GENETIQUE CELLULAIRES DU CNRS (LP8201)
ROUTE DE NARBONNE 118
F 31062 TOULOUSE
Tel.: 3361559657
Telex: 3361559500
E-mail: RITZENTH@FRCICT81.BITNET

DR. S. RIVA
ISTITUTO DI GENETICA BIOCHIMICA ED EVOLUZIONISTICA
CNR
ABBIATEGRASSO 207
I 27100 PAVIA
Tel.: 39382422384
Telex: 39382422286
Fax: 322883IGBEPVI
E-mail: GENPOP@IPVCCN

DR. M. RIVIERE
LAB. IFFA / DIRECTION DES RECHERCHES
DEP. VIROLOGIE, UNITE DE BIOCHIMIE ANA
RHONE MERIEUX
RUE MARCEL MERIEUX 254
B.P. 7009
F 69342 LYON 07
Tel.: 3372723453
Telex: 3372723480
Fax: 375887

DR. K. ROBERTS
HEAD: DEPARTMENT OF CELL BIOLOGY
JOHN INNES INSTITUTE
JOHN INNES CENTRE
NORWICH RESEARCH PARK
COLNEY LANE
UK NR4 7UH NORWICH
Tel.: 4460352571
Telex: 4460356844
Fax: 975L22JIINORG

DR. C. RODRIGUES-POUSADA
INSTITUTO GULBENKIAN DE CIENCIA
APARTADO 14
P 2781 OEIRAS CODEX
Tel.: 35114431331 / 351443
Telex: 35114431643
Fax: 12345 GULBENP

DR. E. RODRIGUEZ-FARRE
HEAD OF THE DEPARTMENT OF PHARMACOLOGY
DEPARTMENT OF PHARMACOLOGY AND TOXICOL
DEPARTMENT OF NEUROCHEMISTRY
CSIC
JORDI GIRONA 18-26
E 08034 BARCELONA
Tel.: 3432040600
Telex: 3432045904
Fax: 97977

PROF. A.M. ROLLIER
ASSOCIATE PROFESSOR UNIVERSITY OF MILA
DIPARTIMENTO DI BIOLOGIA E GENETICA PER LE SCIENZE MEDICHE
UNIVERSITA DEGLI STUDI DI MILANO
VIA VIOTTI 3/5
I 20133 MILANO
Tel.: 39226680356

PROF. J. ROMMELAERE
PROFESSOR
LABORATOIRE DE BIOPHYSIQUE ET RADIOBIO
UNIVERSITE LIBRE DE BRUXELLES
RUE DES CHEVAUX 67
B 1640 RHODE ST GENESE
Tel.: 3226509723
Telex: 326509744

DR. L.J. ROSS
RESEARCH SCIENTIST
DIVISION OF MOLECULAR BIOLOGY
INSTITUTE FOR ANIMAL HEALTH
HOUGHTON LABORATORY
HOUGHTON
UK PE17 2DA HUNTINGDON
Tel.: 4448064101
Telex: 4448067870
Fax: 329154 HPRS

DR. C. ROSSI
ISTITUTO ZOOPROFILATTICO
LABORATORIO DI BIOTECHNOLOGIA
I 25125 BRESCIA
Tel.: 39302290309
Telex: 3930225613

DR. V. RUBIO SUSAN
HEAD OF FILAMENTOUS FUNGI LABORATORY
INSTITUTO DE INVESTIGACIONES BIOMEDICA
C.S.I.C.
ARTURO DUPERIER 4
E 28029 MADRID
Tel.: 3413975408
Telex: 3415854015

DR. P. RUDELSHEIM
FIELD TRIALS SUPERVISOR
PLANT GENETIC SYSTEMS N.V.
JOZEF PLATEAUSTRAAT 22
B 9000 GENT
Tel.: 3291358440
Telex: 3291240694
Fax: 11361 PGSGEN

DR. T. RUZZON
BS RESPONSIBLE FOR SERVIZIO TECNOLOGIE
IST - ISTITUTO NAZIONALE PER LA RICERC
VIALE BENEDETTO XV 10
I 16132 GENOVA
Tel.: 3910354027
Telex: 3910352888
Fax: 286353 ISTEX I
E-mail: TELECOM GOLD 10075:DB10503

PROF. M.P. RYAN
PROFESSOR / HEAD OF DEPARTMENT
DEPARTMENT OF PHARMACOLOGY
UNIVERSITY COLLEGE DUBLIN
FOSTERS AVENUE BLACKROCK
IRL CO DUBLIN
Tel.: 35317061558
Telex: 35312837211
Fax: 32693 EI

DR. H.-J. RZIHA
SCIENTIFIC DIRECTOR, SENIOR SCIENTIST
INSTITUT FUER IMPFSTOFFE
FEDERAL RESEARCH CENTRE FOR VIRUS
DESE
PAUL-EHRLICH-STRASSE 28
D 7400 TUEBINGEN
Tel.: 497071603324
Telex: 497071603201
Fax: 17707131
Teletex: 707131

PROF. C. SACCONE
FULL PROFESSOR IN MOLECULAR BIOLOGY
DIPARTIMENTO DI BIOCHIMICA
E BIOLOGIA MOLECOLARE
UNIVERSITA DI BARI
TRAVERSA 200 RE DAVID 4
I 70125 BARI
Tel.: 3980482100
Telex: 3980482607
E-mail: SACCONE@MVX36.CSATA.IT

PROF. H.-G. SAHL
PROFESSOR OF MICROBIOLOGY, RESEARCH
GR
INSTITUTE FOR MEDICAL MICROBIOLOGY
AND
IMMUNOLOGY
RHEINISCHE FRIEDRICH-WILHELMS
UNIVERSI
SIEGMUND-FREUD-STRASSE 25
D 5300 BONN-VENUSBERG
Tel.: 492282802704 / 49228
Telex: 4922828803763

PROF. H. SAHM
HEAD OF IBTI
INSTITUT FUER BIOTECHNOLOGIE I
FORSCHUNGSZENTRUM (KFA) JUELICH
P.O. BOX 1913
D 5170 JUELICH
Tel.: 492461613294
Telex: 492461612710
Fax: 833556b KFA D

DR. G. SAKELLARIS
RESEARCH SCIENTIST
THE NATIONAL HELLENIC RESEARCH
FOUNDAT
VASSILEOS CONSTANTINOU AVENUE 48
GR 116 35 ATHENS
Tel.: 3017239965
Telex: 3017212729
Fax: 224064

PROF. F. SALA
PROFESSOR
DEPARTMENT OF GENETICS AND
MICROBIOLOG
UNIVERSITY OF PAVIA
VIA S. EPIFANIO 14
I 27100 PAVIA
Tel.: 39382391533
Telex: 39382528496

PROF. J.A. SALAS
PROFESOR TITULAR DE UNIVERSIDAD
DEPARTAMENTO DE BIOLOGIA FUNCIONAL
AREA MICROBIOLOGIA
UNIVERSIDAD DE OVIEDO
JULIAN CLAVERIA S/N
E 33006 OVIEDO
Tel.: 3485103652
Telex: 3485232255
Fax: 84322

PROF. M. SALAS
PROFESSOR OF RESEARCH
CENTRO DE BIOLOGIA MOLECULAR
UNIVERSIDAD AUTONOMA
CSIC
CANTO BLANCO
E 28049 MADRID
Tel.: 3413975070251
Telex: 3413974799
Fax: 27810 EDUCI E

DR. M. SALDEN
DIRECTOR R&D
EURO DIAGNOSTICS B.V.
WILMERSDORF 24
NL 7327 AC APELDOORN
Tel.: 3155422433
Telex: 3155425017
Fax: 52452 RCN

DR. C. SANDER
PROGRAMME COORDINATOR
BIOCOMPUTING GROUP
EUROPEAN MOLECULAR BIOLOGY
LABORATORY
MEYERHOFSTRASSE 1
D 6900 HEIDELBERG
Tel.: 496221387361
Telex: 496221387306
Fax: 461613 EMBL D
E-mail: SANDER@EMBL-HEIDELBERG.DE

DR. M.H. SANTOS
ASSISTENT PROFESSOR/DIRECTOR, MOL. BIO
INSTITUTO DE BIOLOGIA EXPERIMENTAL E
TECNOLOGICA (IBET)
ESTACAO AGRONOMICA NACIONAL
APARTADO 12
P 2780 OEIRAS
Tel.: 35114427787/6146
Telex: 35114421173

PROF. L. SARDA
PROFESSOR
INSTITUT DE CHIMIE BIOLOGIQUE
UNIVERSITE D'AIX-MARSEILLE
PLACE VICTOR HUGO 3
F 13331 MARSEILLE 3
Tel.: 3391959071/514
Telex: 3391501300

DR. B. SAVAKIS
ASS PROF UNIV CRETE, IMBB RESEARCH SCI
INSTITUTE FOR MOLECULAR BIOLOGY
AND BIOTECHNOLOGY
GR 71110 HERAKLION-CRETE
Tel.: 3081212647
Telex: 3081231308
Fax: 262896 IMBB GR
E-mail: SAVAKIS@GRIMBB.BITNET

PROF. C. SCAZZOCCHIO
PROFESSOR
INSTITUT DE GENETIQUE ET
MICROBIOLOGIE
CNRS URA 1354
CENTRE D'ORSAY - UNIVERSITE PARIS-SUD
BATIMENT 409
F 91405 ORSAY CEDEX
Tel.: 33169416356
Telex: 33169417808

DR. M. SCHARTL
RESEARCH GROUP LEADER
PHYSIOLOGISCHE CHEMIE I
BIOZENTRUM DER UNIVERSITAET
WUERZBURG
THEODOR-BOVERI-INSTITUET FUER
BIOWISSE
AM HUBLAND
D 8700 WUERZBURG
Tel.: 499318884148
Telex: 499318884150
Fax: 521740

DR. TH. SCHEPER
UNIVERSITY ASSISTENT
INSTITUT FUER TECHNISCHE CHEMIE
UNIVERSITAET HANNOVER
CALLINSTRASSE 3
D 3000 HANNOVER
Tel.: 495117622509
Telex: 495117623004
Fax: 923868 UNIHN D

PROF. K.-H. SCHLEIFER
FULL PROFESSOR
LEHRSTUHL FUER MIKROBIOLOGIE
TU MUENCHEN
ARCISSTRASSE 21
D 8000 MUENCHEN 2
Tel.: 498921052375
Telex: 498921052000
Fax: 522854 TUMUE D

PROF. R.D. SCHMID
HEAD ENZYME TECHNOLOGY DIVISION
DIVISION OF ENZYME TECHNOLOGY
GESELLSCHAFT FUER BIOTECHNOLOGISCHE
FORSCHUNG MBH
MASCHERODER WEG 1
D 3300 BRAUNSCHWEIG
Tel.: 495316181300
Telex: 495316181302
Fax: 952667 GEBIO D

PROF. A. SCHOUSBOE
PROFESSOR
DEPT. OF BIOLOGY
ROYAL DANISH SCHOOL OF PHARMACY
UNIVERSITETSPARKEN 2
DK 2100 COPENHAGEN
Tel.: 4531370850
Telex: 4535375744

DR. Z. SCHWARZ-SOMMER
SENIOR SCIENTIST
MAX-PLANCK-INSTITUT FUER
ZUECHTUNGSFOR
MAX-PLANCK-GESELLSCHAFT FUER
FOERDERUN
DER WISSENSCHAFTEN
CARL-VON-LINNE-WEG 10
D 5000 KOELN 30
Tel.: 492215062140
Telex: 492215062113
Fax: 8881 028 MPIZD

DR. P. SHELDRICK
DIRECTOR, LABORATOIRE DE VIROLOGIE DE
LAB. DE VIROLOGIE DE L'HERPES
INSTITUT DE RECHERCHES SCIENTIFIQUES S
CNRS - UPR 272
RUE GUY MOCQUET 7
F 94801 VILLEJUIF
Tel.: 33147264658355
Telex: 33147268836

DR. M.W. SHIRLEY
PRINCIPAL SCIENTIST
DIVISION OF MOLECULAR BIOLOGY
HOUGHTON LABORATORY
INSTITUTE FOR ANIMAL HEALTH
UK PE17 2DA HUNTINGDON
Tel.: 4448064101
Telex: 4448067870
Fax: 329154 HPRS

DR. R.J. SIEZEN
HEAD OF DEPARTMENT
NEDERLANDS INSTITUUT VOOR
ZUIVELONDERZ
KERNHEMSEWEG 2
P.O. BOX 20
NL 6710 BA EDE
Tel.: 31838059511
Telex: 31838050400
E-mail: NIZO@CAOS.CAOS.KUN.NL

DR. G. SILVESTRI
PRESIDENT
COSPAG
CENTRO COOPERATIVO SPERIMENTAZIONE
AGR
CABRIOLO 93
I 43036 FIDENZA
Tel.: 3952482763

PROF. H. SIMON / DR. H. GUENTHER
LEHRSTUHL FUER ORGANISCHE CHEMIE
UND BIOCHEMIE
TU MUENCHEN
LICHTENBERGSTRASSE 4
D 8046 GARCHING
Tel.: 498932093340
Telex: 17898174TUMGAR
Fax: 498932093345

DR. P. SIMONET
CHERCHEUR
LABORATOIRE DE MICROBIOLOGIE DES SOLS
URA CNRS 1450
UNIVERSITE DE LYON I
BOULEVARD DU 11 NOVEMBRE 1918 43
F 69622 VILLEURBANNE
Tel.: 3372448289
Telex: 3372448466
Fax: 330722 F
E-mail: NORMAND@CISM.UNIV-LYON1.FR

DR. M.A. SKINNER
RESEARCH SCIENTIST
DIVISION OF MOLECULAR BIOLOGY
HOUGHTON LABORATORY
INSTITUTE FOR ANIMAL HEALTH
HOUGHTON
UK PE17 2DA HUNTINGDON
Tel.: 4448064101
Telex: 4448067870
Fax: 329154HPRS
E-mail: SKINNER@UK.AC.AFRC.IRAD

PROF. DR. P.P. SLONIMSKI
CENTRE DE GENETIQUE MOLECULAIRE DU CNR
CENTRE NATIONAL DE LA RECHERCHE SCIENT
AVENUE DE LA TERRASSE
F 91198 GIF-SUR-YVETTE CEDEX
Tel.: 33169823208
Telex: 33169075322
Fax: 1603579
E-mail: SLONIMSKI@FRCGM51

DR. J.A. SMITH
LECTURER
DEPT. OF PLANT SCIENCES
UNIVERSITY OF OXFORD
SOUTH PARKS ROAD
UK OX1 3RB OXFORD
Tel.: 44865275009
Telex: 44865275074

DR. W. SOMERS
SCIENTIST
DEPARTMENT OF BIOTECHNOLOGY
TNO - NETHERLANDS ORGANIZATION FOR APPLIED SCIENTIFIC RESEARCH
UTRECHTSEWEG 48
P.O. BOX 48
NL 3700 AJ ZEIST
Tel.: 31340444144718
Telex: 31340454186
Fax: 40022 CIVO NL

DR. F. SOUQ
STAFF SCIENTIST
DIRECTION DU DEVELOPPEMENT
LVMH RECHERCHE
AVE. HOCHE 30
F 75008 PARIS
Tel.: 33145630101 EXT. 231
Telex: 33142258407

DR. A. SPENA
STAFF SCIENTIST/GROUP LEADER
MAX-PLANCK-INSTITUT FUER ZUECHTUNGSFOR
CARL-VON-LINNE-WEG 10
D 5000 KOELN 30
Tel.: 492215062329
Telex: 492215062413
Fax: 8 881 028 MPIZ D

PROF. I. SPENCER-MARTINS
DIRECTOR PYCC
PORTUGUESE YEAST CULTURE COLLECTION
GULBENKIAN INSTITUTE OF SCIENCE
APARTADO 14
RUA DA QUINTA GRANDE
P 2781 OEIRAS CODEX
Tel.: 35114431431
Telex: 35114431631
E-mail: TELECOM GOLD 10075:DBI0261

PROF. F. SPENER
PROFESSOR
INSTITUT FUER BIOCHEMIE
WESTFAELISCHE WILHELMS UNIVERSITAET
WILHELM-KLEMM-STRASSE 2
D 4400 D MUENSTER
Tel.: 49251833100
Telex: 492518358
Fax: 892 529 UNIMS D

DR. J.-L. STAMPF
STAFF RESEARCHER
SANOFI RECHERCHE
RUE DU PROFESSEUR J. BLAYAC 371
F 34184 MONTPELLIER CEDEX 04
Tel.: 33674001333815
Telex: 3367405513
Fax: 480240 F

DR. P. STEEN
RESEARCH MANAGER
MARIBO SEED
DANISCO A/S
HOSBYGAARDVL 4
P.O. BOX 29
DK 4960 HOLEBY
Tel.: 4553906031 EXT. 244
Telex: 2553907419
Fax: 31162 MARIBODK

DR. H.Y. STEENSMA
CLUSIUS LABORATORIUM
RIJKSUNIVERSITEIT LEIDEN
WASSENAARSEWEG 64
NL 2333 AL LEIDEN
Tel.: 3171274947
Telex: 3171274863 / 3171274

DR. M.W. STEER
HEAD OF DEPARTMENT
UNIVERSITY COLLEGE DUBLIN
IRL 4 DUBLIN
Tel.: 35317062254
Telex: 3531694409
Fax: 32693 EI

DR. W.J. STIEKEMA
HEAD DEPARTMENT OF MOLECULAR BIOLOGY
DEPARTMENT OF MOLECULAR BIOLOGY
CENTRE FOR PLANT BREEDING RESEARCH CPO
DROEVENDAALSESTEEG 1
P.O. BOX 16
NL 6700 AA WAGENINGEN
Tel.: 31837041276
Telex: 31837014435
Fax: 45856 ITAL NL

PROF. H. STOLTE
PROFESSOR OF MEDICINE, HEAD OF EXPERIM
ARBEITSBEREICH EXPERIMENTELE NEPHROLOG
ABTEILUNG FUER NEPHROLOGIE
MEDIZINISCHE HOCHSCHULE HANNOVER
KONSTANTY-GUTSCHOW-STRASSE 8
D 3000 HANNOVER 61
Tel.: 495115323780
Telex: 49511537898
Fax: 922044 MHHWI D

PROF. J.C. STREIBIG
ASSOCIATE PROFESSOR
SECTION OF CROP SCIENCE
DEPT. OF AGRICULTURAL SCIENCES
THE ROYAL VETERINARY AND
AGRICULTURAL
THORVALDSENSVEJ 40
DK 1871 FREDERIKSBERG C
Tel.: 4531351788 EXT. 2613
Telex: 4531390423

PROF. J.E. SUAREZ
CATEDRATICO DE UNIVERSIDAD
DEPARTAMENTO DE BIOLOGIA FUNCIONAL
AREA DE MICROBIOLOGIA
FACULTAD DE MEDICINA
JULIAN CLAVERIA S/N
E 33006 OVIEDO
Tel.: 3485103559
Telex: 3485232285

DR. S. SUHAI
PROJECT LEADER
MOLECULAR BIOPHYSICS
GERMAN CANCER RESEARCH CENTRE
(DKFZ)
IM NEUENHEIMER FELD 280
D 6900 HEIDELBERG
Tel.: 496221422369
Telex: 496221401271
E-mail: DOK252@CVX12.DKFZ-HEIDELBERG.DE

DR. M. SUNDVALL
COMPUTER DEPARTMENT
UPPSALA BIOMEDICAL CENTRE
BOX 570
S 751 23 UPPSALA
Tel.: 4618174909
Telex: 4618551759
E-mail: MATS.SUNDVALL@BMC.UU.SE

DR. I. TAYLOR
LECTURER IN PLANT GENETICS
DEPT. OF PHYSIOLOGY & ENVIRONMENTAL
SC
FACULTY OF AGRICULTURAL AND FOOD
SCIEN
UNIVERSITY OF NOTTINGHAM
UK LE12 5RD LOUGHBOROUGH
Tel.: 44602484848 EXT. 825
Telex: 44509673917
Fax: 37346 UNINOT G

DR. J. TEMPE
DIRECTEUR DE RECHERCHE, GROUP
LEADER
INSTITUT DES SCIENCES VEGETALES
C.N.R.S.
AVENUE DE LA TERRASSE
F 91198 GIF-SUR-YVETTE CEDEX
Tel.: 33169823689
Telex: 33169823695

PROF. M. TERZI
PROFESSOR OF GENETICS
DIPARTIMENTO DI BIOLOGIA
UNIVERSITA DEGLI STUDI DI PADOVA
VIA TRIESTE 75
I 35121 PADOVA
Tel.: 39498286284
Telex: 39498072213

DR. K. THERES
DIRECTOR
INSTITUT FUER GENETIK
AM MPI FUER ZUECHTUNGSFORSCHUNG
CARL-VON-LINNE-WEG 10
D 5000 KOELN 30
Tel.: 492215062670
Telex: 492215062513
Fax: 8882291 UNIK

DR. L. THIM
NOVO NORDISK A/S
NOVO ALLE
DK 2880 BAGSVAERD
Tel.: 4544448888/2176
Telex: 4544444565
Fax: 37173

DR. G. THIREOS / DR. E. ALEXANDRAKI
DRS.
INSTITUTE OF MOLECULAR BIOLOGY AND
BIO
FOUNDATION OF RESEARCH AND
TECHNOLOGY
P.O.BOX 1527
GR 71110 HERAKLION, CRETE
Tel.: 3081210361 / 3081210
Telex: 3081238824 / 3081239
Fax: 262860 RCC GR
E-mail: THIREOS@GGRIMBB

DR. CH. THOMPSON
DEPARTEMENT DES BIOTECHNOLOGIES
UNITE DE GENIE MICROBIOLOGIQUE
INSTITUT PASTEUR
RUR DU DOCTEUR ROUX 25
F 75724 PARIS CEDEX 15
Tel.: 3345688828
Telex: 334568843
Fax: PASTEUR 250.609 F
E-mail: DAVIES@PASTEUR

DR. R.D. THOMPSON
MPG-STIPENDIAT
MAX-PLANCK-INSTITUT FUER
ZUECHTUNGSFOR
CARL-VON-LINNE-WEG 10
D 5000 KOELN 30
Tel.: 492215062439
Telex: 492215062413
Fax: 8881028 MPIZ D

DR. J.M. THORNTON
SENIOR LECTURER
DEPARTMENT OF BIOCHEMISTRY AND
MOLECULAR BIOLOGY
UNIVERSITY COLLEGE LONDON
GOWER STREET
UK WC1E 6BT LONDON
Tel.: 44713807048
Telex: 44713807193
E-mail: THORNTON@CR.BBK.AC.UK

DR. A. TIEZZI
RESEARCHER
DIPARTIMENTO DI BIOLOGIA AMBIENTALE
UNIVERSITA' DEGLI STUDI DI SIENA
VIA P.A. MATTIOLI 4
I 53100 SIENA
Tel.: 39577298856
Telex: 39577298860

DR. K.N. TIMMIS
DIRECTOR OF DIVISION AND
DEPARTMENT OF
ABTEILUNG MIKROBIOLOGIE
GBF
MASCHERODER WEG 1
D 3300 BRAUNSCHWEIG
Tel.: 495316181400
Telex: 495316181411
Fax: 952667 GEBIO D

DR. C. TONELLI
SENIOR SCIENTIST
DPTO DI GENETICA E DI BIOLOGIA DEI MIC
UNIVERSITA' DEGLI STUDI DI MILANO
CELORIA 26
I 20133 MILANO
Tel.: 3922663498
Telex: 3922664551
E-mail: GENETIC@IMIUCCA.BITNET

DR. M. TRICK
SENIOR SCIENTIFIC OFFICER
CAMBRIDGE LABORATORY
JOHN INNES CENTRE
COLNEY LANE
UK NR4 7UJ NORWICH
Tel.: 4466352571
Telex: 44603502270
Fax: 975122 JIINOR G

PROF. A. TSIFTSOGLOU
PROFESSOR PHARMACOLOGY
LABORATORY OF PHARMACOLOGY
DEPT. OF PHARMACEUTICAL SCIENCES
ARISTOTLE UNIVERSITY OF THESSALONIKI
SCHOOL OF HEALTH SCIENCES
GR 54006 THESSALONIKI
Tel.: 3199147332
Telex: 3031991492

DR. E.M. TUCKER
HEAD OF DEPARTMENT OF MOLECULAR
PHYSIO
DEPT. OF MOLECULAR PHYSIOLOGY
CAMBRIDGE RESEARCH STATION
AFRC INSTITUTE OF ANIMAL PHYSIOLOGY
AN
UK CB2 4AT BABRAHAM, CAMBRIDGE
Tel.: 44223832312
Telex: 44223836122

PROF. G. TURNER
PROFESSOR
DEPARTMENT OF MOLECULAR BIOLOGY
AND
BIOTECHNOLOGY
THE UNIVERSITY OF SHEFFIELD
UK S10 2UH SHEFFIELD
Tel.: 44742768555
Telex: 44742728697
Fax: 347216 UGSHEF G

DR. H.A. ULLITZ-MOELLER
CAND. SCIENT. SYSTEM MANAGER
BIOBASE
AARHUS UNIVERSITET
OLE WORMS ALLE
BYGNING 170
DK 8000 AARHUS C
Tel.: 4586202711/2776
Telex: 4586131160
E-mail: HUM@BIOBASE.AAU.DK

PROF. F. URUBURU
DIRECTOR OF DEPARTMENT OF
MICROBIOLOGY
COLLECION ESPANOLA DE CULTIVOS TIPO
DEPARTAMENTO DE MICROBIOLOGIA,
FACULTA
UNIVERSIDAD DE VALENCIA
E 46100 BURJASOT (VALENCIA)
Tel.: 3463864612
Telex: 3463864372
E-mail: TELECOM GOLD 10075:DBI0428 /
BELLOCH@E

DR. A. VAGNO PEDERSEN
HEAD OF APPLIED TECHNOLOGY
LABORATORY
CHRISTIAN HANSEN'S LABORATORIUM
DANMAR
BOEGE ALLE 10
DK 2970 HORSHOLM
Tel.: 4545767676 EXT. 4022
Telex: 4545765455
Fax: 19184 HANSEN DK

DR. E.W. VAN BREUKELEN
COORDINATOR SPECIAL PROJECTS
NICKERSON-ZWAAN B.V.
GEBROKEN MEELDIJK 74
P.O. BOX 19
NL 2990 AA BARENDRECHT
Tel.: 31180613277
Telex: 31180614079
Fax: 20117 ZWAV NL

PROF. W.J.M. VAN DE VEN
RESEARCH PROFESSOR
CENTER FOR HUMAN GENETICS
K.U. LEUVEN
HERESTRAAT 49
B 3000 LEUVEN
Tel.: 3216215987
Telex: 3216215997

DR. A.R.G. VAN DE WEGHE
RESEARCHER
FACULTY OF VETERINARY MEDICINE
DEPT. OF ANIMAL GENETICS AND BREEDING
RIJKS UNIVERSITEIT GENT
HEIDESTRAAT 19
B 9820 MERELBEKE
Tel.: 3291521941
Telex: 3291525304

DR. H.W.J. VAN DEN BROEK
SENIOR LECTURER
SECTION MOLECULAR GENETICS
DEPT. OF GENETICS
AGRICULTURAL UNIVERSITY WAGENINGEN
DREIJENLAAN 2
NL 6703 HA WAGENINGEN
Tel.: 31837083141
Telex: 31837083146

DR. P.J.M. VAN DEN ELZEN
LABORATORY OF MOLECULAR BIOLOGY
MOGEN INTERNATIONAL NV
EINSTEINWEG 97
NL 2333 CB LEIDEN
Tel.: 3171258282
Telex: 3171221471

DR. N. VAN DER HOEVEN
SCIENTIST
DEPARTMENT OF BIOLOGY
TNO - NETHERLANDS ORGANIZATION FOR APP
SCIENTIFIC RESEARCH
SCHOEMAKERSTRAAT 97
NL 2628 VK DELFT
Tel.: 3115696900
Telex: 3115616812
Fax: 38071 ZPTNO NL

DR. D. VAN DER MEI
DIRECTOR CBS
CENTRAALBUREAU VOOR SCHIMMELCULTURES
P.O. BOX 273
NL 3740 AG BAARN
Tel.: 31215481238
Telex: 31215416142
E-mail: TELECOM GOLD 10075:DBI0548

DR. C. VAN DIJK
HEAD OF THE DIVISION 'BIOCHEMICAL TREA
AGROTECHNOLOGICAL RESEARCH INSTITUTE
ATO
HAAGSTEEG 6
NL 6708 PM WAGENINGEN
Tel.: 31837075054
Telex: 31837012260

DR. J.D. VAN ELSAS
RESEARCH SCIENTIST-SOIL BIOTECHNOLOGY
DEPARTMENT OF SOIL BIOLOGY
INSTITUTE FOR SOIL FERTILITY RESEARCH
KEYENBERGSEWEG 6
NL 6700 AA WAGENINGEN
Tel.: 31837091202
Telex: 31837014435
Fax: 45856 ITAL NL

DR. A.A. VAN LAMMEREN
SENIOR RESEARCHER
DEPARTMENT OF PLANT CYTOLOGY AND MORPH
AGRICULTURAL UNIVERSITY WAGENINGEN
ARBORETUMLAAN 4
NL 6703 BD WAGENINGEN
Tel.: 31837084318
Telex: 31837084761
Fax: NL 45015
E-mail: KIEFT@RCL.WAU.NL

DR. M. VAN MONTAGU
LABORATORIUM VOOR GENETICA
RIJKSUNIVERSITEIT GENT
K.L. LEDEGANCKSTRAAT 35
B 9000 GENT
Tel.: 3291645170
Telex: 3291645349
Fax: 11995GENGENB
E-mail: JECOP@GENGENP.RUG.AC.BE

DR. J.A. VAN MOURIK
HEAD OF DEPARTMENT OF BLOOD COAGULATIO
DEPARTMENT OF BLOOD COAGULATION
CENTRAL LABORATORY OF THE NETHERLANDS
RED CROSS TRANSFUSION SERVICES
PLESMANLAAN 125
NL 1066 CX AMSTERDAM
Tel.: 31205123120
Telex: 31205123332
Fax: 13159 BLOOD NL

DR. J.T. VAN OIRSCHOT
HEAD OPEN VIROLOGY DEPARTMENT
CENTRAL VETERINARY INSTITUTE
EDELHERTWEG 15
P.O. BOX 65
NL 8200 AB LELYSTAD
Tel.: 31320073308
Telex: 31320073473
Fax: 4027 CDI NL

DR. H. VAN ONCKELEN
RESEARCH DIRECTOR OF THE BELGIAN NATIO
DEPT. OF BIOLOGY
UNIVERSITY OF ANTWERP
UNIVERSITEITSPLEIN 1
B 2610 WILRIJK
Tel.: 3238202267
Telex: 3238202249

PROF. J.L. VAN WENT
HEAD OF DEPARTMENT, PROFESSOR IN BOTAN
DEPARTMENT OF PLANT CYTOLOGY AND MORPH
AGRICULTURAL UNIVERSITY WAGENINGEN
ARBORETUMLAAN 4
NL 6703 BD WAGENINGEN
Tel.: 31837083499
Telex: 31837084761
Fax: NL 45015
E-mail: PLANTCM@RCL.WAU.NL

DR. A. VASQUEZ
DEPT. DE GENETICA
FAC. DE BIOLOGIE
UNIVERSIDAD COMPLUTENSE DE MADRID
E 28040 MADRID
Tel.: 3415496785
Telex: 3413941638

PROF. G. VENEMA
FULL PROFESSOR
DEPARTMENT OF GENETICS
UNIVERSITY OF GRONINGEN
KERKLAAN 30
NL 9751 NN HAREN
Tel.: 3150632093
Telex: 3150632348

DR. M.A. VENIS
HEAD OF PHYSIOLOGY
AFRC INSTITUTE OF HORTICULTURAL RESEAR
EAST MALLING
BRADBOURNE HOUSE EAST MALLING
UK MEI9 6BJ MAIDSTONE
Tel.: 44732843833
Telex: 44732849067
Fax: 957251

DR. J.M.A. VERBAKEL
RESEARCHMANAGER
UNILEVER RESEARCH LABORATORIUM
OLIVIER VAN NOORTLAAN 120
NL 3133 AT VLAARDINGEN
Tel.: 31104606933
Telex: 31104605383
Fax: 23261

DR. A. VERBERT
PROFESSEUR, DIRECTEUR DE LUMR NO. 111
LABORATOIRE DE CHIMIE BIOLOGIQUE
UMR C.N.R.S NO. 111
CITE SCIENTIFIQUE BATIMENT C-9
F 59655 VILLENEUVE D'ASCQ X
Tel.: 3320434883
Telex: 3320436555
Fax: USTL 136339 F

PROF. G. VERDIER
PROFESSOR
CENTRE DE GENETIQUE MOLECULAIRE ET
CELLULAIRE C.N.R.S. - UMR 106
UNIVERSITE CLAUDE BERNARD LYON - I
BOULEVARD DU 11 NOVEMBRE 1918 43
F 69622 VILLEURBANNE
Tel.: 3372448379
Telex: 3372440555

DR. R. VERGER
DIRECTEUR DE RECHERCHE
C.N.R.S.-C.B.B.M. 5-31
CHEMIN JOSEPH-AIGUIER 31
B.P. 71
F 13402 MARSEILLE 9
Tel.: 3391164093
Telex: 3391717896

DR. H. VERHOEVEN
SENIOR SCIENTIST
CENTRE FOR PLANT BREEDING RESEARCH 3
P.O. BOX 16
NL 6700 AA WAGENINGEN
Tel.: 31837077144
Telex: 31837018094

PROF. W. VERSTRAETE
PROFESSOR
LABORATORY OF MICROBIAL ECOLOGY
RIJKSUNIVERSITEIT GENT
COUPURE LINKS 653
B 9000 GENT
Tel.: 3291645977
Telex: 3291646248

DR. E.M. VEYS
HEAD OF DEPARTMENT OF
RHEUMATOLOGY, UN
UNIVERSITAIR ZIEKENHUIS RUG
POLIKLINIEK 5 REUMATOLOGIE
RIJKS UNIVERSITEIT GENT
DE PINTELAAN 185
B 9000 GENT
Tel.: 3291402230
Telex: 3291404975

DR. J. VISSER
SENIOR STAFF MEMBER MOLECULAR
GENETICS
DEPARTMENT OF GENETICS
SECTION MOLECULAR GENETICS
AGRICULTURAL UNIVERSITY WAGENINGEN
DREIJENLAAN 2
NL 6703 HA WAGENINGEN
Tel.: 31837082150
Telex: 31837083146
Fax: NL45015BLHWG

DR. A. VIVIAN
READER IN BIOTECHNOLOGY
BRISTOL POLYTECHNIC HIGHER EDUCATION
CORPORATION
COLDHARBOUR LANE FRENCHAY
UK BS16 1QY BRISTOL
Tel.: 44272656261
Telex: 44272583758

DR. J.M. VLAK
SENIOR SCIENTIST
DEPARTMENT OF VIROLOGY
AGRICULTURAL UNIVERSITY
BINNENHAVEN 11
NL 6709 PD WAGENINGEN
Tel.: 31837083090
Telex: 31837084820
Fax: 45015
E-mail: BACULO@RCL.WAU.NL

PROF. J.F.G. VLIEGENTHART
HEAD OF DEPARTMENT
DEPARTMENT OF BIO-ORGANIC CHEMISTRY
UNIVERSITY OF UTRECHT
PADUALAAN B 8
NL 3584 CH UTRECHT
Tel.: 3130532168
Telex: 3130540980
E-mail: KUIK@RUUCMR.CHEM.RUU.NL

PROF. DR. D. VON WETTSTEIN
DEPARTMENT OF PHYSIOLOGY
CARLSBERG LABORATORY
GAMLE CARLSBERG VEJ 10
DK 2500 COPENHAGEN-VALBY
Tel.: 4531221022
Telex: 4531211366
Fax: 15434 CARLS DK
E-mail: OKICARLK@NEUVM1

DR. R. WALLSGROVE
ROTHAMSTED EXPERIMENTAL STATION
THE LAWES AGRICULTURAL TRUST
UK AL5 2JQ HARPENDEN
Tel.: 44582763133
Telex: 44582760981

DR. P.J. WARNER
LECTURER, BIOTECHNOLOGY
CRANFIELD INSTITUTE OF TECHNOLOGY
CRANFIELD
UK MK43 OAL BEDFORD
Tel.: 44234750111256
Telex: 44234750907
Fax: 825072 CITECH G

DR. L. WARNGARD
RESEARCH SCIENTIST
INSTITUTE OF ENVIRONMENTAL MEDICINE
KAROLINSKA INSTITUTET
TOMTEBODAVAGEN 30
P.O. BOX 60208
S 10401 STOCKHOLM
Tel.: 4687286400
Telex: 468343849

DR. S.P. WATERS
FIELD DEVELOPMENT AND REGISTRATION MAN
PLANT SCIENCES DIVISION
MONSANTO EUROPE S.A.
AVENUE DE TERVUREN 270
P.O. BOX 1
B 1150 BRUSSELS
Tel.: 3227614156
Telex: 3227614040
Fax: 62927

DR. U. WEIDLE
GROUP LEADER
WERK PENZBERG
BOEHRINGER MANNHEIM GMBH
IM NONNENWALD 2
D 8122 PENZBERG
Tel.: 498856602801
Telex: 498856302659
Fax: 59323 BMDPZ

DR. M.C. WEISS
DIRECTEUR DE RECHERCHE I CNRS, CHEF DE
INSTITUT PASTEUR
RUE DU DOCTEUR ROUX 25
F 75724 PARIS 15
Tel.: 33145688500
Telex: 33145688521
Fax: 250609F

DR. E. WELLINGTON
UNIVERSITY LECTURER
DEPARTMENT OF BIOLOGICAL SCIENCES
UNIVERSITY OF WARWICK
GIBBET HILL ROAD
UK CV4 7AL COVENTRY
Tel.: 44203523184
Telex: 44203523568

DR. G. WERSUHN
FACHBEREICH BIOLOGIE
HUMBOLDT UNIVERSITAET
INVALIDENSTRASSE 43
D O-1040 BERLIN
Tel.: 37228972640
Telex: 37228972641

DR. R. WIERENGA
GROUP LEADER
EUROPEAN MOLECULAR BIOLOGY LABORATORY
MEYERHOFSTRASSE 1
D 6900 HEIDELBERG
Tel.: 496221387256
Telex: 496221387306
Fax: 461613 EMBL D
E-mail: WIERENGA@EMBL-HEIDELBERG.DE

PROF. K. WILLECKE
PROFESSOR OF GENETICS
INSTITUT FUER GENETIK
ABT. MOLEKULARGENETIK
UNIVERSITAET BONN
ROEMERSTRASSE 164
D 5300 BONN 1
Tel.: 49228550210
Telex: 49228550263
Fax: 886657 UNIBO D
E-mail: BONNGEN@DBNINF5.BITNET

DR. D.C. WILLIAMS
LECTURER IN BIOCHEMISTRY AND COLLEGE F
BIOCHEMISTRY DEPARTMENT
TRINITY COLLEGE
UNIVERSITY OF DUBLIN
IRL 2 DUBLIN
Tel.: 35317021802
Telex: 3531772400
Fax: 93782TCDEI

DR. L. WILLMITZER
PLANT TISSUE CULTURE LABORATORY
INST. FUER GENBIOLOGISCHE FORSCHUNG
IHNESTRASSE 63
D 1000 BERLIN
Tel.: 493083000760
Telex: 493083000736

PROF. U. K. WINKLER
FULL PROFESSOR
RUHR UNIVERSITAET BOCHUM
UNIVERSITAETSSTRASSE 150
P.O. BOX 102148
D 4630 BOCHUM
Tel.: 49234700/3100
Telex: 492347002001
Fax: 0049234825860

PROF. S.J. WODAK
ASSISTANT PROFESSOR
UNITE DE CONFORMATION DES MACROMOLECUL
BIOLOGIQUES (UCMB)
UNIVERSITE LIBRE DE BRUXELLES
AVENUE PAUL HEGER - P2 - 16
CP160
B 1050 BRUXELLES
Tel.: 3226485200
Telex: 3226488954
Fax: 23069 UNILIB B
E-mail: SHOSH%RUBENS.UUCP.BLEKUL60

DR. G. WRICKE
INST. FUER ANGEWANDTE GENETIK
UNIVERSITAET HANNOVER
HERRENHAEUSER STRASSE 2
D 3000 HANNOVER
Tel.: 495117623603
Telex: 495117623015

PROF. G.J. WULLEMS
PROFESSOR AND HEAD OF THE DEPARTMENT
FACULTY OF SCIENCES
UNIVERSITY OF NIJMEGEN
TOERNOOIVELD 1
NL 6525 ED NIJMEGEN
Tel.: 3180652688
Telex: 3180553450
Fax: 48228 WINA NL

PROF. M. YANIV
PROFESSOR, HEAD TUMOR VIRUS UNIT
TUMOR VIRUS UNIT
DEPT. OF BIOLOGY
INSTITUT PASTEUR
RUE DU DOCTEUR ROUX 25
F 75724 PARIS 15
Tel.: 33145688512
Telex: 33145688521
Fax: 250609 F
E-mail: YANIV@PASTEUR

DR. M. ZABEAU
KEYGENE N.V.
KEYENBERGSEWEG 6
NL 6704 PJ WAGENINGEN
Tel.: 31837024141
Telex: 31837024939

PROF. V.I. ZANNIS
PROFESSOR OF BIOCHEMISTRY
INSTITUTE OF MOLECULAR BIOLOGY AND BIOTECHNOLOGY (IMBB)
KNOSSOS AV.
GR 71410 IRAKLION
Tel.: 3081231199
Telex: 3081238824
Fax: 262860 RCC-GR
E-mail: N206@GRIMBB

PROF. DR. F.K. ZIMMERMANN
INSTITUT FUER MIKROBIOLOGIE
TECHNISCHE HOCHSCHULE DARMSTADT
SCHNITTSPAHNSTRASSE 10
D 6100 DARMSTADT
Tel.: 496151162855
Telex: 496151165489
Fax: 41957THD

European Communities — Commission

EUR 14298 — Biotechnology Research for Innovation, Development and Growth in Europe (1990-1993): progress report 1992

Edited by A. Vassarotti

Luxembourg: Office for Official Publications of the European Communities

1992 — VIII, 528 pp. — 14.8 X 21.0 cm

ISBN 92-826-3997-5

Price (excluding VAT) in Luxembourg: ECU 48

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 at eliminating bottlenecks which prevented the 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 ECU. The research activities in the new 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 and 1991. 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 early 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 '1992 report' which presents, for the first time since the programme started, a complete overview of research progress achieved under the EC BRIDGE programme over the period 1991-March 1992.

Venta y suscripciones • Salg og abonnement • Verkauf und Abonnement • Πωλήσεις και συνδρομές
Sales and subscriptions • Vente et abonnements • Vendita e abbonamenti
Verkoop en abonnementen • Venda e assinaturas

BE G Q E BELGIË

Moniteur belge /
Belgisch Staatsblad
Rue de L uvain 42 Louvenseweg 42
1 0 Bruxe e 1 00 Brusse
Té 02 512 26
Fax 511 1 84
CP P streken ng 000 20 5502 27

Autres d trib teurs
Overige verk oppunten

Librairie européenne/
Europese Boekhandel
Avenue Abert nnart 50
Abert nnart aan 50
1200 Bruxe es 1200 Brusse
Te 02 734 02 81
Fax 735 08 60

Jean De Lannoy
Avenue du R 2 Kon ngs aan 202
1 6 Bruxe e 1060 Brus e
Te 2 538 69
Télex 6322 NBOOK B
Fax 2 538 8 41

CREDOC
Rue de a M ntagne 34 Bergstraat 34
Bte 11 Bus 11
1000 Bruxe es 1000 Brusse

DANMARK

J. H. Schultz Information A/S
EF-Publikationer
t a e 18
25 Va by
Tf 36 44 22 66
Fax 36 44 01 41
Gr kont 6 0 08 86

BR DE T HLAND

Bundesanzeiger Verlag
B e te straße
P stfa h 1 80 6
5 K n 1
Te 2 21 2 29
Te ex ANZE GER B NN 8 882 595
Fax 2 29 78

GREECE ΕΛΛΑΔΑ

G.C. Eleftheroudakis SA
nternat na B kstore
Nik treet 4
1 563 Athens
Te 01 322 6 23
Te ex 21941 E EF
Fax 323 98 21

ESPANA

Boletin Oficial del Estado
Trafalga 27
28 1 Madrid
Te 91 44 82 135

Mundi-Prensa Libros, S A.
aste 17
28 01 Madrid
Te 91 431 33 99 L br
431 32 22 Su p ones
435 36 37 D rec n
Te ex 49377 MPL E
Fax 91 575 39 98

u rsal
Librería Internacional AEDOS
n e de C e t 391
8 9 Bar o a
Te 93 1 86 15
Fax 93 317 1 41

Libreria de la Generalitat
de Catalunya
Rambla de s Estud s, 118 Pa au Mu a
80 2 Bar e na
Te 93 3 2 68 35
3 2 64 62
Fax 93 3 2 12 99

FRANCE

Journal officiel
Service des publications
des Communautés européennes
26 rue Desa x
75727 Par s Cedex 15
Te 1 40 58 75
Fax 1 4 58 75 74

IRELAND

Government Supplies Agency
4 5 Harcourt Road
Dub n 2
Te 1 61 31 11
Fax 1 78 06 45

ITA IA

Licosa Spa
Va Du a d aabra 1 1
a e a p sta e 552
5 1 5 Frenze
Te 55 64 54 15
Fax 64 12 57
Te ex 570466 LICOSA I
CCP 343 509

GRAND DUCHÉ DE LUXEMBOURG

Messageries Paul Kraus
11 rue Chr st phe P ant n
2339 Luxemb urg
Té 499 88 88
Te ex 2515
Fax 499 88 84 44
CCP 49242 63

NEDERLAND

SDU Overheidsinformatie
Externe F nossen
P tu 2 14
25 EA s Gravenhage
Te 7 37 89 911
Fax 07 34 75 778

P RT GAL

Imprensa Nacional
asa da Moeda EP
Rua D Fran s Manue de Me o 5
1 92 sb a dex
Te 01 69 34 14

Distribuidora de Livros
Bertrand, Ld.ª
Grupo Bertrand, SA
Rua das Terras d s Va es 4 A
Apartado 37
27 Amad ra C dex
Te 01 49 59 5
Te ex 15798 BERDI
Fax 49 6 255

UN TED K NGD M

HMSO Books (PC 16)
HM O Pub cat ns Centre
51 N ne E ms Lane
Lond n SW8 5DR
Te 071 873 20
Fax GP3 873 8463
Te ex 29 71 138

ÖSTERREICH

Manz'sche Verlags-
und Universitätsbuchhandlung
Koh markt 16
1014 W en
Te 0222 531 61-0
Te ex 11 25 00 BOX A
Fax 0222 531 61-39

SUOMI

Akateeminen Kirjakauppa
Keskuskatu 1
PO Box 128
00101 Helsinki
Te 0 121 41
Fax (0 121 44 41

NORGE

Narvesen information center
Bertrand Narvesens ve 2
PO B x 6125 Etterstad
0602 Os 6
Te 2 57 33 00
Te ex 79668 NIC N
Fax 2 68 19 01

SVERIGE

BTJ
Box 200
22100 Lund
Te 046 18 00 00
Fax 046) 18 01 25

SCHWEIZ SUISSE SVIZZERA

OSEC
Stampfenbachstraße 85
8035 Zur ch
Te 01 365 54 49
Fax 01 365 54 11

CESKOSLOVENSKO

NIS
Have k va 22
1300 Praha 3
Te 02) 235 84 46
Fax 42 2 264775

MAGYARORSZÁG

Euro-Info-Service
Budapest I Kr
Att a ut 93
1012 Budapest
Te (1 56 82 11
Te ex 22) 4717 AGINF H 61
Fax 1 17 59 031

POLSKA

Business Foundation
Kr za 38 42
00 512 Warszawa
Te 22) 21 99 93, 628 28 82
International Fax&Phone
0 39 12 00 77

JUGOSLAVIJA

Privredni Vjesnik
Bu evar Len na 171/XIV
11 70 Be grad
Te 11 123 23 40

CYPRUS

Cyprus Chamber of Commerce and Industry
Chamber Bu ld ng
38 Gr vas Dh genis Ave
3 De g org s Street
PO B x 1455
N cos a
Te 2 449500 462312
Fax (2) 458630

TURKIYE

Pres Gazete Kitap Dergi
Pazarlama Dağitim Ticaret ve sanayi AŞ
Nar bahçe Sokak N 15
stanbu -Cağa ğlu
Te (1) 520 92 96 - 528 55 66
Fax 520 64 57
Telex 23822 DSVO-TR

CANADA

Renouf Publishing Co. Ltd
Mail orders — Head Office
1294 A goma Road
Ottawa Ontario K1B 3W8
Te (613) 741 43 33
Fax (613) 741 54 39
Te ex 0534783

Ottawa Store
61 Sparks Street
Tel (613) 238 89 85

Toronto Store
211 Yonge Street
Tel (416) 363 31 71

UNITED STATES OF AMERICA

UNIPUB
4611-F Assembly Dr ve
Lanham MD 20706-4391
Te Toll Free (800) 274 4888
Fax (301) 459 0056

AUSTRALIA

Hunter Publications
58A G pps Street
Co ngwood
Victoria 3066

JAPAN

Kinokuniya Company Ltd
17 7 Sh njuku 3-Chome
Sh njuku-ku
Tokyo 160 91
Te 03 3439-0121

Journal Department
PO Box 55 Chitose
Tokyo 156
Te 03) 3439-0124

AUTRES PAYS
OTHER COUNTRIES
ANDERE LÄNDER

Office des publications officielles
des Communautés européennes
2, rue Mercier
2985 Luxembourg
Tél 49 92 81
Télex PUBOF LU 1324 b
Fax 48 85 73/48 68 17
CC bancaire BIL 8-109/6003/700

12'91

CD-NA-14298-EN-C

NOTICE TO THE READER

All scientific and technical reports published by the Commission of the European Communities are announced in the monthly periodical **'euro abstracts'**. For subscription (1 year: ECU 110) please write to the address below.

The BRIDGE Programme (*B*iotechnology, *R*esearch for *I*nnovation, *D*evelopment and *G*rowth in *E*urope) covers the period 1990-93, with a budget of 100 million ECU. The research activities in the BRIDGE programme are implemented 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 and 1991. These encompass 579 participating organizations (388 for the N-projects and 191 for the T-projects) from 11 Member States and five EFTA countries. This first BRIDGE progress report covers the period 1991 - March 1992 and gives for each project the results achieved in the different laboratories, the evidence of an increasing integration of work and additional relevant information.

Price (excluding VAT) in Luxembourg: ECU 48

ISBN 92-826-3997-5

OFFICE FOR OFFICIAL PUBLICATIONS
OF THE EUROPEAN COMMUNITIES
L-2985 Luxembourg