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)

Catalogue of contracts
with project descriptions

Edited by
B. Nieuwenhuis

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INTRODUCTION

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 contributed to the construction of a supportive infrastructure for biotechnology research in Europe and the elimination of barriers to knowledge and know-how which prevent exploitations of the results originating from fundamental research [1]. To achieve this specific Community structures (ELWW = European Laboratory Without Walls) were created for organising transnational research funded by the programmes and the transfer of technology from academic laboratories to industry [2].

The current programme BRIDGE (Biotechnology Research for Innovation, Development and Growth in Europe) covers the period 1990-1993, with a budget of 100 million ecu. The research activities in BRIDGE are conducted via two different types of projects : N-projects and T-projects. N-projects, implemented by European Laboratories Without Walls (ELWW's, see above), have been extended from BAP into BRIDGE. The T-projects, larger and targeted towards the elimination of specific bottlenecks resulting from structural or scale constraints, have been initiated in BRIDGE. A more detailed description of both types of projects and their implementation has been given elsewhere [3,4].

Following three successive calls for proposals in 1989 and 1990, 69 N-projects and 7 T-projects were launched during 1990. These encompass 579 participating organisations (387 for the N-projects and 192 for the T-projects) from 11 Member States and 5 EFTA countries. These projects were selected from 461 submitted proposals from a total of 2189 organisations. Table 1 gives an overview of the results of the selection.
The N-projects and T-projects are described in two different parts of this catalogue of contracts which provides, for each of these projects, a title, the contract number, a summary of the objectives, as well as a brief description and a number of keywords. This outline is followed by a list of names and

addresses of each of the participants in the projects. Indexes of participants, contractnumbers and keywords are given at the end of the catalogue.

TABLE 1

BRIDGE calls for proposals				
	Number of proposals	Number of partners	cost proposed (million ecu)	contribution requested (million ecu)
Entire call	461	2189	596	430
Selected	112	581	164	114

BRIDGE project funded		
Number of projects	Number of partners	EC contribution (million ecu)
91	579	70

Detailed information on these projects can be found in brochures describing each of the T-projects and the ELWW's constructed around the N-projects. These brochures are available from the Division Biotechnology of the Commission upon request. Annual reports giving full details of the results are foreseen to appear towards the end of 1992 and 1993; a Final Report will be published in 1994.

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References :

[1] Vassarotti, A. and Magnien, E. (1991) Biotechnology R&D in the European Communities, vol. I, Catalogue of BAP Achievements, Elsevier, Paris
[2] Magnien, E., Aguilar, A., Wragg, P. and de Nettancourt, D. (1989) Biofutur, November, 17-30
[3] De Nettancourt, D. (1991) Chimicaoggi *9*, 9-11
[4] De Nettancourt, D. (1991) Agro-Industry Hi-Tech, March/April, 3-9

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BRIDGE

N-PROJECTS

AREA: A

INFORMATION INFRASTRUCTURE

Processing and analysis of bio(techno)logical data

CONTINUATION OF THE EXPANDED SERVICES OF THE EMBL DATA LIBRARY

CONTRACTNUMBER: BIOT CT-910254
START: 1991-03-01 *DURATION*: 24 months
EC CONTRIBUTION: 1,000,000 ECU

Objectives:

Production and distribution of the EMBL Nucleotide Sequence Database in a period of rapid growth. Data will be distributed to users on magnetic tape and CD-ROM and over computer networks. Development work will be undertaken for future nucleotide sequence databases.

Brief Description:

The main task of the Data Library is the production, maintenance and distribution of the nucleotide sequence database. This is done in close collaboration with GenBank in the USA and the DNA Database of Japan. In the last few years some effort has also been dedicated to the provision of other data - protein sequences, the Eukaryotic Promoter Database and restriction enzyme information for example. Data are collected from journals and direct submissions by researchers. Entering biological sequences into the database is only a small part of the work. The largest single kind of work is annotation of these sequences - extracting the biological information from the literature and casting it in a form suitable for inclusion in the database. There is also a substantial administrative workload in communicating with researchers about their submissions and with users about their requirements.

The data are distributed to users:

(1) by quarterly releases on magnetic tape and CD-ROM,

(2) using a network fileserver which allows users to request the latest data over computer network, and

(3) by daily updates of remote copies at nodes on EMBNet, a computer network connecting a number of European molecular biological computer centres.

During the contract period these services will continue and the development work will be undertaken for a future database which:

(1) takes account of requirements generated by genome scale sequencing and mapping,

(2) provides better linkage to other related databases, and

(3) is well adapted to inclusion of data from automated sequencing machines.

Keywords:
DATABASE, DNA, BIOSEQUENCES, MOLECULAR BIOLOGY, SEQUENCE ANALYSIS, NUCLEOTIDE SEQUENCE, DATA COLLECTION, SOFTWARE

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CARBBANK - A COMPLEX CARBOHYDRATE STRUCTURAL DATABASE

CONTRACTNUMBER: BIOT CT-900184
START: 1991-02-01 *DURATION*: 36 months
EC CONTRIBUTION: 342,000 ECU

Objectives:

The objective of the research program is to create and maintain a Complex Carbohydrate Structural Database (CCSD) and a database management program (CarbBank). The database will contain all published carbohydrate structures larger than disaccharides to enable scientists to search for carbohydrate structures in a systematic and rapid fashion. Currently, the database contains about 8000 records which will be extended in the next years to about 20000.

Brief Description:

In cooperation with scientific groups worldwide a survey of the existing literature is performed. In addition to collecting structural data manually, Chemical Abstracts (CA) services are used to provide carbohydrate structures and thus allowing for an easy access to the existing literature in that field. The CCSD contains information about complex carbohydrates larger than disaccharides with biological importance. The full primary structure, the reference, keywords and supplementary information are given for each record. Using the management program CarbBank the database can be edited and searched for whole and partial structures and text entries. Due to the menu-driven CarbBank program a search can be performed easy and quickly.

In addition to the survey of the literature the structural database will be linked to spectroscopic information, like ^{1H}- and ^{13}C-NMR data. This has already been accomplished using the preliminary version of the program SUGABASE. The development of tools to analyse the spectroscopic data utilising Neural Network methods are planned. Links and cross-references to other databases, i.e. protein sequence databases will be established.
Currently the program is available for IBM compatible PCs running DOS. Development of a program version running under UNIX is in progress.

Keywords:
COMPLEX CARBOHYDRATE STRUCTURAL DATABASE, CARBBANK, NUCLEAR MAGNETIC RESONANCE

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PROTEIN SEQUENCE DATABANK

CONTRACTNUMBER: BIOT CT-900170
START: 1991-01-01 *DURATION*: 36 months
EC CONTRIBUTION: 300,000 ECU

Objectives:

Development of tools to support the rapid distribution of protein sequence data within Europe.

Brief Description:

The Protein Sequence Databank at the Max-Planck-Institute for Biochemistry is responsible for the data collection and distribution of protein sequence data within Europe. Approximately 10,000 new sequences are collected by PIR-International annually. The goal of the closely cooperating databases in Europe, the US and Japan is to provide high quality up-to-date data to a user community that is estimated larger than 50,000 scientists in life sciences, bioindustry, and, more recently in the patent business.

Distribution through 'hard' media such as magnetic tapes or CD-ROMs is too slow to satisfy the need for access to the latest data. On-line access to molecular sequences and sequence data analysis software is more appropriate, but a single node in Europe is not sufficient to provide satisfactory services.

Based on the European Bioinformatics Network, a distribution scheme is developed to forward transactions to national nodes that act directly on an indexed database. The implementation of such a method will allow frequently updates, including corrections and will improve the European infrastructure in bioinformatics.

Keywords:
PROTEIN SEQUENCE DATABANK, DATA DISTRIBUTION, DATA ACCESS

Coordinator

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THE PROMOTION OF EMBNET: COMPUTER NETWORK FOR BIOINFORMATICS IN EUROPE

CONTRACTNUMBER: BIOT CT-910273
START: 1991-04-01 *DURATION*: 36 months
EC CONTRIBUTION: 650,000 ECU

Objectives:

The main objectives of this BRIDGE funded programme are all relevant
the reinforcement of the European Molecular Biology Network (EMBnet
EMBnet was established in 1988, initially, as a data collection backbone
EMBL (European Molecular Biology Laboratory). Thanks to the improv
ment of computer network communications, its scope and mandate h
greatly enlarged; and national EMBnet nodes have been entrusted
manage biocomputing services in their own countries.

Brief Description:

The European scope of this project has been declared ever since its begi
nings; and the underlying concept, that each country may profit from th
expertise of any other national node, has made it natural for it to b
included in a EC project. Institutions from Denmark, Finland, France, Ge
many, the United Kingdom, Greece, Italy, The Netherlands, Norway, Spai
Sweden and Switzerland take part in this project. BRIDGE funding will b
shared between the scientific coordination by CNR, Bari, Italy and th
administrative coordination by EMBL, Heidelberg, Germany. Each node w
report directly to the scientific coordinator for scientific matters and
EMBL for any administrative question. Funds will be employed in particul
to improve computer network connections and to organize training course
Much effort will be done during the first year of the project towards standa
dization of communication protocols, which is instrumental to the develo
ment of new products in Nucleic Acid Sequence Data Banks. To this pu
pose, regular and specialized courses will be organized to train bo
biologists and computer analysts in biocomputing and bioinformatics, ar
business meetings will be held to allow the exchange of expertise betwee
the nodes. With a view to this also Bulletin Board and Conferencing systen
will be implemented.

Keywords:
BIOCOMPUTING, BIOINFORMATICS, MOLECULAR BIOLOG
BIOSEQUENCES, DATABASES, EMBL DATA LIBRARY, NETWORI
ING, CONNECTIVITY

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INTEGRATED DATA AND KNOWLEDGE BASE OF PROTEIN STRUCTURE AND SEQUENCE

CONTRACTNUMBER: BIOT CT-910271
START: 1991-04-01 *DURATION*: 36 months
EC CONTRIBUTION: 875,000 ECU

Objectives:

The general objective is to develop an integrated European protein structure data- and knowledge-base. This will be achieved through the integration of data, algorithmic and knowledge-based computational approaches.

Brief Description:

The project is concerned with the development of an integrated protein structure environment and with the development of new computer methods for relating protein sequence, structure and function, by exploiting methods of logic programming, automatic learning procedures, and models of expert problem solving. Data, algorithmic and knowledge based computational approaches will be pooled, cross validated and applied to improve protein structure analysis, prediction methods and computer aided protein design techniques.

Among the more specific goals are the following important developments:

1. Deriving standard forms for manipulating and exchanging data entities among the project partners and between software developed by them or commercially.

2. Cross validation of data and software developed by the different partners through direct comparisons facilitated by the use of a common data schema and storage environment. This includes development of data validation software, testing data programs and scientific methodology in the context of structure prediction and modelling.

3. Shared development of user interfaces (front end), efficient data storage (back-end), as well as programs and methods.

4. Improved integration of advanced methods of database management and AI technology into the field of molecular biology.

5. Improved knowledge based methods for protein structure prediction.

Keywords:
SEQUENCE, PROTEIN STRUCTURE, PROTEIN MODELLING, PROTEIN ENGINEERING, DATABASE, KNOWLEDGE BASE, LOGIC PROGRAMMING, EXPERT SYSTEMS, INFORMATION TECHNOLOGY

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IMMUNOCLONE AND HYBRIDOMA DATABASE NETWORK FOR EUROPE

CONTRACTNUMBER: BIOT CT-910257
START: 1991-04-01 *DURATION*: 24 months
EC CONTRIBUTION: 730,000 ECU

Objectives:

Set up of a database on immunoclone descriptions from scientific literature, patent applications registered at the European Patent Office (Den Haag, Netherlands), commercial catalogues of biotechnology firms world-wide and public laboratories' collections.

Brief Description:

Organisation:
This consortium of 6 European centres (including 5 research laboratories) is based on the following rules:

— production: each record produced (full coding and update) entitles the owner (producing centre) to a part of the copyright on the database. The production planning is managed by the coordinator (CERDIC for the first two years);

— use: each production centre has total access to the database for its production activity and its personal use;

— distribution: each centre has the right to distribute information, but the distribution rights will be managed, at the consortium level, by the coordinator.

Methodology:

a) Sharing of data entry by geographical origin of sources

b) Systematic search in bibliographical databases

c) Agreement with the European Patent Office (EPO, Den Haag, The Netherlands)

d) Use of a reference thesaurus: EMTREE of ELSEVIER's EMBASE database

e) Full scientific responsibility of the centres

f) A common record format including the descriptions of the hybridoma, of the monoclonal antibody and the source of information. Procedures for control, checking, updates and activity reports are fully computerized and file exchange is planned on a weekly basis, by online procedures.

g) Computerized decentralized management: each centre can have total access to the database on its work stations.

h) Access to information: a user-friendly software is developed by the DIMDI bank host (Köln, FRG), on which the Immunoclone Database is loaded. The database is also available on Data-Star (Bern, Switzerland) and via Minitel on the host of the Conseil General des Alpes-Maritimes (Nice, France).

Keywords:
MONOCLONAL ANTIBODY, HYBRIDOMA, IMMUNOLOGY, INFORMATION PROCESSING

Coordinator

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ELECTRONIC LINKING SERVICE FOR BIOTECHNOLOGISTS AND MICROBIOLOGISTS IN EUROPE

CONTRACT: GRANT
START: 1991-01-01 *DURATION*: 36 months
EC CONTRIBUTION: 100,000 ECU

Objectives:

The MSDN aims to link and harmonise biotechnology and microbiology information resources in Europe and worldwide. An integrated bioinformatics service will be established, providing databases, bulletin boards and communications.

Brief Description:

The MSDN plans a series of activities that will strengthen the bioinformatics base in the Community. The following activities are planned:

Linking additional individuals, scientific groups and organisations to the network for communications and information exchange

Providing international access to European databases (linking databases and networks)

Developing the MSDN Directory of laboratories recording data on specific microbial properties

Organising workshops, training courses and individual training in the use of the MSDN network.

These activities will be implemented through the expansion of the existing network, drawing in new groups and initiatives and ensuring proper training in the use of the services. The result will be the integration of currently separate information resources, the establishment of a trained body of users and a greatly enhanced volume of biotechnology and microbiology information available in a readily accessible form. Close collaboration with the partners in the original MSDN proposal will ensure that different biological user-groups (serology, genetics, industrial microbiology, animal cell technology and culture collections) will be incorporated into the network.

Keywords:
BIOINFORMATICS, DATABASES, NETWORKS, TRAINING, COMMUNICATIONS

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AREA : A

INFORMATION INFRASTRUCTURE

Culture Collections

INFORMATION CENTRE FOR EUROPEAN CULTURE COLLECTIONS (ICECC)

CONTRACTNUMBER : BIOT CT-900162
START : 1991-01-01 *DURATION* : 36 months
EC CONTRIBUTION : 135,000 ECU

Objectives :

Despite the existence of a number of important national collections of biological materials within Europe, industry and the scientific community are often unaware of their existence or of the range of services offered. It is, therefore, an important task for the European culture collections to draw attention to themselves and to the facilities they have to offer. An important stage in their future development is considered to be the coordination of the exchange of information between culture collections and the provision of an information service to users.

Brief Description :

The major tasks of the Information Centre for European Culture Collections (ICECC) are:

— To provide a permanent secretariat for all European culture collections and a central contact point for European scientists and any institution seeking advice and information on cultures and culture collection related matters. To act as a public relations office on behalf of culture collections in dealing with regulatory bodies and the media. To publish regularly an Information Newsletter. To cooperate closely with the CEC supported Microbial Information Network Europe (MINE).

— To collect information on the strains, cell lines and services offered by the European culture collections to users and to publicise the resources within the culture collections in terms of materials (preparation of printed and visual material for distribution) and scientific expertise. By participation at technical and scientific exhibitions, conferences, and congresses the ICECC demonstrates the importance of the services offered by European collections of biological material.

— To hold catalogue from the various participating culture collections and to consider development of a European catalogue.

— To keep close contacts with European research and biotechnology in order to inform or advise culture collections on new developments and requirements.

— To encourage scientists to deposit microbial strains and other biological specimens of biotechnological and/or of general scientific significance in European culture collections as an important future resource.

— To keep up-to-date information on national and international patent regulations and treaties for the benefit of depositors and depositories. To advise depositors on the requirements for the deposit of cultures for patent purposes. To advise culture collections on changes in patent regulations and to act as an information centre on patent matters in general.

— To publicise the services and activities of the European culture collections throughout the world. To assist in the planning and running of national training courses within Europe and to act as a training centre.

Keywords :
CULTURE COLLECTIONS, COLLECTIONS OF BIOLOGICAL MATERIAL, INFORMATION AND MICROORGANISMS, PLASMIDS AND CELL LINES

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DEVELOPMENT OF MICROBIAL INFORMATION NETWORK IN EUROPE (MINE) TO A CENTRALIZED EUROPEAN CULTURE COLLECTIONS DATA BASE SYSTEM

CONTRACTNUMBER : BIOT CT-910280
START : 1991-06-01 *DURATION* : 36 months
EC CONTRIBUTION : 765,000 ECU

Objectives :

To make information about the strains of microorganisms in the European Culture Collections more easily available to the users by establishing an integrated and centralized database.

Brief Description :

Culture collections from 11 countries cooperate to make their microbial strain data available on-line in one centralized and integrated database.

In the previous phase of the project, formats have been developed for fungal and bacterial data. Collections entered (part of) their data in these agreed formats. At some collections, the databases can be accessed on-line.

In the present phase, data entry will continue, and the data from the various collections will be integrated into one database. The integration is carried out by two Data Integrating Nodes (one for fungi and yeasts, and one for bacteria) in two steps: firstly of the minimal data set(extended catalogue data), then of the full data set. Responsible committees of microbiologists have been established to fully harmonize the data during this process.

The integrated database will be accessible at DIMDI (Köln), a well-known host in the fields of biology and chemistry.

Keywords :
CULTURE COLLECTION, DATABASE, MINE, MICROBIOLOGY

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AREA: B

ENABLING TECHNOLOGIES

Protein design / molecular modelling

ENGINEERING OF MICROBIAL PEPTIDE LANTIBIOTICS FOR USE IN AGRO-FOOD AND BIOMEDICAL INDUSTRY

CONTRACTNUMBER: BIOT CT-910265
START: 1991-05-01 *DURATION*: 36 months
EC CONTRIBUTION: 680,000 ECU

Objectives:

The aim of the collaborative research project is to unravel the structure function relationships of lantibiotics and the development of lantibiotics with improved properties with respect to the solubility and bactericidal activity. The development of molecules with relaxed bactericidal activity is also anticipated.

Brief Description:

Lantibiotics are a recently defined family of post-translationally modified peptide antibiotics that possess bactericidal activity against a wide variety of Gram-positive bacteria and their spores. They are produced by Gram-positive bacteria of different genera and are characterized by a high content of unusual aminoacids especially, lanthionine, thioether-bridged aminoacids and 2,3-dihydro aminoacids. The lantibiotics exhibit extremely low toxicity. The best studied lantibiotic, nisin, produced by lactic acid bacteria, occurs naturally in dairy products and is used as a preservative in the food industry. It is anticipated that other lantibiotics will find their use as a medicine against bacterial and viral infections or as regulator of blood pressure.

Lantibiotics are synthesized on the ribosome and post-translationally processed and modified. Although the chemical structures of some lantibiotics are available knowledge of the mechanism of their biosynthesis and post-translational modification is very limited. Several hypothesis have been put forward for their mechanism of action including an effect on the potential of the bacterial membrane by pore formation, but nothing is known about their structure-function relationships.

In the project four different research groups, with complementary know how, expertise and laboratory infrastructure, are collaborating in an effort to unravel the structure-function relationships of lantibiotics and to describe and develope a second generation of these molecules. To this end, modified and mutant lantibiotics will be constructed, isolated and synthesized. Moreover, new lantibiotics will be isolated from other natural sources. The relationship between structure and function (or activity) will be investigated through elucidation of the three-dimensional structure of wildtype and mutant lantibiotics with the aid of NMR spectroscopy combined with studies of the processess involved in bacteriocine biosynthesis, post-translational processing, modification and secretion, and of the mechanism responsible for cell killing and immunity.

Keywords:
LANTIBIOTICS, 3-D STRUCTURE, STRUCTURE-FUNCTION, PROTEIN ENGINEERING, HIGH RESOLUTION NMR, MODELLING, INTERDISCIPLINARY

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MULTIDISCIPLINARY APPROACH TO THE ANALYSIS OF ENZYME CATALYSIS, PROTEIN STABILITY AND FOLDING

CONTRACTNUMBER: BIOT CT-910270
START: 1991-06-01 *DURATION*: 36 months
EC CONTRIBUTION: 681,000 ECU

Objectives:

In this multidisciplinary cooperation the methods of molecular modelling, structure determination and microbial techniques are applied to the analysis of protein stability, folding and functional properties, with the aim of understanding the underlying mechanism at the atomic level.

Brief Description:

The RNase Barnase from the microorganism *Bacillus amyloliquefaciens* is taken as the target enzyme in a multidisciplinary protein engineering approach.

As can be seen from numerous published investigations Barnase is an excellent system to study the stability and folding of proteins in general and also the interactions of this enzyme with different nucleotides or other proteins. During this project the knowledge about the mechanism of protein folding and stability should be improved. Also investigations about the interactions between Barnase and its substrates, substrate analogoues and proteinaceous inhibitors like Barstar will be made.

The results of that project should lead to an improvement of the experimental and theoretical techniques in protein engineering.

To achieve this goal a series of protein design cycles will be performed. In the first cycle suggestions for the production of mutant enzymes should be derived from molecular modelling studies based on the existing 3-D structures of Barnase and related RNases. The structures and properties of the wild type and mutant enzymes will be studied by NMR, enzyme kinetics and microcalorimetry techniques. The results of the experiments will than be

evaluated with the theoretical methods of protein engineering and should influence the subsequent protein design cycles of this project.

Keywords:
BARNASE, PROTEIN ENGINEERING, NMR, PROTEIN 3-D STRUCTURE, CALORIMETRY, MOLECULAR MECHANICS, MOLECULAR DYNAMICS, FREE ENERGY CALCULATION

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STABILITY STUDIES AND PROTEIN - DESIGN STUDIES WITH TRIOSEPHOPHATE ISOMERASES

CONTRACTNUMBER: BIOT CT-900182
START: 1991-01-01 *DURATION*: 36 months
EC CONTRIBUTION: 829,000 ECU

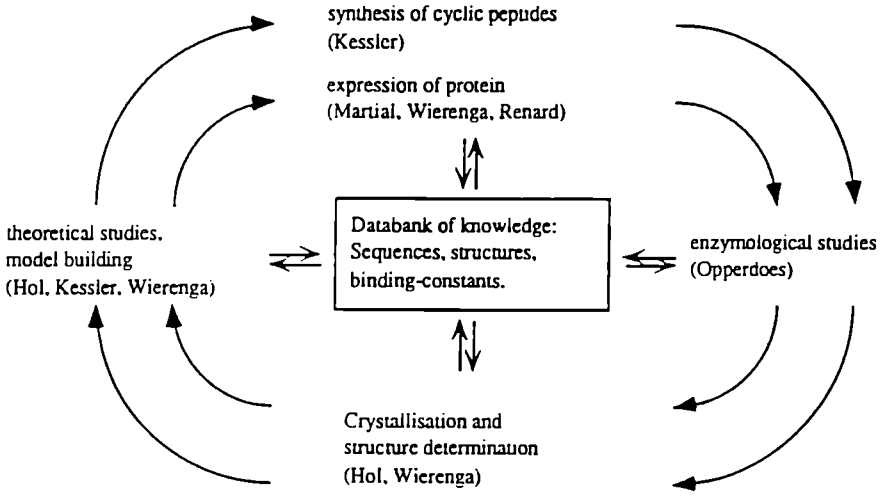
Objectives:

1. Characterization of a range of TIMs which differ widely in stability.
2. Creation of monomeric mutants of TIM by either site directed mutagenesis or complexation with tight-binding cyclopeptides, or via both approaches.
3. Eventually, with the help of model building techniques, the implementation of loop transplantations will be attempted in such a way that single monomers are formed with an altered specificity and/or new catalytic activity.

Brief Description:

A network of European research groups (see figure) has been formed to study and engineer the stability and activity of the dimeric enzyme triosephosphate isomerase (TIM) with the ultimate goal of being able to create new classes of enzymes based on the (βα)8-framework.

Within this collaboration psychhrotrophic and thermophylic TIMs will be studied, including the crystal structure determinations. This data will help to evaluate important characteristics related to the stability of this enzyme.
On the basis of the refined crystal structure of trypanosomal TIM, mutants will be made in which the dimer interface interactions are weakened. The eventual goal is to obtain monomeric TIM (complexed with or without cyclopeptides which mimic the dimer-interface loops) with an altered specificity and/or new catalytic activity.



Keywords:
TIM, STABILITY, CYCLOPEPTIDES, PROTEIN DESIGN, CYCLOPEPTIDE DESIGN, CRYSTAL STRUCTURE

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DESIGN AND ENGINEERING OF ALPHA-HELICAL BUNDLE PROTEINS: MODIFIED STRUCTURES AND NOVEL FUNCTIONS

CONTRACTNUMBER: BIOT CT-910262
START: 1991-05-01 *DURATION*: 36 months
EC CONTRIBUTION: 1,092,000 ECU

Objectives:

The goal is to understand the structural principles of small globular proteins as a basis for engineering novel functions; and, to apply this understanding to the production of specific prototypes of engineered proteins. Functions to be engineered into the helical bundle proteins include metal binding sites, enzyme inhibitors, peptide hormone analogues and epitope carriers.

Brief Description:

In order to develop the full potential of protein engineering, fundamental aspects of protein 3-D structure must be well understood. The structural simplicity and functional diversity of the 4-alpha-helix bundle protein motif makes it an excellent development system for protein engineering. The proteins rop and ferritin will be reengineered, first, to understand their architectural principles, and second, to equip them with desired functions.

In the first phase, parts of the basic protein scaffold will be altered by mutations or deletions, in order to test their contribution to formation and stability of the structure. Subsequently, with work on the basic scaffold continuing, the emphasis will shift to desired functional modifications, e.g. the engineering of binding or catalytic functions.
The project is carried out by an international group of laboratories with complementary expertise in: molecular genetics, protein purification, functional and immunoassays, 3-D structure determination by X-ray crystallography and NMR, light spectroscopy, energetics of protein folding, molecular modelling and protein structure theory - a European laboratory without walls in protein engineering.

Keywords:
FERRITIN, ROP, IRON PROTEINS, PROTEIN 3-D STRUCTURE, PROTEIN DESIGN

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AREA: B

ENABLING TECHNOLOGIES

Biotransformation

NEW WAYS OF BIOTRANSFORMATION IN NON-AQUEOUS SYSTEMS FOR THE SYNTHESIS OF PHARMACEUTICALS. APPLICATION OF SUPERCRITICAL GASES, ORGANIC SOLVENTS, LIQUID MEMBRANES AND MICROEMULSIONS

CONTRACTNUMBER: BIOT CT-900176
START: 1991-02-01 *DURATION*: 36 months
EC CONTRIBUTION: 900,000 ECU

Objectives:

The behaviour of selected hydrolases and dehydrogenases will be studied in four different non-conventional systems: supercritical CO_2 (CO_2SF), low-water content organic phases, microemulsions and liquid membranes. The reactions under investigation will be various enantio/stereo selective reactions for the production of long chain β-hydroxylic acids, terpens, prostaglandin synthons, esters of oligosaccharides and natural glucosides.

Brief Description:

Enzymes represent novel routes to a number of important products ranging from ß-blockers to synthetic cacaobutter. Though they have outstanding catalytic properties, the use of enzymes in organic synthesis is quite limited. The main reason is that most of the organic compounds can not be dissolved in aqueous media used in conventional enzymology. The recent realisation that enzymes can function also in organic solvents has given to the synthetic potential of enzymes a powerful boos for many processes. Other uncommon biocatalysis media are supercritical CO_2(CO_2SF), microemulsions, liquid membranes. Despite the promising future of biocatalysis in these new media the lack of information is still tremendous.

The purpose of this project is to cover the following various topics:

(a) Commercially available and new enzymes will be tested in regard to their synthetic potential.

(b) Enzymes will be modified chemically in order to increase their solubility in the apolar solvents.

(c) Immobilization techniques will be used for activity increase.

(d) Development and adaption of analysis techniques for enzyme reaction monitoring.

(e) Study of enzyme kinetics and thermodynamics in the different solvent.

(f) Modelling of the reaction to enlarge the understanding of enzyme interaction with the given type of solvents.

(g) Optimization of the reactions considering the model as well as the practical experiences.

The enzymes to be used will be selected hydrolases and hydrogenases and the reactions type will be various enantio/stereo selective reactions for the production of important esters in organic solvents, supercritical CO_2 (CO_2SF) microemulsions and liquid membranes. The project will help to increase the potential of enzymes by pursuance of innovative concepts, also, will elucidate basic questions regarding the enzyme action in the new media.

Keywords:
SUPERCRITICAL GASES, MICROEMULSIONS, ORGANIC SOLVENTS, LIQUID MEMBRANES, STEREO/ENANTIOSELECTIVITY

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CHARACTERIZATION AND SURFACE PROPERTIES OF SEMI-SYNTHETIC REDOX ENZYMES FOR THEIR APPLICATION IN BIOSENSOR DEVICES

CONTRACTNUMBER: BIOT CT-910279
START: 1991-10-01 *DURATION*: 36 months
EC CONTRIBUTION: 958,500 ECU

Objectives:

Chemically modifying redox enzymes of analytical interest by covalent attachment of coenzymes (FAD, NAD, NADP) and redox mediators to improve their properties for application in amperometric and/or combined electro-optic biosensors.

Brief Description:

Aim of the project is to contribute to bridge the the existing gap with respect to the lack of knowledge of the particular enzymology concerned with the operational adaption and functioning of redox enzymes in biosensor devices. The results may lead to better functioning enzymes under the harsh conditions that arise from the direct proximity of interfacially acting enzymes to device surfaces, both for amperometric and electro-optic biosensors. The evaluation of various semi-synthetic redox enzymes derived by chemically modifying inherently stable (e.g from thermophile microorganisms) flavoprotein oxidases and NAD(P) dependent dehydrogenases is premised. For covalent FMN- and FAD-oxidase conjugates, synthesized from apo-oxidases that originally bind flavin coenzymes noncovalently, we expect to achieve an improved compatibility with metalized (Ag, Au or Pt), carbon and (semi) conducting surfaces (SiO_2, TiO_2, SnO_2 by avoiding gradual loss of free FMN and FAD under stress conditions. Covalent NAD(P)-dehydrogenase-NAD(P)H oxidator conjugates are anticipated to function as oxidases by intramolecular NAD(P) recycling. Attaching chemical electron relays to unmodified oxidases and all semi-synthetic redox enzymes may result in facilitated direct electron transport to amperometric devices and O^-independency for oxidases.

Finding the most appropriate fixation mode for achieving optimal operational conditions for surface fixed semi-synthetic redox enzymes in

amperometric and/or combined electro-optic biosensors will be supported by computer simulation of enzymatic redox reactions on surfaces.

Keywords:
SEMI-SYNTHETIC REDOX ENZYMES, AMPEROMETRIC AND ELECTRO-OPTIC BIOSENSORS

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NEW TYPES OF REDOX ENZYMES FOR THE PRODUCTION OF CHIRAL SYNTHONS: BASIC RESEARCH, FUNCTIONALISATION AND APPLICATION

CONTRACTNUMBER: BIOT CT-900157
START: 1991-03-01 *DURATION*: 36 months
EC CONTRIBUTION: 753,000 ECU

Objectives:

Understanding of structure-function relationships between redoxenzymes, mediators and electrodes. Synthesis of stable and less toxic mediators. Procedures to allow long term operational stability for chiral product formation in a preparative scale.

Brief Description:

Combinations of redox enzymes, mediators and coenzymes will be tested. The results will be evaluated in order to select a limited number of enzymes, mediators and coenzymes which are to be looked on with respect to homogenous and heterogeneous bioelectrocatalysis.

The group at the university in Delft will screen for new enzymes containing PQQ as a cofactor (=quinoproteins). The group at the Technical University in Munich detected enzymes accepting viologens, which will be studied for their use in electrobiocatalysis. Both universities will try to purify and study the respective enzymes and assess their capability for the production of high added value compounds. Further enzymes will be a 3-ketoacid reductase, discovered at Munich, which forms a chiral synthon for L-carnitin synthesis, and new types of alcohol dehydrogenases. The Dutch university will proof the discovered quinoprotein alcohol dehydrogenase (enantioselective for glycidol or solketal) with novel mediators. New analytical methods will be developed for the determination of the alcohols and amines. Furthermore is planned: i) synthesis of PQQ-analogues and derivatives and recombinations with apoenzymes; ii) interaction studies of the holoenzymes with the mediators developed; iii) coupling of the modified PQQ's to an electrode and reconstitution with apoenzymes. Interactions between mediators and

isolated enzymes will be studied by spectroscopic techniques (mainly NMR, EPR and Moessbauer spectroscopy) by the group at CTQB in portugal. This group with its experience in spectroscopic techniques (mainly NMR and EPR) will collect data for the elucidation of structural properties of the active center and for structure-function relationships of these new enzymes. This knowledge is a prerequisite for a rational cofactor modelling and optimisation procedures. The group will start with enoate reductase and hydroxycarboxylate viologen oxidoreductase, two new enzymes which were purified by the group in Munich, and alcohol- and aldehyde dehydrogenase purified by the group in Delft. The partners from TNO and ATO will use the enzymes to test their electrochemical behaviour and long term stability, with emphasis on modelling for ATO. At first catalytically active enzyme/ mediator and/or coenzyme combinations delivered by the two universities will be tested on electroactivity by cyclic voltammetry (homogeneous bioelectrochemistry).

Keywords:
REDOX ENZYMES, MEDIATORS, PQQ, ELECTRODES, CHIRAL, SYNTHONS, LARGE SCALE PRODUCTION

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ENANTIOSELECTIVE BIOTECHNOLOGICAL RESOLUTION OF RACEMIC EPOXIDES IN THE PRODUCTION OF OPTICALLY PURE EPOXIDES

CONTRACTNUMBER: BIOT CT-910269
START: 1991-06-01 *DURATION*: 36 months
EC CONTRIBUTION: 540,000 ECU

Objectives:

The objective of the project is to gain an understanding of the fundamentals of enantioselective epoxide degradation by microorganisms.

Brief Description:

Recently, a participant of the current project has discovered a stereoselective enzyme in a *Xanthobacter* species. Such novel enzymes resolve cheap racemic epoxide mixtures to very valuable optically pure epoxides to be used in subsequent industrial chemical syntheses of optically pure bio-active compounds.

The three collaborating university participants will study enantioselective epoxide degradation at three distinct levels:

- The Division of Industrial Microbiology, Agricultural University Wageningen, at the whole cell level, will study growth optimization and optimization of enzyme expression in *Xanthobacter*. This participant furthermore will isolate new bacteria with enantioselective epoxide-degrading activities.
- The Centre for Biotechnology, Imperial college, London, at the enzyme level, will study the mechanism and enzymology of epoxide breakdown.
- The Instituto Superior Tecnico, Lisbon, at the level of immobilized cells, will study process productivity and optimization, mainly in organic solvents.

The industrial participant Andeno B.V. supplies model racemic epoxides.

Keywords:
EPOXIDES, ENANTIOSELECTIVE DEGRADATION

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GLYCOSYLTRANSFERASES FROM *STREPTOMYCETES* AS TOOLS IN BIOTRANSFORMATIONS

CONTRACTNUMBER: BIOT CT-900155
START: 1991-03-01 *DURATION*: 36 months
EC CONTRIBUTION: 648,000 ECU

Objectives:

The use of Streptomycete glycosyltransferases (GT) for biotransformations on natural and synthetic aglycones should be explored. The goal is the development of compounds with new biological activities.

Brief Description:

It is planed to isolate and characterize useful GT's and their genes from various Streptomycetes with known glycosylation mechanisms for varying aglycones. Especially GT's for sugar components of antibiotics such as aminoglycosides, macrolides, polyenes, anthracyclines and nucleosides will be searched and cloned into suitable expression hosts. Preparation and testsystems for modified sugars, their activation and transfer will be developed. Natural and synthetic aglycones and planed to be prepared and glycosylated in the last phase of this project.

Keywords:
GLYCOSYLTRANSFERASES, STREPTOMYCETES, ANTIBIOTICS, AGLYCONES, BIOTRANSFORMATION

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AREA: B

ENABLING TECHNOLOGIES

Genome sequencing

ALTERNATIVE METHODS OF DNA SEQUENCING

CONTRACTNUMBER: BIOT CT-900252
START: 1991-02-01 *DURATION*: 36 months
EC CONTRIBUTION: 509,000 ECU

Objectives:

The proposed project aims are two-fold:

1) the development of alternative DNA labelling methodologies that will reduce the complexity and cost of current conventional DNA sequencing systems;

2) the parallel development of a new non-gel principle by which fluorescently labelled DNA (generated by PCR techniques) can be detected allowing the direct sequencing and mapping of DNA fragments in solution.

Brief Description:

The project will run on three fronts.

The Biotechnology Institute (Copenhagen) and sub-contractors will investigate methods of DNA labelling using non-laser excited DNA base-specific photochemical cleavage of DNA will be used as the basis of alternative methods to classical DNA sequencing.

In parallel, a new method for the **direct** sequencing of solution-phase DNA fragments will be developed by **CAMR**. The technique will utilise recent advances made in the production of optical apertures of similar size to DNA fragments. Thereby, the optical detection of individual, solution-phase DNA molecule fragments generated and labelled by the novel chemistries developed at GEG and using PCR methods, will permit non-gel mediated sequencing of DNA.

This work will rely on the second phase (year two) development of comprehensive data analysis systems (developed by the software house,

Optimum Ltd., Greece) employing sophisticated detection (opto)electronics and computer software packages capable of the rapid assimilation of real-time generated from solution phase sequencing.

Keywords:
SOLUTION PHASE DNA SEQUENCING, DNA LABELLING, FLUOROPHORES, CAPILLARY ELECTROPHORESIS, SIGNAL PROCESSING

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AREA: C

CELLULAR BIOLOGY

Physiology and molecular genetics of industrial microorganisms

INTEGRATION OF PRIMARY METABOLISM, SECONDARY METABOLISM AND DIFFERENTIATION IN *STREPTOMYCES COELICOLOR*: A BIOCHEMICAL, PHYSIOLOGICAL AND GENETICAL APPROACH

CONTRACTNUMBER: BIOT CT-910255
START: 1991-04-01 *DURATION*: 36 months
EC CONTRIBUTION: 975,000 ECU

Objectives:

The objective is to analyse the switch between primary metabolism during vegetative growth, and secondary metabolism and differentiation, in a model antibiotic-producing actinomycete, *Streptomyces coelicolor* A3(2). Understanding of this switch will have important biotechnological implications for the rational development of more efficient antibiotic-producing cultures.

Brief Description:

During vegetative growth of an actinomycete, glucose is taken up by the cells and used primarily for energy production and the synthesis of cellular components. At a certain stage in the life cycle, unknown nutritional triggers cause a switch to the use of glucose for the production of antibiotics, storage carbohydrates, and the reproductive phase of the colony. An integrated view of the metabolic controls responsible for this switch is sought in a single genetically amenable streptomycete. The routes of glucose uptake and metabolism, the control of flux through these routes, and the mechanism of catabolite repression (possibly involving glucose kinase) will be studied by physiology, biochemistry, and molecular genetics. Interactions between central metabolic regulation (catabolite repression and the stringent response) and two kinds of secondary metabolism (the antibiotic actinorhodin, and storage compound synthesis) will be analysed by: two-dimensional protein gel electrophoresis; analysing carbon flux during secondary metabolism; and studying glycogen and trehalose metabolism during differentiation and antibiotic production. The regulatory pathway for actinorhodin synthesis will be mapped by analysing the effects of the *act*II pathway-specific regulatory gene and by seeking the targets of the pleiotropic regulatory gene *bldA*. The

regulation and roles of glycogen and trehalose will be studied by cloning the relevant genes. Genes for ribosomal and other proteins shown by two-dimensional gel analysis to have interesting regulation during growth and differentiation will be cloned and their expression studied.

Keywords:
ANTIBIOTICS, CATABOLITE REPRESSION, FLUX CONTROL, DIFFERENTIATION, GLUCOSE METABOLISM, GLYCOGEN, QUEST, RIBOSOME, *STREPTOMYCES*, STRINGENT RESPONSE

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THE PRODUCTION AND RECOVERY OF BIOTECHNOLOGICALLY IMPORTANT PROTEINS FROM THE YEAST *SACCHAROMYCES CEREVISIAE*

CONTRACTNUMBER: BIOT CT-900165
START: 1991-01-01 *DURATION*: 36 months
EC CONTRIBUTION: 659,000 ECU

Objectives:

This project is aimed at investigating a number of key aspects of the production and recovery of foreign proteins from *S. cerevisiae*, where gaps in basic knowledge are limiting the effectiveness of the yeast as a production host for biotechnology.

Brief Description:

Protein production is governed in the first instance by transcription of the encoding gene. The regulation of that transcription is therefore important. Of particular interest to this project is the growth-phase-specific regulation of some genes, as the elements that regulate their transcription can be used to regulate the expression of recombinant cloned genes to a particular pattern. In this way, gene expression could be targeted to the most productive phase, or phases, of host cell growth.

One of the important features of yeast as a biotechnology host is that it can secrete certain proteins, although only a small number are actually secreted. Thus, if a 'foreign' protein were targeted to be secreted from yeast, it could be recovered from the culture medium in the presence of relatively few other proteins, giving an advantage of immediate enrichment as a step towards purification. However, it has become clear that there are 'bottlenecks 'in the secretion pathway. Consequently, another aspect of this project is to isolate mutants that overcome these bottlenecks. These will improve secretion yield and genetically define important components of the secretion pathway.

Unfortunately not all proteins are capable of being secreted, reflecting features of their biochemical composition and the requirement in secretion

for proteins to pass through cell membranes. There are many such proteins of biotechnological interest which may therefore only be recovered by breaking open the yeast cells. However, yeast cells are covered with a strong, rigid, cell wall and, consequently, are very difficult to break open. Because of this, one further component of the research programme is a study of aspects of cell wall composition, and, in particular, the development of a system that eases cell breakage. A prototype for such a system under investigation is based upon a temperature-sensitive autolysis mutant of yeast. The cells grow normally at low temperature; however, if the temperature is raised, the cell wall weakens and the cells lyse.

Biotechnological applications can be seen in the production of pharmaceutical products, veterinary or animal husbandry products, and improvements in traditional fermentative processes, such as brewing or spirit production.

Keywords:
YEAST, BIOTECHNOLOGY, GENE EXPRESSION, SECRETION, CELL WALL

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WIDE DOMAIN CONTROL OF PRIMARY AND SECONDARY METABOLISM IN ASPERGILLI

CONTRACTNUMBER: BIOT CT-900169
START: 1991-02-01 *DURATION*: 36 months
EC CONTRIBUTION: 696,000 ECU

Objectives:

To elucidate the mechanisms of carbon catabolite repression and pH regulation in controlling expression of genes involved in both primary and secondary metabolism in two *Aspergillus* species for both scientific and practical benefit.

Brief Description:

Two wide domain regulatory systems, carbon catabolite repression and pH regulation, will be investigated in the filamentous fungus *Aspergillus nidulans* and the results extended to the more industrially important species *Aspergillus niger* and from primary to secondary metabolism. A powerful combination of physiological and non-invasive spectroscopic studies and classical and molecular genetics will be used to investigate regulatory genes mediating each of these important forms of regulation and the receptor sites for their products. The interaction of carbon catabolite repression with pathway-specific induction mechanisms, a frequent obstacle to using inexpensive substrates for industrial fermentation, will also be investigated. The choice of these two wide domain regulatory systems is based on their involvement in regulation of industrially important products such as pharmaceuticals, fine chemicals, primary metabolites and heterologous and homologous proteins. *A. nidulans* is an ideal organism for an in depth investigation of molecular interactions underlying carbon catabolite repression and pH regulation and accompanying pathway-specific regulation. These fundamental studies pave the way for exploitation of corresponding regulatory systems in the most industrially important fungi such as *A. niger* and *Penicillium chrysogenum* where their molecular and physiological analysis will greatly contribute to strain improvement.

Keywords:
ASPERGILLI, CARBON CATABOLITE REPRESSION, PH REGULATION, rDNA, PRIMARY METABOLISM, SECONDARY METABOLISM

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STABILITY OF GENETIC INFORMATION IN BACILLUS

CONTRACTNUMBER: BIOT CT-910268
START: 1991-04-01 *DURATION*: 36 months
EC CONTRIBUTION: 1,005,000 ECU

Objectives:

Characterisation of processes which underlie segregational and structural genetic instability in *Bacillus subtilis* and decreasing the instability to industrially acceptable levels.

Brief Description:

The project consists of five main tasks:

(A) Characterisation of DNA replication. Three representative replication systems will be studied: (i) small single-stranded DNA plasmids which replicate as rolling circles; (ii) large plasmids which replicate as Θ-forms; (iii) bacteriophage Φ which replicates as a linear molecule.

(B) Characterisation of the role of topoisomerases. Roles of topoisomerase I and DNA gyrase in structural and segregational stability will be determined.

(C) Characterisation of the partition systems. Active partition systems will be sought, identified and used to stabilise heterologous replicons.

(D) Construction of post-segregational killing systems. These will be based on the repressor and lysis functions of the phage PBSX. Lysis gene will be integrated in the chromosome and the repressor gene will be carried on plasmids, such that the loss of plasmids provokes lysis of the host cell.

(E) Characterisation of the stability of genes inserted in the chromosome. The effects of neighbouring structures and functions as well as the chromosomal location will be determined.

Segregationally and structurally stable cloning systems should result from these studies.

Keywords:
DNA REPLICATION, RECOMBINATION, TOPOISOMERASES, PLASMIDS, SEGREGATION

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PHYSIOLOGY AND MOLECULAR GENETICS OF AMINO ACID PRODUCTION AND SECRETION BY CORYNEBACTERIA: FLUX OF INTERMEDIATES AND FEEDBACK CONTROL MECHANISMS

CONTRACTNUMBER: BIOT CT-910264
START: 1991-07-01 *DURATION*: 36 months
EC CONTRIBUTION: 882.200 ECU

Objectives:

The main objective of this collaborative research proposal is to develop the molecular genetics of corynebacteria in order to understand the physiology of this group of microorganisms which are used industrially for the production of amino acids, flavour enhancing agents and in bioconversions of steroids, vitamins and a variety of other compounds.

Brief Description:

Corynebacteria are widely used in the production of amino acids and flavour enhancing agents, in bioconversions of steroids and intermediates in vitamin C biosynthesis and in the cheese industry. Some corynebacteria are also important plant pathogens.

The aim of this collaborative research between 5 groups already established in the field of amino acid production by corynebacteria is to further develop the molecular genetics of corynebacteria in order to understand the physiology of this group of microorganisms which are used industrially for production of amino acids in Europe, in Japan and the USA.

The main objectives of the collaborative research project are:

1. Development of phage-derived genetic tools for corynebacteria, particularly construction of cosmid vectors, transduction systems and characterization of the integration sequences of temperate bacteriophages.
2. Characterization of DNA sequences required for plasmid replication and plasmid stability in liquid cultures (fermenters) and construction of integrative vectors.

3. Development of genetic methods for the analysis and manipulation of corynebacteria genes relevant for amino acid production (gene disruption and gene replacement, transposon mutagenesis, construction of physical maps and cosmid libraries).
4. Regulation of the TCA cycle enzymes in order to increase the availability of the glycolysis and TCA cycle-derived precursors for amino acid biosynthesis.
5. Characterization of promoters of the amino acid biosynthetic genes and feedback control mechanisms of the amino acid biosynthetic pathways in order to remove bottlenecks in the pathways for overproduction of amino acids.

The project is an integrated approach (genetics and physiology) to study amino acid biosynthesis in corynebacteria. The results of this research will also be useful in other applications of corynebacteria, e.g. in biotransformations and in the interaction of pathogenic corynebacteria with plants.

Keywords:
AMINO ACID PRODUCTION, CORYNEBACTERIA, DNA SEQUENCES, VECTOR STABILITY, PRECURSORS FOR AMINO ACID BIOSYNTHESIS, PROMOTERS

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VALORISATION OF NON-CONVENTIONAL YEASTS OF INDUSTRIAL INTEREST: EXPLORATION AND MOLECULAR ENGINEERING OF THEIR GENETIC CONSTITUENTS

CONTRACTNUMBER: BIOT CT-910267
START: 1991-04-01 *DURATION*: 36 months
EC CONTRIBUTION: 902,000 ECU

Objectives:

General objectives are:

(i) to bring non-conventional yeast species to be genetically manipulatable organisms,

(ii) to develop expertise for the control of their physiology and

(iii) to create new combinations of genetic elements suitable for the production of medical and agricultural interest.

Brief Description:

Specific objectives are (i) to develop genetics and physiology of selected yeast species of industrial interest (in particular, *Kluyveromyces lactis*, *Hansenula polymorpha* and *Yarrowia lipolytica*), (ii) to identify the molecular basis of their specific physiological properties, and (iii) to engineer genetic elements into industrially exploitable forms.

High level production of proteins, especially of heterologous origins, requires the techniques of molecular engineering of genes. Amongst yeasts, *Saccharomyces cerevisiae* has been practically the only choice in which gene manipulation systems were available. Over the last several years the possibility of using the species other than *S. cerevisiae* has gained an increasing interest in the industrial sector as a tool for the secreted production of proteins. The species mentioned above have been known to secrete or transport specific proteins. The project is a converged effort to develop physiological and molecular studies of these species, by various approaches, for a better control of their biology.

Methods used by the participants include:

(i) classical chromosomal and cytoplasmic genetics of yeast,

(ii) molecular cloning of specific genetic elements,

(iii) molecular characterization of these elements,

(iv) modification of these elements by *in vitro* DNA engineering,

(v) introduction of these elements into yeast cells by means of various genetic transformation techniques,
(vi)determination of cellular activities of the transformed yeasts at laboratory level and in pilot fermentors.

Keywords:
YEAST, *KLUYVEROMYCES*, *HANSENULA*, *YARROWIA*, GENE ENGINEERING, PROTEIN SECRETION, FERMENTATION

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AREA: C

CELLULAR BIOLOGY

Basic biotechnology of plants and associated microorganisms

MOLECULAR AND GENETIC ANALYSIS OF GENES CONTROLLING FLOWER DEVELOPMENT

CONTRACTNUMBER: BIOT CT-900171
START: 1991-01-01 *DURATION*: 36 months
EC CONTRIBUTION: 743,400 ECU

Objectives:

To isolate and study key genes controlling flower development by establishing a range of molecular and genetic tools in the model species, *Antirrhinum*. The aims include: isolation of new transposons by trapping them in genes, construction of a combined RFLP and genetic map, genetic characterisation of floral mutants and development of a transformation system. The possibility of using genes from *Antirrhinum* to study their counterparts in other species, such as pea, will also be investigated.

Brief Description:

The mechanisms which control the production and development of flowers have been a major concern for plant research for many years. However, an important gap remains in our basic understanding of how floral development is genetically controlled because of major obstacles to the molecular isolation and analysis of the genes involved. In a recent breakthrough, several of the key floral genes have been isolated by exploiting a model species, *Antirrhinum*. This plant can grow throughout Europe, has a good genetic map, well-characterised transposons, large flowers and an extensive range of well documented mutations affecting floral development. The aim of this proposal is to develop further tools in *Antirrhinum* which will facilitate the isolation and study of key genes and so provide a molecular foundation to our general understanding of floral development. In addition, the possibility of using genes from *Antirrhinum* to study their counterparts in other species, such as pea, will be investigated.

One feature of the *Antirrhinum* system which has been vital for gene isolation is the availability of cloned transposons. If a gene mutation is caused by insertion of a well-characterised transposon, the affected gene can ususally be isolated. So far seven different transposons have been isolated from

Antirrhinum and one aim is to clone further transposons by trapping them in previously defined genes. This will provide a "library" of transposons, available for general use. A further resource will be the construction of a restriction fragment length polymorphism (RFLP) map. This has already been initiated and, as other groups isolate various clones, a coordinated map will be established. A transformation/regeneration system in *Antirrhinum* will also be developed to enable genes isolated to be introduced into mutants and wild-types to study their mode of action. In addition, genetic crosses will be carried out to establish complementation groups and to construct double mutants.

Keywords:
MORPHOGENESIS, HOMEOTIC GENE, FLOWERING, TRANSPOSON, *ANTIRRHINUM*, PEA, TRANSFORMATION, RFLP, GENE ISOLATION

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THE MOLECULAR BASIS OF CELL-CELL INTERACTIONS IN SELF-INCOMPATIBILITY

CONTRACTNUMBER: BIOT CT-900172
START: 1990-12-01 *DURATION*: 36 months
EC CONTRIBUTION: 714,600 ECU

Objectives:

To elucidate the molecular basis of gametophytic self-incompatibility in crop plants

Brief Description:

The mechanism of action of the *S* (self-incompatibility) locus is being studied in diploid potato species. The major pistil *S*-locus component has been cloned, and antibodies generated from *E. coli* expressed polypeptides. The cloned genes are being used to investigate at the biochemical and ultrastructural levels the pollen-pistil interaction which takes place in an incompatible reaction. The chromosomal region surrounding the pistil *S*-locus sequence is being characterised for the presence of other transcripts which may play a role in self-incompatibility, for example, those expressed in the male gametophyte. Tobacco and tomato plants have been transformed with *S*-gene constructs in order to identify sequence elements responsible for pollen and pistil-specific expression. The application of these results to SI-based breeding systems in tomato and rye will be pursued.

Keywords:
PLANT BREEDING, MOLECULAR RECEPTOR GENE, INCOMPATIBILITY

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GAMETE DIFFERENTIATION AND FERTILIZATION (CONCERTED ACTION)

CONTRACTNUMBER: BIOT CT-900180
START: 1990-11-01 *DURATION*: 36 months
EC CONTRIBUTION: 30,000 ECU

Objectives:

Elucidation of the fertilization process of *Petunia hybrida*. Analysis of the genetical, molecular and cell biological aspects of gamete transfer, recognition and fusion.

Brief Description:

mRNAs are collected from isolated embryo sacs and sperm cells of *Petunia hybrida*, in order to construct representative cDNA libraries. From these libraries gamete-specific genes will be selected, the expression of which in developing male and female gametophytes will be analyzed by in-situ hybridization. Proteins from isolated embryo sacs and sperm cells are used to produce monoclonal antibodies. Gamete-specific monoclonals will be selected by immuno-labelling, and used for the immuno-localization of gamete-specific protein synthesis. Plasma membrane specific antibodies will be used in *in vitro* fertilization experiments with isolated egg and sperm cells, to identify membrane-bound recognition and fusion proteins. Sperm and egg cell development will be analyzed ultrastructurally to elucidate the cytological aspects of gamete differentiation. This will provide the cytological basis for the analysis of specific gene expression by in situ-hybridisation and the analysis of specific protein synthesis by immuno-labelling. Fertilization-defective mutants of *Petunia hybrida* will be searched for, to be used as experimental tools to establish possible functions of selected genes and gene products in gamete transfer, recognition and fusion.

Keywords:
PETUNIA HYBRIDA, FERTILIZATION, GAMETE, DIFFERENTIATION, TRANSFER, RECOGNITION, FUSION

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MOLECULAR GENETICS AND PHYSIOLOGY OF SELF INCOMPATIBILITY IN BRASSICA CROPS

CONTRACTNUMBER: BIOT CT-900174
START: 1991-02-01 *DURATION*: 36 months
EC CONTRIBUTION: 1,102,900 ECU

Objectives:

Characterisation of the molecular and physiological events comprising the self-incompatibility mechanism operating in the breeding system of *Brassica oleracea*.

Brief Description:

The mechanism by which *Brassica* plants recognise and reject their own pollen is of scientific and commercial importance. The self-incompatibility (SI) response occurs on the dry surface of the female stigma and, as such, has proved difficult to study using conventional molecular technology.

Using a multidisciplinary approach, aspects of the structure, physiology and molecular biology of SI are under investigation in 10 institutional and commercial laboratories throughout Europe.Individual 'Target Themes' are addressed by smaller groups of participants, each coordinated by a 'managing Contractor'. Structure and physiology are being studied in Oxford (UK), Lyon (F), Danisco (DK) while a group at Nijmegen (NL) are examining the system *in vitro*. The molecular studies are concentrated in Norwich (UK), Birmingham (UK), Lyon (F) and Durham (UK), with HRI (UK) focusing on the generation of S-allele probes. The industrial participants, Nickerson-Zwaan (NL), Zaadunie (NL) and Royal Sluis (NL) collaborate actively in the programme, provide a valuable commercial perspective, and supply essential technical support, especially in the area of field trials.

The project is coordinated from Oxford, and is integrated, as far as is possible, with a parallel programme on SI in *Solanum* coordinated from Cologne. A joint meeting is held yearly, but individual sub-groups meet more frequently. A regular newsletter SINEWS provides a useful channel of communication between participants - in addition to fax and E-mail.

Keywords:
BRASSICA OLERACEA, PLANT REPRODUCTION, POLLEN / STIGMA INTERACTION, SELF-INCOMPATIBILITY

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MOLECULAR CONTROL OF GENETIC INSTABILITY IN REGENERATION OF CROP PLANTS

CONTRACTNUMBER: BIOT CT-900154
START: 1991-01-01 *DURATION*: 36 months
EC CONTRIBUTION: 30,000 ECU

Objectives:

The objective of this concerted action was to co-ordinate research on molecular mechanisms of genetic instability in plant tissue culture and regeneration and to concentrate European efforts into an integrated approach which combines the expertise, materials and facilities from the different participating groups.

Brief Description:

Uncontrolled instability can arise through the culture of plant cells and their regeneration into whole plants. Although a well described phenomenon, it's causes are not yet known. Factors that affect the frequency of changes include *in vitro* culture conditions, tissue source, ploidy and genotype indicating that whilst variation is influenced by culture conditions, it originates both from the differentiated donor tissue and from the that different genomes respond to the stress of culture. Some genomes are more unstable irrespective of ploidy, suggesting that genome composition is important. Evidence indicates that repetitive sequences have an important role in changes induced in both coding and non-coding regions of the genome.

This proposal is a concerted approach to determine the molecular basis of genetic instability in plant regeneration through inter-relating studies on (1) the stability of endogenous and transformed genes (2) the behaviour of sequences in euchromatin vs heterochromatin (3) amplification of coding and non-coding sequences (4) changes in mitochondrial DNA that occur during somatic embryogenesis and (5) aberrations in cell division. Instability will be studied in relation to states of differentiation, somatic embryogenesis compared with organogenesis, *in vitro* culture conditions and changes in selective constraints imposed during cell division and morphogenesis. An understanding of how the genome responds in relation with *in vitro* culture

conditions will provide the vital logic necessary for control over instability in plant regeneration. Fundamental knowledge on how plant genomes change and of the constraints imposed during differentiation and morphogenesis will also be gained.

Keywords:
GENETIC INSTABILITY, REGENERATION, *IN VITRO* CULTURE, MOLECULAR MECHANISMS

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THE MOLECULAR BIOLOGY OF THE CELL-TO-CELL MOVEMENT OF PLANT VIRUSES IN RELATION TO PLASMODESMATAL FUNCTION

CONTRACTNUMBER: BIOT CT-900156
START: 1991-01-01 *DURATION*: 36 months
EC CONTRIBUTION: 819,000 ECU

Objectives:

The main objectives are: (a) To determine how many apparently different mechanisms of cell-to-cell movement of viruses there are; (b) To study the basic interactions between the virus, the movement protein and plasmodesmata; (c) To use viral movement proteins to examine the physical structure of plasmodesmata and how plasmodesmata function; (d) To analyse the infection units of viruses which move through plasmodesmata; (e) To determine if the interactions between movement proteins and plasmodesmata can be interfered with and thus confer resistance.

Brief Description:

This project brings together five laboratories to study how plant viruses move from cell to cell through plasmodesmata and to use this information to gain an insight as to how plasmodesmata function. Many plant viruses encode proteins which facilitate their cell-to-cell movement by gating plasmodesmata to enable the infection unit to pass through. From comparisons of the amino acid sequences of movement proteins and their ultrastructural properties it is possible that there are several mechanisms by which they operate. The movement proteins of several viruses are being compared on features such as tissue localization, time of expression in the plant and properties of the *in vitro* expressed protein to look for commonalities and differences. From this approach and from studies on the properties of the proteins an understanding will be gained of how movement proteins function. Plasmodesmata are one of the least studied of plant organelles because of difficulties in accessing them. As one of the functions of viral movement proteins is the interaction with plasmodesmata these proteins are considered as useful probes for the study of the structure and function of these organelles. There are two main questions being addressed in this part of the project. What are

the interactions between movement proteins and plasmodesmata and what is the molecular structure and function of plasmodesmata? The answers to these questions should throw light on how the virus gene product gates the plasmodesmata to enable the movement of the infection unit, which it is proposed to characterize, between cells. From this information on function of viral movement proteins constructs, which express mutants of the protein which interfere with their function, will be introduced into plant genomes to determine if this approach can be used to confer virus resistance. This latter aspect will focus on three economically important viruses, beet necrotic yellow vein virus, cucumber mosaic virus and grapevine fanleaf virus.

Keywords:
VIRUS MOVEMENT PROTEIN FUNCTION, PLASMODESMATAL STRUCTURE AND FUNCTION, NON-CONVENTIONAL RESISTANCE

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GENES REQUIRED FOR PATHOGENICITY OF BACTERIA TO PLANTS AND APPLICATION OF KNOWLEDGE IN BIOLOGICAL CONTROL OF DISEASES OF CROPS

CONTRACTNUMBER: BIOT CT-900168
START: 1991-02-01 *DURATION*: 36 months
EC CONTRIBUTION: 824,000 ECU

Objectives:

Three classes of gene which are important in determining pathogenicity and host specificity of bacteria for plants are being studied. Molecular profiles of diseases caused by related *Pseudomonas* and *Xanthomonas* species will be constructed within which possibilities for novel crop protection strategies and improved diagnostic procedures can be devised.

Brief Description:

Three classes of gene essential for pathogenicity of the bacteria *Pseudomonas* and *Xanthomonas* to plants are being studied. Avr genes determine the inability to cause disease in plant hosts carrying certain resistance genes, rpf genes control coordinately production of pathogenicity determinants, probably in response to factors in the plant, and hrp genes are needed both for pathogenicity and for ability to incite plant resistance responses. Aspects of genomic organisation, sequence, function, and expression of the genes are being investigated, particularly the interdependence of expression of the several classes, together with the control of hrp and rpf expression and function by substances from the plant environment. X-ray crystal structure of a regulatory Hrp protein is being determined. Pre-inoculation with certain hrp mutants (which are non-virulent) can protect plants against subsequent infection by virulent strains, and the potential of this for biological disease control in a situation where little useful genetic resistance is available is being studied. The collaborative project is based on the already demonstrated similarities of some pathogenicity mechanisms and genes in bacteria causing diseases of different classes, including some of the economically most serious crop diseases. Comparative studies will demonstrate the extent of functional equivalence and perhaps verify the con-

cept that a consensus set of related essential pathogenicity genes is found in many pathogens irrespective of their host plant and infection strategy. This information is essential for the development of advanced disease control strategies based upon interference with the normal development of the host-pathogen complex.

Keywords:
BACTERIA, PLANT PATHOGEN, *PSEUDOMONAS*, *XANTHOMONAS*, GENES, DISEASE CONTROL

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MOLECULAR BASIS OF SIGNALLING IN *RHIZOBIUM MELILOTI-MEDICAGO* INTERACTIONS AND GENETIC IMPROVEMENT OF NODULATION ABILITY

CONTRACTNUMBER: BIOT CT-900159
START: 1990-12-01 *DURATION*: 36 months
EC CONTRIBUTION: 624,000 ECU

Objectives:

The aim of this project is to elucidate how the signal molecules determined by the *Rhizobium* nodulation (*nod*, *nol* and*nfe*) genes evoke responses in the plant host leading to root nodule development. Based on the these molecular genetic studies, methods for improving the efficiency and competitiveness of *Rhizobium* strains to be used as field inoculants will be developed.

Brief Description:

The induction of root nodules on leguminous plants by rhizobia is a highly complex process, requiring multiple interactions between the two partners. Numerous bacterial (*nod* genes) and plant genes (nodulin genes) are specifically expressed during the development of symbiosis. The two partners interact also at the level of gene expression by transmitting signal molecules to activate genes in the other partner. The initial step of nodulation is triggered by flavonoids exuded by the roots of the plant host which activate the expression of the *nod* genes. The *nod* gene products participate in the production of a family of return signals (lipo-oligosaccharides) which induce root hair curling and cortical cell division leading to nodule development.

The structure and regulation of *R. meliloti nod* genes as well as the involvement of the various *nod* gene products in the production of the lipo-oligosaccharide *Nod* factor family will be studied. The chemical structures of the *Nod* factors will be determined and the question how the specific signal molecules are recognized by the plant host will be addressed. Plant genes activated by these signal molecules will be identified and their regulation will be studied by constructing fusions with reporter genes and transforming into

the host plant *Medicago*. Then, the effect of purified *Nod* signal molecules on the expression of these gene fusions will be investigated in these transgenic plants. In order to test whether this new class of bacterial factors could also play a role in non-legumes, the *nod* genes involved in lipooligosaccharide synthesis will be introduced and expressed in tobacco. The plants will be analyzed for morphological abnormalities and alterations in the phytohormone balance. The expression of genes of the phenylpropanoid pathway determining flavonoid biosynthesis and plant defense against pathogenes will be studied which could lead to the elucidation of their possible involvement in nodule initiation. For future genetic studies of *Medicago* symbiotic genes, RFLP mapping of diploid *Medicago* will be carried out in the framework of an international collaboration, involving also laboratories not participating in this project.

Studies on the *Rhizobium* genes controlling nodulation has led to the identification of genes influencing nodule formation efficiency and competitiveness. These genes (*nfe* genes) will be characterized and their involvement in the expression of competitiveness will be studied, with the final aim of improving nodulation competitiveness and efficiency of *Rhizobium* strains for future agricultural use.

Keywords:
SYMBIOTIC NITROGEN FIXATION, *NOD* GENES, EARLY NODULIN GENES, NODULATION COMPETITIVENESS

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THE MOLECULAR BASIS OF PATHOGENICITY, AVIRULENCE AND RESISTANCE IN THE INTERACTION BETWEEN THE FUNGAL PATHOGEN CLADOSPORIUM FULVUM AND TOMATO

CONTRACTNUMBER: BIOT CT-900163
START: 1991-04-01 *DURATION*: 36 months
EC CONTRIBUTION: 967,000 ECU

Objectives:

Genes conferring pathogenicity and avirulence in the fungal pathogen *Cladosporium fulvum*, the causal agent of tomato leaf mould, will be cloned. The function and regulation of those genes will be studied. The final objective is to clone the resistance genes in the tomato plant complementary to the fungal avirulence genes. Both resistance genes and avirulence genes can be exploited to obtain broad spectrum resistance to plant pathogens.

Brief Description:

Adopting an integrated approach involving biochemical genetic and molecular techniques the structure, function and regulation of genes and gene products controlling the pathogenicity and race-specificity of *C. fulvum* and the resistance of tomato (*Lycopersicon esculentum*) will be investigated. Two approaches will be employed.

Firstly, the proteins isolated from apoplastic fluid of infected tomato leaves will be characterized. Their genes (whether fungal or plant) will be cloned and their functions determined by transformation of tomato or *C. fulvum* as appropriate. The model for this approach is the successful purification of the *avr9* elicitor peptide and the cloning of its encoding avirulence gene. The role of apoplastic plant proteins such as 1,3-beta-glucanases, chitinases and proteinaceous inhibitors of endopolygalacturonases as well as oligosaccharides released by some of these enzymes will be studied. Also the structure and function of these oligosaccharides in inducing and enhancing defence responses will be objects of study.

The second approach is an unbiased search for fungal gene products controlling pathogenicity and avirulence. This will involve characterization of

nonpathogenic mutants created by integrative transformation and the use of promoter probe vectors based on the beta-glucuronidase reporter gene.

The regulation and function of the isolated fungal genes will be studied. To this end, wild type genes will be replaced by mutated forms of the genes by homologous recombination and double cross-over. A receptor for the *avr9* peptide in tomato, the putative product of resistance gene Cf9 will be isolated by binding studies, or characterized by functional cloning.

Keywords:
PATHOGENICITY GENES, AVIRULENCE GENES, ELICITORS, DISEASE RESISTANCE GENES, GENE DISRUPTION, PR-PROTEINS, FUNGAL GENETICS

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GENETIC AND MOLECULAR APPROACHES OF THE PHYSIOLOGY OF BACTEROIDS IN RELATION TO THE PLANT NODULE METABOLISM

CONTRACTNUMBER: BIOT CT-900166
START: 1990-12-01 *DURATION*: 36 months
EC CONTRIBUTION: 857,925 ECU

Objectives:

To get a better understanding of the relationships between oxygen, nitrogen and carbon metabolism in the nodule and the expression and activity of the nitrogen fixing apparatus of bacteroids and evaluate the possibilities to manipulate regulatory circuits and metabolic fluxes in order to increase the symbiotic nitrogen fixation potential.

Brief Description:

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 indicated 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 and 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 study 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.

Keywords:
SYMBIOTIC NITROGEN FIXATION, CARBON METABOLISM, NITROGEN METABOLISM, MICRO-AEROBIOSIS, *RHIZOBIUM MELILOTI*, *RHIZOBIUM LEGUMINOSARUM*, SIGNAL TRANSDUCTION

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TOMATO TRANSPOSON TAGGING: ISOLATION OF GENES INVOLVED IN DISEASE RESISTANCE, HORMONE BIOSYNTHESIS AND PLANT CELL DEVELOPMENT

CONTRACTNUMBER: BIOT CT-900192
START: 1991-01-01 *DURATION*: 38 months
EC CONTRIBUTION: 695,000 ECU

Objectives:

The joint project is to develop a tomato transposon tagging system in order to enable each group to clone different genes. To this end it is aimed to jointly obtain a series of plants each carrying a transposable element insertion (either Ac or a derivative) in different positions that are equally spaced over the twelve chromosomes of tomato and to demonstrate the feasibility of this series for cloning specific genes by targeted transposon tagging.

Brief Description:

Transposon insertion has permitted the isolation of genes from bacteria, Drosophila and plants. In tomato, there exists a wealth of genetic variation in genes affecting sexual reproduction, in disease resistance, in the perception or synthesis of plant hormones and genes which are thought to be involved in plant development. In each of the participating laboratories there is interest in one or more of these areas.
The project consists of two parts:

(1) developing an efficient transposon tagging procedure in tomato and

(2) tagging desired tomato genes.

The first part is largely additive and is an essential prerequisite to the second part. Each lab undertakes to map 50 T-DNA insertions (containing either Ac or Ds). Furthermore, Ac-derivatives will be constructed that provide transposase in trans but can not themselves transpose. This involves fusion of transposase to various constitutive and tissue specific promoters.

During the development of these transposon tagging procedures all four participating groups are sharing the work between them and are exchanging all information and materials. The second part of the project involves tagging of desired genes. These range from disease resistance genes (Cf, Norwich; Asc, Amsterdam) to genes involved in hormone biosynthesis (sitiens, notabilis and flacca, Nottingham) and to genes involved in plant development (Cologne). Clearly, such experiments will be initiated as soon as the stocks are generated in which they can be carried out.

Keywords:
TOMATO, *LYCOPERSICON ESCULENTUM*, TRANSPOSON TAGGING, PLANT-PATHOGEN INTERACTIONS, PLANT HORMONES, NEW PLANT GENES

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IDENTIFICATION AND FUNCTIONAL ANALYSIS OF GENES CONTROLLING MAJOR METABOLIC PATHWAYS IN HIGHER PLANTS

CONTRACTNUMBER: BIOT CT-900164
START: 1991-02-01 *DURATION*: 36 months
EC CONTRIBUTION: 1,151,000 ECU

Objectives:

Modification of the flow of metabolic pathways by the genetic engineering of regulatory genes coding for transcription factors controlling the expression of the enzymes of these pathways.

Brief Description:

The flow of metabolic pathways is regulated by the availability of metabolic precursors and enzymes involved in these pathways. In the perspective of increasing the flow of a pathway, modifying a catalytic step may reveal another bottleneck in this pathway. It is therefore desirable to find other ways to modify coordinately several steps of a pathway. The purpose of the project is to evaluate the possibility to do this by identifying regulatory genes coding for transcription factors, and by modifying the expression of these genes. Three types of pathways will be studied: the anthocyanin metabolic pathway, the nitrate assimilatory pathway and the carbon assimilatory pathway. In a first step genetic and molecular studies will be developed to study whether the different steps are under the control of a same regulatory circuit, and to which extent the regulation is specific to the pathway or over-imposed on different pathway. As an example light may regulate the three pathways under study by common or independent mechanisms. In a second step, transcription factors controlling the expression of different enzymes involved in these pathways will be cloned and characterized. In a third step the corresponding genes will be modified and reintroduced back in their host to evaluate a consequence of their modification on the functioning of the pathway. Ultimately these approaches will be used in collaboration with two companies AGC and SEITA to modify flower pigmentation or nitrate storage according to the needs of their utilization.

Keywords:
REGULATORY GENE, TRANSCRIPTION FACTOR, LIGHT, NITRATE, CARBON, ANTHOCYANIN, MAIZE, TOBACCO, PETUNIA, *ARABIDOPSIS*

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THE PLASMALEMMA AND THE TONOPLAST OF PLANT CELLS AS TARGETS TO INCREASE PLANT PRODUCTIVITY

CONTRACTNUMBER: BIOT CT-900175
START: 1991-01-01 *DURATION*: 36 months
EC CONTRIBUTION: 900,000 ECU

Objectives:

The program is aimed at the identification of several membrane proteins directly involved in the distribution of assimilates and ions in plants, and at the cloning of the corresponding genes. These proteins include the sucrose carrier, the amino acid carriers and the ion carriers of the plasmalemma, and the malate transporter of the tonoplast.

Brief Description:

The project involves a combination of various approaches: biochemistry, immunology, electrophysiology, molecular biology, mutagenesis. Biochemical work on the sucrose transporter and on the amino acid transporters is conducted on sugar beet leaves, while the genetic approach on the ion transporters is conducted on *Arabidopsis*. Cell cultures (*Catharanthus roseus*) or leaves of crassulacean plants (*Kalenchoe daigremontiana*) are used for the work on the malate transporter.

A putative sucrose transporter of the plasma membrane has been identified by differential labeling. Polyclonal antibodies directed against fractions enriched with this polypeptide inhibit selectively the uptake of sucrose into protoplasts or into plasma membrane vesicles. The sucrose transporter has been partially purified in a functional state by high performance liquid chromatography and reconstituted into proteoliposomes (Poitiers). These antibodies are being used to screen a cDNA library from sugar beet leaf in attempts to clone the corresponding cDNA. The purified fraction has been analyzed by two-dimensional gel elecrophoresis, and the relevant spots will be microsequenced in order to synthesize oligonucleotide probes (Berlin). A similar approach will be developed for the amino acid transporters. In addition, polymerase chain reaction using primers and bacteria from fungal

amino acid permease is used to amplify sequences coding for the amino acid carriers, starting from poly-A+ RNA barley root as a template. Expression of size-fractionated mRNA in Xenopus oocytes, associated with electrophysiological recordings, will be carried out to identify the mRNA corresponding to the amino acid transporters (Rothamsted).

For the ion transporters, the procedure used involves the isolation and characterization of transport mutants altered in specific transport processes or resistant to chemical reagents influencing transport (Milan + Fidenza).

The malate transporter is characterized by a combination of biochemical (photoaffinity labeling, Toulouse), electrophysiological and molecular approaches (patch-clamp, polymerase chain reaction, Oxford). Comparison of the properties of the malate transporter from vacuoles of C3 plants (*Catharanthus*) and from Crassulacean plants (*Kalenchoe*) suggests important differences in the functioning and in the structure of the transporters.

Keywords:
PLANT MEMBRANES, SUCROSE CARRIER, AMINO ACID CARRIER, ION CARRIER, MALATE TRANSPORTER

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AREA: C

CELLULAR BIOLOGY

Biotechnology of animal cells

CONSTRUCTION OF ARTIFICIAL CHROMOSOMES FOR HIGHER EUKARYOTIC CELLS

CONTRACTNUMBER: BIOT CT-910259
START: 1991-04-01 *DURATION*: 36 months
EC CONTRIBUTION: 1,080,000 ECU

Objectives:

The aim of our project is the construction of artificial chromosomes to be used for directed engineering of higher eukaryotic cells. This will be accomplished by assembling biologically relevant DNA sequences (replication origins, telomeric and centromeric sequences) from eukaryotic chromosomes and naturally occurring linear plasmids.

Brief Description:

The reason for creating artificial chromosomes in higher eukaryotic cells is that to date no satisfactorily autonomously replicating vectors exist for such cells and for higher eukaryotic organisms and it may well be that the ideal eukaryotic vector may have to mimic a chromosome. A vector of this type will allow directed genetic engineering of higher eukaryotes, will permit the isolation of other biologically important molecules, will be useful in the production of medically important molecules, will help to understand differentiation processes, and may prove to be a safe and reproducible method for the somatic therapy of some genetic diseases. The minimal requirements for an artificial chromosome are sequences to allow and regulate its autonomous replication, centromeric sequences to guarantee the mitotic and meiotic stability of the chromosome, and telomeric sequences needed for its replication as a linear DNA molecule. Sequences involved in the replication and amplification of the chromosome will be isolated from mouse and the naturally occurring linear plasmids of ciliates. They can be fuctionally tested in such different organisms as yeast, *Xenopus*, and mammalian cells. The functional role of telomeric and subtelomeric sequences will be studied in yeast and in mammalian cells. Particular attention will be given to sequences isolated from human DNA. The approach for isolating centromeric sequences will be based on the fact that some repeated satellite DNA sequences have been found to be located in the centromeric regions of eukaryotic

chromosomes. Appropriate expression cassettes will also be included in the constructs. All these sequences will be isolated from different sources, characterized structurally, tested in homologous and heterologous systems, and assembled into artificial chromosomes functioning in a variety of higher eukaryotic cells.

Keywords:
ARTIFICIAL CHROMOSOMES, DNA STRUCTURE, TELOMERES, CENTROMERES, REPLICATION ORIGINS, VECTORS

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IMPROVED TECHNIQUES FOR ESTABLISHING A HIGH EXPRESSION PRODUCTION SYSTEM FOR RECOMBINANT PROTEINS FROM ANIMAL CELLS

CONTRACTNUMBER: BIOT CT-900185
START: 1991-04-01 *DURATION*: 36 months
EC CONTRIBUTION: 720,000 ECU

Objectives:

Construction or selection of mammalian cell lines which allow by genetic engineering stable and high expression of recombinant proteins.

Brief Description:

To obtain cell lines allowing the stable expression of recombinant proteins highly active chromosomal loci in several cell types (CHO, BHK, myeloma) which promote the transcription of any integrated transgene will be defined. These sites will be targeted with the gene of interest in appropriate vectors by means of homologous recombination. Physiological and physical properties in high density floculated and immobilized culture systems will be tested in order to select cell clones which favour the intended expression.

Keywords:
MAMMALIAN CELLS, CELL PHYSIOLOGY, GENE TARGETING, RECOMBINANT PROTEINS

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STRUCTURAL AND FUNCTIONAL ANALYSIS OF REGULATORY GENES CONTROLLING LIVER SPECIFIC PROTEIN

CONTRACTNUMBER: BIOT CT-910260
START: 1991-12-01 *DURATION*: 36 months
EC CONTRIBUTION: 700,000 ECU

Objectives:

Investigation of the mechanisms involved in the control of liver specific gene expression and hepatocyte differentiation.

Brief Description:

Liver is the site of synthesis and secretion of a number of proteins of major pharmalogical importance. Several of these proteins require specific folding and several posttranslational modifications. The mechanisms which control the expression of several liver-specific genes (alpha l-AT, HP, RBP, ALB, apoAI, coagulatory factor IX) are the subject of intensive studies by us and by others. Some of the regulatory genes which control liver specific expression have been cloned (LF-BI, HNF1, VHNF1, NF-1) and others (LF-A1, LF-A2, LF-B2) are the topic of ongoing research by several of the participants. 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 defferentiation. 2) To study the mechanism of transcriptional 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 study the role of extracellular signals (matrix components and growth factors) in the regulation of liver-specific genes in advanced tissue culture systems employing different extracellular matrices. The role of liver regulatory proteins in maintaining high rate of synthesis of liver proteins in hepatocyte cultures would also be investigated.

Keywords:
GENE REGULATION, LIVER, DIFFERENTIATION, TRANSCRIPTION

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THE DEVELOPMENT OF A GENETIC AND PHYSICAL MAP OF THE PORCINE GENOME (PIG GENE MAPPING PROJECT - PIGMAP)

CONTRACTNUMBER: BIOT CT-900187
START: 1991-02-01 *DURATION*: 36 months
EC CONTRIBUTION: 1,200,000 ECU

Objectives:

To produce a genetic map with markers spaced at approximately 20 centiMorgan intervals over at least 90% of the pig genome. 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.

Brief Description:

At the initiation of the project the gene map of the pig was rudimentary, with only 38 genes assigned to 17 linkage or synteny groups and only 27 tentative chromosomal assignments.
A genetic map with markers spaced at approximately 20 centiMorgan intervals over at least 90% of the pig genome will be produced. The map will include a mixture of genes previously mapped in man and mouse, porcine coding sequences and hypervariable loci with high polymorphic information content (PIC). Diverse genetic stocks European commercial pigs, European wild boar and Chinese Meishan pigs) are being used to provide crosses informative for linkage analysis and known to be segregating for quantitative trait loci (QTLs) of economic value. Purebred, F1 and F2 animals are being genotyped for the markers isolated within the project and for known polymorphisms (biochemical, blood groups, etc.). The data are being analysed with the LINKAGE computer package. Between species synteny and DNA sequence conservation means that cloned human and murine genes can provide dispersed markers to build a basic porcine map to be gradually fleshed out with more variable markers. Probes detecting variable number tandem repeat (VNTR) or minisatellite loci in the pig are being developed to allow isolation of locus specific probes with high PIC values. Highly polymorphic microsatellite loci formed from simple oligonucleotide repeats dispersed throughout the genome are also being used as they offer

the means of rapid, automated genotyping using the polymerase chain reaction (PCR) technique.
A physical map with at least one distal and one proximal landmark locus mapped on each porcine chromosome arm and also genetically mapped will be produced. The porcine karyotype is uniquely suited among farm animals for physical mapping studies. Hybrid cell lines and *in situ* hybridisation techniques are being used to assign genes to chromosomes and chromosomal regions. Further stable and well characterised hybrid cell lines will be developed within the project. Participants with access to fluorescence activated cell sorters (FACS) will develop a flow karyotype and procedures for sorting individual chromosomes as alternative to hybrid cell lines and as sources of single chromosomes for the creation of chromosome-specific libraries. In addition to complementing the genetic map the physical map will allow the conservation of synteny between pigs, man, mice and cattle to be evaluated.
The statistical techniques required to analyse data from QTL mapping experiments are being developed and evaluated. Studies for the detection of quantitative trait loci will be designed but only implemented after the completion of this initial gene mapping project.

Keywords:
PIG, GENE MAPPING, LINKAGE ANALYSIS, CHROMOSOME, PIGMAP

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A STUDY OF FISH GENES AND THE REGULATION OF THEIR EXPRESSION

CONTRACTNUMBER: BIOT CT-900188
START: 1991-01-01 *DURATION*: 36 months
EC CONTRIBUTION: 942,760 ECU

Objectives:

The scientific aim is the isolation and characterisation of fish genes and their promoters, which will contribute to the understanding of the regulation of fish gene expression.

Brief Description:

Various fish genes are under study by eight research groups. The prime target is the molecular, biological, physiological and genetical characterisation of a suite of well-selected genes.

The complementary DNA (cDNA) of tilapia prolactin (PRL) and thymidine kinase genes from a salmonid herpes virus which infects several cell types have been cloned. The corresponding genes of tilapia PRL, the cDNA of seabass prolactin, the oncogenes of *Xiphophorus*, cod immunoglobin genes and various genes abundantly expressed in the liver and other tissues of salmon (including smoltification genes) are being cloned.

Moreover, regulatory elements of trout metallothionein, tilapia prolactin, thymidine kinase of a salmonid herpes virus, various liver specific genes and fish oncogenes are being cloned. The resulting constructs will be characterised in molecular terms, and linked to reporter genes, they will be introduced into cultured fish cell lines from various fish tissues and in fish eggs. These *in vitro* and *in vivo* systems provide excellent models to monitor the expression of the hybrid genes and/or their modulation under various physiological conditions.

Important is the establishment of uniform test organisms. Therefore protocols for the cloning of various fish are being developed in addition to the already existing ones. Finally, a test case of this research includes the

evaluation of various strategies for conferring genetic resistance to trout against viral infection.

The companies Eurogentec SA and Sepia International provide essential support.

Keywords:
CLONING, FISH, GENE, GENE REGULATION, GENE SEQUENCING, GENETIC ENGINEERING

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STUDY OF THE AVIAN HERPESVIRUS GENOME MAREK'S DISEASE VIRUS

CONTRACTNUMBER: BIOT CT-900173
START: 1991-08-01 *DURATION*: 36 months
EC CONTRIBUTION: 540,000 ECU

Objectives:

To identify and sequence glycoprotein genes of Marek's disease virus (MDV) and non-essential genes of herpesvirus of turkeys (HVT) with the main aim of constructing recombinant HVT viruses which express MDV glycoprotein genes and genes of other avian pathogens.

Brief Description:

Glycoprotein genes of MDV and non-essential genes of HVT will be identified by random sequencing and comparing the translated sequences to protein databases of herpes simplex and varicella zoster viruses. This approach has been used previously in our laboratory to identify several MDV homologues of alphaherpesvirus genes including glycoprotein B (gB) which has already been sequenced. MDV glycoproteins gD and gH and their homologues in HVT will be sequenced. Knowledge of the deduced amino acid sequences will allow the preparation of MDV-specific anti-peptide sera which will be required to monitor expression of the MDV genes by HVT recombinants.

Initially, the thymidine kinase (TK) locus of HVT will be used as an insertion site for expressing MDV glycoprotein genes particularly gB as this is an important immunogen. Virus recombinants will be generated by homologous recombination following co-transfection of infectious HVT DNA and plasmid constructs consisting of MDV genes flanked by HVT TK sequences. Recombinants will be selected and tested for safety, replication *in vivo* and efficacy as vaccines. Alternative sites for gene insertion in HVT will be identified and those that allow efficient replication of recombinants *in vivo* and optimal expression of the genes of interest will be finally selected for constructing recombinant vaccines.

Keywords:
AVIAN HERPESVIRUS, RECOMBINANT VACCINES, GENE MAPPING

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DEVELOPMENT OF SECOND GENERATION VACCINES AGAINST PARVOVIRUSES

CONTRACTNUMBER: BIOT CT-910256
START: 1991-02-01 *DURATION*: 36 months
EC CONTRIBUTION: 545,800 ECU

Objectives:

The first object of this project is to identify the key antigenic sites in the porcine parvovirus and to test peptides representing these sites for their ability to induce immunity.
The second object of this project is to produce the designed new proteins in bulk to scale-up the bacterial or eukaryotic fermentation process, to select the proper down stream purification methods, to formulate the vaccines, to test the vaccines on a laboratory scale followed by field trials to assess the protection in target animals.

Brief Description:

In the modern pig industry of the EC the control of porcine parvovirus (PPV) is mandatory. Vaccination against this disease is a prerequisite for a profitable production of pig meat. Currently available vaccines are produced by conventional techniques. However, PPV is difficult to grow in cell culture, which makes the vaccine production technically demanding, laborious and expensive. It is doubtful whether the current, first generation vaccine production based on fast growing cells will be able to cope with the increasing demand, caused by the increasing industrialization of pig farming. Thus, we propose to develop a second generation vaccine using recombinant DNA technology, gene expression, epitope scanning by multiple overlapping peptides (PEPSCAN technique, mimotopes), monoclonal antibodies (MAbs) and modern adjuvants (i.e. ISCOMS). The object is to define the key antigenic sites necessary for protection and to construct expression systems able to produce proteins exposing these sites in the best possible way. Finally, the new proteins will be incorporated into vaccines. The vaccines will be tested in the target animal (field trials). On the basis of the experience obtained with PPV it is envisaged that second generation vaccines against closely related viruses of large economic and social importance (Mink

enteritis virus, Canine parvovirus and Feline panleukopenia virus) can be produced along the same lines. These parvoviruses are similar to PPV and of great economical and social importance.

Keywords:
PORCINE PARVOVIRUS, SECOND GENERATION VACCINE, RECOMBINANT DNA, PEPSCAN, PARVOVIRUSES

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ENGINEERING AND IMMUNOGENICITY OF FOOT-AND-MOUTH DISEASE VIRUS PROCAPSIDS PRODUCED IN INSECT CELLS

CONTRACTNUMBER: BIOT CT-900190
START: 1991-07-01 *DURATION*: 36 months
EC CONTRIBUTION: 560,000 ECU

Objectives:

Recombinant baculoviruses will be constructed containing cDNA cassettes of foot-and-mouth disease virus (FMDV) which produce the procapsid proteins. The baculovirus system will be optimized for expression of these FMDV proteins and procapsid assembly. The antigenicity and immunogenicity of these products will be compared with conventional FMDV vaccines.

Brief Description:

cDNA cassettes encoding the P1-2A structural protein precursor linked to the 3C protease, required for processing of the precursor to the mature procapsid components 1AB, 1C and 1D, will be produced. Similar constructs will be made using cDNA from each of the O,A and C serotypes of foot-and-mouth disease virus (FMDV) encountered in Europe. Following characterization of the cassettes they will be transfered into baculovirus transfer vectors and then recombinant baculoviruses containing these cassettes will be isolated. Modifications to the basic baculovirus technology may be required to optimize the expression level of the FMDV proteins. The initial characterization of the FMDV proteins produced will address issues of the myristoylation of 1AB, the degree of assembly into procapsids and the antigenicity of these particles. Medium scale production of the FMDV proteins within insect cells will be undertaken to provide material for biological characterization of the material. In particular the ability of the procapsids to induce protective immunity against FMDV in animals will be assessed. Modifications to the initial cDNA cassettes will be made with the idea of improving the yield and stability of the product. Chimeric capsids could also be designed which contain epitopes from different serotypes on a single procapsid particle.

Keywords:
BACULOVIRUS, RECOMBINANT VACCINE, MYRISTOYLATION, ANTIGENICITY

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TOWARDS A SECOND GENERATION VACCINE AGAINST BOVINE HERPESVIRUS TYPE 1 (BHV1): IMMUNOLOGICAL CHARACTERIZATION OF HERPESVIRAL GLYCOPROTEINS AND CONSTRUCTION OF BHV1 DELETION MUTANTS

CONTRACTNUMBER: BIOT CT-900191
START: 1991-02-01 *DURATION*: 36 months
EC CONTRIBUTION: 720,000 ECU

Objectives:

The aim of the project is to define the contribution of individual glycoproteins to protective immunity to herpesviruses. Central is the development of an engineered vaccine to bovine Herpesvirus type 1 (BHV1). Antigenic and immunogenic properties of herpesviral glycoproteins will be tested following expression of individual genes by vaccinia recombinants and subsequent immunization of cattle.

Brief Description:

Herpesvirus associated diseases cause high economic losses in the fram animal husbandry and are highly prevalent in the human community. Virus diseases of the respiratory tract present a threat to cattle husbandry. Frequently bovine Herpesvirus type 1 is involved and is considered the major infectious agent, capable of producing alone a respiratory disease. This herpesvirus causes two clinical syndromes:

1.) a disease (infectious bovine rhinotracheitis, IBR) characterized by rhinitis and tracheitis, fever, a drop in milk production and abortion, and

2.) a veneral disease (infectious pustular vulvovaginitis/balanopostitis, IPV) characterized by pustular lesions of the genital tract of cows and bulls.

The goals of the project are to determine the prerequisites for the development of an engineered vaccine to bovine Herpesvirus type 1 (BHV1) by defining the contribution of individual glycoproteins to protective immunity and to construct deletion mutants of BHV1 that provide the basis for a safe

and efficacious vaccine. The immunological role (isotype specific systemic and mucosal antibody response, cell mediated immunity) of isolated herpesviral glycoproteins will be studied *in vitro* and *in vivo* using small laboratory animals and cattle. Each of the genes coding for the probably nonessential glycoproteins gC, gE, gG and gI (nomenclature of the herpes simplex homologs) will be deleted from the viral genome and the resulting viral variants will be tested for virulence in specified pathogen free calves. The combined immunological, pathological and molecular biological results will be used to fotmulate the requirements for a second generation vaccine against BHV1 infections of cattle.

Keywords:
SECOND GENERATION VACCINE, DELETION MUTANT, HERPESVIRAL GLYCOPROTEINS, CELLULAR IMMUNE RESPONSE, VACCINIA RECOMBINANTS, LATENCY

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AREA: D

PRE-NORMATIVE RESEARCH

In vitro evaluation of the toxicity and pharmacological activity of molecules

DEVELOPMENT OF CULTURED HUMAN ENDOTHELIAL CELL LINES, RETAINING THEIR DIFFERENTIATED PROPERTIES, FOR *IN VITRO* PHARMACOLOGY AND TOXICOLOGY TESTING

CONTRACTNUMBER: BIOT CT-900195
START: 1991-02-01 *DURATION*: 36 months
EC CONTRIBUTION: 365,000 ECU

Objectives:

The objective of the current proposal is to produce stable, immortalized lines of human endothelial cells, safe for use, which retain their functional properties and have been fully evaluated for pharmacological and toxicological testing. This objective will require innovative strategies for 'minimal immortalization 'where metabolic disruption induced by the immortalizing gene product is engineered down to a minimum.

Brief Description:

Human endothelial cells (HEC) in culture are a vital cellular model in the study of vascular diseases. The development of HEC lines retaining their differentiated characteristics would prove an invaluable tool for pharmacological and toxicological studies. previous attempts have resulted in HEC lines either with substantially altered properties or, when amphotropic pseudotypes of murine sarcoma virus were used, not fully safe in terms of infection of the workers. The current proposal describes innovative molecular biology strategies that will be used to test the effect of a series of genes with the potential to immortalize/transform cells. Three different approaches will be followed. The first two will use either retroviral or EBV vectors which will allow efficient introduction of the gene of interest into HEC and provide a screening method for a variety of oncogenes, growth factor genes and receptor genes. The third approach will use integrative plasmides which will allow the manipulation of gene expression using heterologous promoters to achieve minimal immortalization i.e. where metabolic disruption induced by the immortalizing gene product is engineered down to a minimum. The HEC lines will be then characterized for their growth characteristics and specific endothelial markers. In addition

a full pharmacological and toxicological assessment of the Hec lines' responses will be performed.

Keywords:
HUMAN ENDOTHELIAL CELLS, IMMORTALIZED LINES, PHARMACOLOGY, TOXICOLOGY

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DEVELOPMENT OF *IN VITRO* NEURAL SYSTEMS FOR THE IDENTIFICATION OF AGENTS WITH TOXICOLOGICAL AND PHARMACOLOGICAL POTENTIAL

CONTRACTNUMBER: BIOT CT-900183
START: 1991-01-01 *DURATION*: 36 months
EC CONTRIBUTION: 760,030 ECU

Objectives:

To utilise the *in vitro* systems developed in BAP to clarify the mechanisms of action of compounds particularly analogues of endogenous neuroactive aminoacids with pharmacological and toxicological properties involved with brain cell synaptic functioning, proliferation and differentiation, in order to evaluate their potential as anticonvulsants; neuroprotective agents in excitotoxin-mediated neurodegenerative disease e.g. ischaemia and epilepsy; anti-gliosis agents and anticancer drugs; and their effects as causative agents in the processes of cytotoxicity, epilepogenicity and other convulsive states.

Brief Description:

Neonatal rodent primary culture systems of different neurons and astrocytes may provide better screening systems for the development of new drugs for use in the CNS. The brain slice system allows validation in a multicellular system. Cultured neutrons and astrocytes will be used to obtain basic knowledge about structure activity relationships for the neuronal and glial GABA high affinity transport systems. As the balance between neuronal and glial uptake is one of the factors regulating the size of the neurotransmitter GABA pool in neurons this screening system may provide a simple *in vitro* model to predict if a given compound may be of interest as an anticonvulsant drug or a convulsant and therefore toxic compound with an action based on interference with GABA neurotransmission. An assay system (developed under BAP) for cytotoxic agents based on the release of cytoplasmic LDG from cultured neural cells has been used to monitor neurotoxic actions of excitatory amino acids and will be used in studying neurotoxic actions of a family of amino acids structurally related to glutanate.

Some sulphur amino acids (SAAs), with neuroexcitant and convulsant properties, are attracting attention in the study of neurological and affective disorders. The mechanisms of these effects for a group of acidic SAAs which represent *in vivo* candidates with neuroexcitotoxic potential on the basis of their structural analogy to the major EAA (excitatory amino acid) glutamate will be studied using cultured neurons and astrocytes to test their potential as an initial screening system for evaluating neurotoxic mechanisms designing potential drugs for treatment of disorders of the CNS.

A group of synthetic neuroactive chlorinated compounds (polychlorocycloalkanes, PCCAs) with neurotoxic and convulsant properties, are increasingly being used as pharmacological probes for convulsant/anticonvulsant agents related to GABA funcionality. *in vitro* characterization of PCCAs activity using the systems described above may lead to the development of *in vitro* systems for testing convulsant/anticonvulsant agents.

The effects of GABA and its agonists will be studied at the morphological and functional level by quantitative electron microscopy, transmitter release, GABA receptor expression and oncogene expression to establish systems for identifying compounds with advantageous or deleterious trophic or differentiative properties.

Keywords:
IN VITRO SYSTEMS, GABA, GLUTAMATE, TRANSPORT, EXCITOTOXICITY, CONVULSANTS, ANTICONVULSANTS, NEUROTOXINS

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DEFINITION AND SCIENTIFIC VALIDATION OF AN *'IN VITRO'* TEST, FOR THE SCREENING OF TUMOR PROMOTERS AND PROTECTIVE CHEMICALS, BASED ON GAP JUNCTIONAL INTERCELLULAR COMMUNICATION ASSAYS IN HUMAN AND ANIMAL CELLS

CONTRACTNUMBER: BIOT CT-910261
START: 1991-03-01 *DURATION*: 36 months
EC CONTRIBUTION: 780,000 ECU

Objectives:

Elaboration of an optimal *in vitro* system to detect chemicals interfering with gap junction intercellular communication (GJIC), inhibitors or enhancers, and validation of this system (using human cells, as well as animal cells), to study mechanisms of GJIC and their role in the promotion phase of the carcinogenesis process.

Brief Description:

Published data suggest that alteration of cell to cell communication mediated by gap junctions could play an important role in carcinogenesis, specially in the tumour promotion stage. Therefore, modulation of gap junctional intercellular communication (GJIC) will be used as the endpoint of a screening assay for potential tumour promoters (inhibitor) or potential antipromoters (enhancers). Since most types of human cancers have epithelial origin, our studies will be performed with human epithelial as well as animal cell lines, both at the functional level (direct estimation of cell to cell communication by fluorescent dye transfer) and at the molecular level (gap junction proteins and mRNA expression). We will try to elucidate whether cell expression and function modulated by tumour promoting agents, play a role in the regulation of gap junctional communication.

The scientific program will focus on:

a) Intralaboratory testing of chemicals, using dye coupling in a standardized protocol to measure GJIc in different cells systems.

b) Biochemical (pH, Ca variations, specific membrane binding receptors) and molecular studies on the regulation aspects of the GJIc modulation: Identifications of specific connexins (cDNA probes and antibodies) expressed in the different cell lines types used for this screening ; studies on how the modified corresponding protein may effect the gap junction channel stability, as it seems that tumour promoter chemicals act differently in cells that express different connexins.

c) Interlaboratory testing on the basis of a blind trial in selected cell systems and evaluation of test predictability and validity.

Keywords:
IN VITRO TEST, VALIDATION, CELL TO CELL COMMUNICATION, DYE TRANSFER, GAP-JUNCTION, CONNEXINS, TUMOURS PROMOTERS, ANTIPROMOTERS

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DEVELOPMENT OF A PREDICTIVE *IN VITRO* TEST FOR DETECTION OF SENSITIZING COMPOUNDS

CONTRACTNUMBER: BIOT CT-900186
START: 1991-02-01 *DURATION*: 36 months
EC CONTRIBUTION: 520,000 ECU

Objectives:

The aim of the project is to develop an *in vitro* method for detection of sensitizing compounds. The final objective of the project is to validate an alternative method to *in vivo* sensitization and reduce the number of living animals in predictive tests.

Brief Description:

A panel of known allergenic, irritant and non-allergenic compounds will be tested on bulk epidermal cells, enriched Langerhans cells and endothelial cells of mice, guinea pig and man in *in vitro* conditions. Each team will use the same preparation of xenobiotics (purified or synthesized by one of the contractors). Data will be gathered on three complementary cellular systems with parallel experiments. The murine system is a reference in immunology, most existing *in vivo* data were generated in the guinea pig and finally human cells have been chosen for clinical relevance and final validation of the method.

We will focus on early morphological, biochemical and functional modifications of keratinocytes, Langerhans cells (epidermal antigen-presenting cells) and endothelial cells, that occur specifically with sensitizing molecules. Cell surface markers (class II and adhesion molecules) expression, as well as cytokine (IL-1, IL-6, IL-8, TNFalpha, GM-CSF) production and requirement will be studied in murine, guinea pig and human cellular systems. In some cases ultrastructural (electron microscopy) analysis will be implemented. Conditions for optimal *in vitro* haptenization and antigen-presentation to naive T cells (functional analysis) in these three cellular systems will also be defined.

So far *in vitro* sensitization has remained difficult due to technical pitfalls (insolubility of most haptens in physiological media) and gaps in our knowledge of hapten processing. Therefore hapten-cystein or hapten-S-peptide conjugates (which could generate the original hapten in situ through biochemical transformation) as well as hapten- hydrophobic peptide conjugates (which could associate directly to class II molecules and be presented to T cells) will be prepared and tested as hapten substitutes.

Sharing of reciprocal findings will enable us to progress quickly and remove many bottle-necks that still exist for development of an alternative method to laboratory animals sensitization.

Keywords:
IN VITRO CONTACT, SENSITIZERS, XENOBIOTICS, ALLERGIC DERMATITIS, DELAYED HYPERSENSITIVITY, EARLY EVENTS, HUMAN, GUINEA, PIG, MOUSE, CYTOKINES

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PHARMACOLOGY AND TOXICOLOGY OF DIFFERENTIATED CELL TYPES, THEIR CELL-CELL AND CELL-MATRIX INTERACTIONS IN AN *IN VITRO* RECONSTRUCTED HUMAN SKIN MODEL

CONTRACTNUMBER: BIOT CT-900193
START: 1991-03-01 *DURATION*: 36 months
EC CONTRIBUTION: 660,000 ECU

Objectives:

The aim of this project is to perfect a living human skin equivalent using advanced culture procedures and to validate it as reliable predictive *in vitro* system for human skin pharmaco-toxicology.

Brief Description:

Human skin was the first organ to be reconstructed *in vitro* and is likely to provide a predictive system for evaluating drug efficacy and toxicity, avoiding animal experimentation. organogenesis *in vitro* for pharmacological studies is a very new and promising field of investigation.

Culturing cells in close contact with their physiological matrix molecules and with cell types usually adjacent *in vivo*, cells have been shown to communicate and differentiated in this skin equivalent. These cell-matrix-cell interactions greatly modify the response to pharmaco-toxicological agents, resembling the situation *in vivo* and demonstrating that some pharmaceutical agents operate on the cell-cell and cell-matrix communication system.

To achieve our goal, the collaboration begun during a previous BAP program will be reinforced in order to study the role of cell-matrix and cell-cell interactions in differentiation processes and in pharmacological responses.

The validation of these models with a possible transfer to industry to screen the activity of drugs on a large scale is expected for wound healing promoters, antipsoriatic drugs, pigmentogenic, antineoplastic and anti-ageing substances in the next four years.

In addition, this concept of organogenesis *in vitro*, allowing pharmaco-toxicology at the cellular communication level, will identify pathways that might represent targets for drugs of high socio-economic value.

This first human organ reconstructed *in vitro* can be considered as a prototype. Most of the technical innovations made with the skin model could be applied to the reconstruction of other organs such as blood vessels, ligaments, endocrine glands etc..

Keywords:
SKIN, PHARMACOLOGY, CELL-CELL INTERACTIONS, CELL-MATRIX INTERACTIONS, HUMAN

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ESTABLISHMENT OF IMMORTAL DIFFERENTIATED DIPLOID HEPATOCYTE LINES FROM TRANSGENIC MICE AND THEIR USE FOR STUDYING VIRAL AND CHEMICAL AGENTS

CONTRACTNUMBER: BIOT CT-900189
START: 1991-02-01 *DURATION*: 36 months
EC CONTRIBUTION: 265,000 ECU

Objectives:

Immortal, differentiated hepatocyte lines will be generated from transgenic mouse strains bearing metallithionein (MT-I)-driven SV40 T-antigen constructs for the development of *in vitro* systems for the identification of potentially mutagenic agents. The lines will also be employed for transfecting Hepatitis B virus (HBV) DNA to study HBV gene expression and replication *in vitro*.

Brief Description:

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 differentiated functions for a few days, which limits their usefulness in long-term studies. Also, hepatoma cell lines are available which exhibit only a fraction of hepatic functions. Thus, the goal of this research project is to obtain diploid, immortal hepatocyte lines derived from adult transgenic mice expressing an immortalizing transgene (SV40 virus T- and t-antigens) in the liver. Resulting cell lines will be analyzed for their ability to express hepatic functions including drug metabolizing enzyme systems which are essential for the metabolic activation of indirectly acting mutagens. Attempts will be made to employ such hepatocyte lines for the establishment of an *in vitro* test system for the identification of potentially mutagenic agents in chemically defined culture medium. Also, the cell lines will be employed to evaluate the roles of Hepatitis B Virus (HBV), growth factors & of plasma membrane constituents in mediating contact inhibition phenomena, and in controlling cell growth and the progression of hepatocytes toward malignancy in culture. For this purpose, basic investigations are required to define mechanisms that

control the balance between growth and differentiation of hepatocytes in culture. Furthermore, generated hepatocyte lines will be of broad interest for studies in toxicology and, after appropriate genetic manipulations, and for the production of pharmaceutically important proteins & glycoproteins in large scale.

Keywords:
LIVER, IMMORTAL HEPATOCYTE LINES, CONTACT INHIBITION, GROWTH CONTROL, HEPATITIS B VIRUS, BIOTECHNOLOGY

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DEVELOPMENT OF AN IMMORTALISED HUMAN ARTICULAR CARTILAGE CELL LINE AND ITS USE IN PHYSIOLOGICAL, PHARMACOLOGICAL AND TOXICOLOGICAL *IN VITRO* INVESTIGATIONS

CONTRACTNUMBER: BIOT CT-900196
START: 1991-02-01 *DURATION*: 36 months
EC CONTRIBUTION: 695,000 ECU

Objectives:

Development of an immortalized human articular cell line and investigation of its suitability in physiological, pharmacological and toxicological *in vitro* studies.

Brief Description:

The aim of the study is the development of an immortalized human cartilage cell line, and the investigation of its function with respect to the homeostasis of extracellular matrix macromolecules. The preservation of the original (*in vivo*) functions in an *in vitro* model (culture in different gelified matrices) will be investigated. Functional characterisation of this cell line will involve the study of synthesis, secretion and turnover of proteoglycan and collagen in an artificial extracellular matrix. In order to obtain full control of the environment and complete standardisation of this system, Insulin-like growth factor will replace serum in the incubation medium.

The system will be used to explore the ways in which normal and altered chondrocytes build, degrade and/or repair their environment. The effects of disease mediators (cytokines, 0^{2-}-radicals, co-cultured macrophages) in this model, as well as the effects of some 'pilot' drugs (steroids, non-steroidal anti-inflammatory drugs, interleukin-1-inhibitors) on mediated chondrocyte metabolism will be studied. To this end cells will be obtained from human donors (autopsy). Finally, cartilage cells will be purchased from experimental animal models of degenerative and inflammatory joint diseases in order to determine whether *in vitro* observed abnormalities of these cells are consistent with the pathological changes encountered *in vivo*, and whether their phenotypes are preserved after immortalisation.

Keywords:
CONNECTIVE TISSUE, CARTILAGE, CHONDROCYTES, PROTEOGLYCAN, COLLAGEN, CELL CULTURE, INFLAMMATION, CYTOKINES, IMMORTALISATION, DNA-LIBRARY

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THE RISK POTENTIAL OF BIOTECHNOLOGY-DERIVED POLYPEPTIDES CAUSING NEPHROTOXICITY BASED ON ASSESSMENT IN HUMAN AND ANIMAL RENAL CELL LINES USING MOLECULAR AND CELLULAR BIOLOGY

CONTRACTNUMBER: BIOT CT-910266
START: 1991-03-01 *DURATION*: 36 months
EC CONTRIBUTION: 458,550 ECU

Objectives:

To develop a spectrum of immortalized renal cell lines tailored to combine characteristics that make them sensitive to biomolecules *in vitro*. These will be used to screen novel verterinary and therapeutic biotechnology products to document change that underly adverse effects *in vivo*. The molecular basis of cellular changes will be used for risk assessment to maximise the economic benefits of development.

Brief Description:

We are developing an array of sensitive and selective *in vitro* methods for assessing the potential nephrotoxicity of biotechnology polypeptides and novel molecules that may affect the kidney in the clinical situation by:

1) Tailoring cells by fusion, transfection, nuclear injection and optimizing culture conditions (varying media, changing substrata, etc.), using primary rat, rabbit and pig cells; and continuous cell lines derived from the rat, pig, dog, man that represent each of the different regions of the kidney.

2) Defining functional and structural characteristics of these cells by molecular and cell biology techniques, including immunochemistry, fluorescent probes, hormone responsiveness, handling of macromolecules and ultrastructural assessment.

3) Establishing a full profile of response to biotechnology products by monitoring the secondary messenger systems, ion fluxes and membrane function, secretory proteins, electrophysiology, ultrastructure and immunohistochemistry. Polypeptide handling will be characterised by

radio-, fluorescent or immuno-morphological and receptor binding ligand techniques. Polypeptides will include those with known nephrotoxic potential (eg interferons), those affecting the kidney (ANF, Vassopressin), recombinant products, immunomodulators, neuropeptides, or appropriate biotechnology products will be compared.

4) New cells will be made available to all participants. Validation will include multicentre evaluation of cell culture methods and the correlative evaluation performed using higher order *in vitro* systems, such as the isolated defined nephron segment and the isolated perfused kidney.

5) Transfer technology and use the knowledge base for veterinary and human clinical safety and risk assessment of biotechnology produced polypeptides and novel molecules.

Keywords:
RENAL CELL LINES, NEPHROTOXICITY, SCREENING, THERAPEUTIC BIOTECHNOLOGY POLYPEPTIDES, CELL TAILORING, RECOMBINANT PRODUCTS, MULTICENTRE EVALUATION, RISK ASSESSMENT

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AREA: D

PRE-NORMATIVE RESEARCH

Biosafety

ANALYSIS OF GENE TRANSFER BETWEEN MICROORGANISMS AND PLANTS

CONTRACTNUMBER: BIOT CT-910282
START: 1991-10-01 *DURATION*: 24 months
EC CONTRIBUTION: 523,000 ECU

Objectives:

We propose to investigate the potential for transfer and expression of model genes under conditions that employ different sets of interactions between microbes and plants. We expect to develop new extraction and detection methods appropriate to these studies. Environmental factors (e.g moisture and bacterial motility) will be investigated for potential roles in survival and transfer.

Brief Description:

The research is based essentially on the use of a group of specific marker genes (gentamicin®, phosphinothricin® and bromoxynil®) that are excellent selective markers for a variety of microbes and plants to monitor the transfer of genetic information between species in nature and under natural conditions.

Several aspects of plasmid transfer in soil will be considered; transfer between gram negatives such as *Rhizobium leguminosarum* and *Agrobacterium tumefaciens* into *Pseudomonas syringae* in soil, using the marker genes and detection methods developed, and also transfer between gram positives. In the latter case we have shown transfer of broad host range gram negative plasmids to streptomycetes by conjugation, the plasmids can, surprisingly, replicate and express marker genes. We have designed a system of entrapment by which we will add a selectable, marked streptomycetes to soil and then screen for transfer to this recipient, after growth and selection.

We will also investigate the effects of motility, moisture, soil composition, and other chemical and physical factors. Experiments have been planned to try to estimate how often mating partners make contact, and what factors may influence the efficiency of this contact.

Transfer between microbes in nodules and the possibility of transfer from plant to microbe will be investigated. For these experiments, three different model systems will be used. The first will investigate transfer of a marker gene from transgenic plants into *Rhizobium leguminosarum*. The second will examine the possibility of transfer between transgenic tobacco plants and a) indigenous soil microbes surrounding the roots and b) microbes associated with the tobacco plants during the fermentative processes that take place during curing. Studies investigating gene transfer in plant/microbial interaction will consider transfer from *Rhizobium meliloti* to plant in alfalfa nodules when the marker genes are carried by a promiscuous plasmid. The marker gene will be used in several types of constructions involving both bacterial and plant promoter sequences. Concomitantly, transfer of the same genes from plant to bacteria will be screened in nodules, tumours (*Agrobacterium*), following infections (*Xanthomonas*), and during the normal curing process of tobacco.

Keywords:
GENE TRANSFER, MICROBES, BACTERIA, PLANTS, SOIL, MOTILITY, ANTIBIOTIC RESISTANCE, HERBICIDE RESISTANCE

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FATE OF GENETICALLY ENGINEERED MICROORGANISMS (GEMs) AND GENETICALLY ENGINEERED DNA SEQUENCES (GEDs) IN SOME ENVIRONMENTAL HOT SPOTS

CONTRACTNUMBER: BIOT CT-910284
START: 1991-10-01 *DURATION*: 24 months
EC CONTRIBUTION: 478,500 ECU

Objectives:

The study of the behaviour and influence of GEM's in microbial environmental 'hot spots 'with special emphasis on the role of broad host range plasmids in gene dissemination and the development of bacterial containment systems.

Brief Description:

The behaviour of GEM's and GED's will be assessed in microcosms simulating environmental 'hot spots'. We define environmental 'hot spots 'as sites where gene transfer is likely to occur. The microcosms will simulate soils polluted with xenobiotics, river sediments, diseased plant material and decaying plant roots and plant-soil ecosystems. The survival, mobility of GEM's and the transfer of cloned GED's into an appropriate introduced recipient strain and into the indigenous population will be followed. The cloned GED's are genes which are only well expressed if they have been transferred into an appropriate host such as *czc* (coding for resistance to heavy metals). Special emphasis will be put on the role of broad host range plasmids (BHR) in gene transfer by introducing well known BHR plasmids to the microcosms.

Furthermore, we will assess the presence of natural transfer potential in the environmental hot spots by the exogenous isolation of plasmids from the environmental samples and their further characterization, by retrotransfer of non self transferable environmental plasmids and by DNA-DNA hybridization on total DNA extracted from the samples with appropriate probes, such as oriV and oriT of Incp1 and IncW plasmids.

The possible disturbance of the ecosystem after introduction of GEM's will be investigated by determining changes in different metabolic parameters in the samples, whereas the influence of indigenous organisms on the survival of the introduced GEM will be studied by selective elimination of populations of the natural microbial community.

Different bacterial containment systems will be constructed by construction of strains bearing mutations which affect physiological and genetic adaptation to chemical stress and toxic compounds and by construction of strains deficient in conjugation exchange.

Keywords:
GEM'S, GED'S, DELIBERATE AND ACCIDENTAL RELEASE, BROAD HOST RANGE PLASMIDS, CONJUGATION, EXOGENOUS ISOLATION OF PLASMIDS, HOT SPOTS, CONTAINMENT SYSTEMS

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THE EFFECTS OF SELECTION ON GENE STABILITY AND TRANSFER IN POPULATIONS OF BACTERIA IN SOIL

CONTRACTNUMBER: BIOT CT-910285
START: 1991-10-01 *DURATION*: 24 months
EC CONTRIBUTION: 340,000 ECU

Objectives:

To determine the effects of selection on the stability of antibiotic resistance genes amongst populations of soil bacteria. Resistant phenotypes will be selected for in soil by addition of antibiotics and by their production *in situ*. Subsequent effects on gene stability and transfer will be determined for chromosomal, plasmid and phage borne genes.

Brief Description:

The survival and dissemination of genes in soil microbial populations may be influenced by their selective advantage. The project aims to investigate the ways in which selection might act for antibiotic resistance in soil bacteria using the genes for thiostrepton resistance (*tsr*) and aminoglycoside resistance (*aph I, II, V*). A number of aspects will be considered to determine how these genes might spread in soil populations and if indigenous resistance is affected by the selection pressure imposed. The three participating laboratories will investigate gene stability by introducing resistance genes on plasmids, transposons and within amplified sections of the chromosome. Parallel experiments involving continuous soil columns will be run to allow comparison between the different sources of the genes.

The mobility of *tsr* within populations of introduced and indigenous streptomycetes will be studied at all three laboratories while Tn5 (*aph II*) will be studied in marked plasmids and transposons. The *aph II* gene being selected for work with amplified sequences on the chromosome. The stability of genes *aph I* and *II* will also be investigated in pseudomonad populations introduced into soil. Selection for the genes will be simulated by the enrichment of soil with antibiotic solutions and by introduction of antibiotic-producing streptomycetes (in nutrient-enriched soil). Genes will be tracked

by direct probing and PCR of soil DNA and by analysis of phenotype and genotype of soil isolates.

Our aim is the following:

1. Can selection for antibiotic resistance genes occur in soil?
2. Genes thought to be lost, following die-off through death of unfit inoculants, can reappear with selection?
3. The location of the gene is important for stability and mobility, but to what extent does this matter after several generations in soil?

Keywords:
RESISTANCE GENES, GENE STABILITY, SOIL DNA, GENE TRANSFER, CONTINUOUS SOIL MICROCOSMS

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SAFETY ASSESSMENT OF THE DELIBERATE RELEASE OF TWO MODEL TRANSGENIC CROP PLANTS, OILSEED RAPE AND SUGAR BEET

CONTRACTNUMBER: BIOT CT-910298
START: 1991-10-01 *DURATION*: 24 months
EC CONTRIBUTION: 653,919 ECU

Objectives:

The study of the dispersal of genes via genetic engineering into plants under common agricultural situations in the field. New techniques, assays and probes for the quick and efficient tracking of the transgenes in seeds and plants will be developed.

Brief Description:

The participants assess the entire range of safety issues associated with the deliberate release in the environment of genetically modified plants (GMP), in specific of two model crop species: oilseed rape and sugarbeet.

In addition to earlier defined aspects in a number of BAP-projects - pollen dispersal and outcrossing to wild relatives - descriptions are provided on seed dispersal, population dynamic behaviour of GMP, hybridity and viability of outcrosses, the stability of GMP and the evolution of the expression of the transgenes over generations and time.

The main assessments are performed in the field under natural agricultural conditions. Suitable general procedures and techniques are evaluated and the globalization of the defined parameters is achieved in a definition of a safety prediction model, relating laboratory, greenhouse and field data.

Keywords:
SAFETY ASSESSMENT, PLANTS, OILSEED RAPE, SUGARBEET, GENE TRANSFER

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STABILITY, GENETIC TRANSFER AND ECOLOGY OF FUNGI USED AS BIOCONTROL AGENTS

CONTRACTNUMBER: BIOT CT-910290
START: 1991-10-01 *DURATION*: 24 months
EC CONTRIBUTION: 470,500 ECU

Objectives:

Actually there is no data on the risk assessment linked to the dispersal of fungi in the nature, though there are more perspectives to use such microorganisms.
Objectives are to estimate those risk with *Beauveria* and *Fusarium* strains used as pests or disease control agents.

Brief Description:

The program is divided in 4 complementary parts:

1) Genetic stability of heterologous DNA will be estimated in *Beauveria*:
   *Influence of vector, insertion site, etc.
   *Stability of transformants in different soils, microcosm or mass production process

2) Stability of homologous recombinants obtained with nitrate reductase or ATP sulphurylase genes of *B. bassiana* 147

3) Natural genetic transfer
   *Influence of genetic distances between strains
   *Parasexuality of *Fusarium* and *Beauveria* under natural conditions
   *Transposition in *Fusarium* with transposons already isolated
   *Research of transposon in *Beauveria*

4) Methodology for soil ecology
   *Methodology to track strains
   *Ability of *Fusarium* strains to colonize soils

Keywords:
HYPHOMYCETE, PARASEXUALITY, TRANSPOSON, HETEROLOGOUS DNA, TRANSFORMATION, TRACKING, HOMOLOGOUS RECOMBINATION

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GENETIC TOOLS FOR CONSTRUCTING GENETICALLY-ENGINEERED MICROORGANISMS (GEMs) WITH HIGH PREDICTABILITY IN PERFORMANCE AND BEHAVIOR IN ECOLOGICAL MICROCOSMS, SOILS, RHIZOSPHERES AND RIVER SEDIMENTS

CONTRACTNUMBER: BIOT CT-910293
START: 1991-10-01 *DURATION*: 24 months
EC CONTRIBUTION: 480,000 ECU

Objectives:

The development of genetic tools for constructing GEMs with high predictability in performance and behaviour in ecological microcosms, soils, rhizospheres and river sediments.

Brief Description:

The development of (GEMs) for biotechnological applications, involving their deliberate release, particularly in the agricultural and environmental protection sectors, is growing in importance. Concerns about possible risks, mainly the lack of predictability of GEMs behaviour in natural habitats, transfer of recombinant information to other organisms, and impact on natural populations, has become apparent. The goal of our current work is to make available a collection of genetic tools and resources for engineering GEMs with a high degree of ecological predictability. Specific approaches are being developed to assure (a) the stable inheritance of cloned genes, (b) their efficient expression where and when desired, (c) death of the GEM when its application is completed, and (d) depression of the level of lateral transfer of recombinant genes of the GEM to indigenous bacteria. As a model microorganism we use *Pseudomonas* bacteria engineered to degrade toxic aromatic chemicals (alkyltoluenes and alkyl- and chlorobenzoates). Within the context of this project 'new 'bacteria able to eliminate polychlorinated biphenyls (PCBs) will be constructed. The construction of such bacteria will involve the transference of a genetic module containing PCB-degradation pathway of a PCB-degrading bacteria that survives poorly

in soil to indigenous gram-negative ones from the plant root rhizosphere, which are more likely to survive in soils and to remove PCB's more efficient in situ.

Keywords:
SUICIDES GENES, *PSEUDOMONAS*, MICROCOSMS

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EXPERIMENTAL AND MODELLING STUDIES ON THE FATE IN SOIL OF INTRODUCED BIOLOGICALLY-CONTAINED BACTERIA

CONTRACTNUMBER: BIOT CT-910288
START: 1991-10-01 *DURATION*: 24 months
EC CONTRIBUTION: 316,500 ECU

Objectives:

Development and testing of safe carrier organisms for the application of beneficial genes to soil.

Brief Description:

The broad objective of this project is to provide a way for the safe introduction of genetically-engineered microorganisms (GEMs) into soil. The project is linked to an Agricultural Research Organization Wageningen project aimed at developing biological alternatives (based on *Bacillus thuringiensis* crystal protein genes) for the control of insect larvae feeding on grass and cereal roots. To improve the biosafety of such releases, an intrinsic control system based on host-killing genes will be inserted into soil bacteria and the behaviour of these in soil situations will be tested. Special focus will be on the development of a regulatory system of the suicide system which, even though killing off introduced bacterial cells, will permit survival of the bulk of the introduced population in soil for an adequate period of time (controlled survival). Suitable marker genes will also be inserted into the soil bacteria for the purpose of ecological testing; if time and regulatory constraints permit, the target bacteria, i.e. strains carrying the aforementioned *Bacillus thuringiensis* crystal protein genes will be used. As a corollary, mathematical models predictive of such behaviour will be developed and validated against data obtained from soil microcosm and, if possible, field experiments.

Keywords:
BIOLOGICAL CONTAINMENT, BACTERIA, SOIL, SURVIVAL, MODELLING

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AN EXPERIMENTAL APPROACH TO INVESTIGATE HORIZONTAL GENE TRANSFER BETWEEN ORGANISMS

CONTRACTNUMBER: BIOT CT-910287
START: 1991-10-01 *DURATION*: 24 months
EC CONTRIBUTION: 240,000 ECU

Objectives:

The objective of this research is to measure experimentally the frequency of horizontal gene transfer between eukaryotic organisms. The model system we have chosen involves the plant pathogen *Cladosporium fulvum* and its host species, tomato. The genes that will be studied will be plant and fungal retrotransposons, as we consider these genes are best equipped to invade non-host genomes.

Brief Description:

Tobacco, tomato and *C. fulvum* all contain retrotransposons in their genomes. These elements have no known role beneficial to their hosts; their sequence homology and distribution provides strong circumstantial evidence of horizontal gene transfer. The rationale behind this project is to establish the situation in which horizontal gene transfer might take place and to be able to detect such an event even if it is very rare. The first condition is provided by the intimate relationship between a biotrophic plant pathogen and its host plant. The second condition is provided by the creation of test genes whose transfer can be detected even amongst a vast excess of cells lacking the transferred genes. Engineered copies of the retrotransposon *Cf*T-1, from*C. fulvum*, will be created by including chimeric genes for either hygromycin phosphotransferase or β-glucuronidase using either the CaMV 35S or *A. nidulans* gpd promoters. Similarly, copies of the tobacco retrotransposon Tnt-1 will be created with the chimeric genes. Control experiments will establish whether the chimeric genes are active in both genomes. Next, the marked transposons will be transformed into both *C. fulvum*, and tomato. Ultimately we would infect marked *C. fulvum* strains onto tomato and select plant cells carrying the marker gene. Conversely,

marked strains of tomato will be infected With wild-type *C. fulvum*. Fungal spores, from the infected leaves, carrying the marker-gene will be sought.

Keywords:
PLANT PATHOGEN, TOMATO, TOBACCO, *CLADOSPORIUM FULVUM*, RETROTRANSPOSON

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SAFETY OF GENETICALLY ENGINEERED RETROVIRUSES USED FOR GENE TRANSFER

CONTRACTNUMBER: BIOT CT-910286
START: 1991-10-01 *DURATION*: 24 months
EC CONTRIBUTION: 533,300 ECU

Objectives:

The objective of the project is to accumulate basic information regarding cellular and viral mechanisms involved in modulating the behaviour of retroviral vector systems and to use this knowledge (i) as a basis for an assessment of the risk of using retroviral vectors for the production of transgenic animals or for human gene therapy and (ii) to construct safe, efficient and targeted retroviral vector systems.

Brief Description:

Genetically engineered DNA molecules and viruses are being increasingly used for biotechnological applications. Among the most widely utilized engineered molecules are retroviral vectors - viruses which have been modified to provide a highly efficient means of transporting genes into various kinds of cells. Such genetically engineered retroviruses are gaining usage in the medical field for vaccine production and gene therapy, in research to study gene function and regulation, and in industrial applications for the production of transgenic farm-animals and bio-reactors.

The overall aim of the project is to collect basic information regarding the safety of retroviral vectors. Retroviral vector systems consist of two parts - the packaging cell line, providing the retroviral proteins *in trans* and the retroviral vector construct supplying the recombinant RNA to be packaged into the retroviral particle. One part of the work addresses questions concerning the stability of the packaging cell to identify cellular factors causing either appearance of wild-type virus or shut-down of protein expression. The stability of the second retroviral vector system component, the vector construct itself is investigated. These studies attempt to identify cellular factors affecting the expression of the retroviral vector, and to assess the frequency of recombination of retroviral vectors with endogenous sequences in the

target cell. An important aspect of retroviral vector design is the ability to target expression of introduced genes to specific tissues or organs. The infectivity and expression of the vectors among various cell types are investigated both for avian vectors and for murine vectors. With an emphasis on murine vectors, the expression spectrum mediated by retroviral signals or site of integration are investigated. The introduction of tissue specific expression elements resulting in an alteration of the expression spectrum is explored with the aim of targeting vector expression to specific cell types. New types of vectors are also being designed to target specific integration sites using homologous recombination approaches. The potential risks of germ line infection in animals after somatic gene transfer using retroviral vectors will be assessed and the stability, spread, and infectiousness of different types of retroviral vectors systems will be evaluated with respect to the infection of human cells.

Keywords:
RETROVIRAL VECTORS, PACKAGING CELL LINES, RECOMBINATION, MUTATION FREQUENCY, TARGETING, RISK ASSESSMENT, TISSUE SPECIFIC VECTOR EXPRESSION

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ASSESSMENT OF ENVIRONMENTAL IMPACT FROM THE USE OF LIVE RECOMBINANT VIRUS VACCINES

CONTRACTNUMBER: BIOT CT-910289
START: 1991-10-01 *DURATION*: 24 months
EC CONTRIBUTION: 592,622 ECU

Objectives:

The programme aims to assess the environmental impact (if any) of the use of live recombinant virus vaccines. The host and tissue tropism of the recombinant vaccines will be determined and compared to those of the parent viruses. Possible evolution of an altered vaccine, following recombination of the recombinant vaccine and wild type viruses present in the environment, will be assessed.

Brief Description:

The development of recombinant vaccines has made rapid progress in the last few years. Environmental aspects related to the use of such vaccines are unknown and the safety regulations for the use of such vaccines are largely undefined. This collaborative programme aims to address the following aspects:

(i) To provide a scientific basis to be able to predict and evaluate potential environmental problems involved in the use of these vaccines by
   (a) identifying the factors that are pertinent to the safety of the vaccines and
   (b) developing the techniques necessary to evaluate the effect of the use of these vaccines prior to release.

(ii) Identification of factors that must be considered to develop the techniques necessary to evaluate the effect of the use of these vaccines prior to release.

(iii) To provide a basis for the establishment European and International Legislation for the use of the recombinant vaccines.

Studies will concentrate on possible changes in tissue and host tropism of the vector system as a result of the expression of the heterologous genes, and any associated pathogenic alterations. In addition we will study the possibility of recombination between live recombinant vaccine virus and wild type field virus. The virus vector systems to be studied, capripoxvirus, fowlpoxvirus, orthopoxvirus and herpes virus are expected to be applied in the field in the near future as multivalent recombinant vaccines.

Keywords:
RECOMBINANT VACCINES, POXVIRUSES, HERPES

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IDENTIFICATION OF GENES INVOLVED IN LATENCY AND REACTIVATION OF PSEUDORABIES VIRUS, USE IN BIOLOGICAL CONTAINMENT STUDY OF VIRAL GENOMES IN PIGS

CONTRACTNUMBER: BIOT CT-910297
START: 1991-10-01 *DURATION*: 24 months
EC CONTRIBUTION: 437,700 ECU

Objectives:

The joint proposal aims at the identification of viral genes influencing establishment of and reactivation from latency of pseudorabies virus (PRV); Aujeszky's disease virus; herpes suid 1) in the **natural** host, swine. The major objective will be a detailed analysis on the biosafety of PRV mutants specifically manipulated in latency-associated (LA) functions.

Brief Description:

LA transcripts (LAT's) will be identified, characterized, and the molecular biological studies (in situ hybridization, Northern blotting, protection assay, PCR, cDNA bank) will be done in a convergent and complementary way between the different laboratories. The results will enable construction of viable PRV mutants. After *in vitro* assays, those mutants can be immediately investigated in pigs to analyse their organ tropism and latency characteristics. *In vitro* culture systems of porcine neural cells reflecting a natural target cell of PRV shall be established. Finally, identification of PRV gene(s) specific for latency would offer for the first time the availability of a latency-marker in PRV-infection, and its applicability for different diagnostic purposes will be tested. Sensitive detection of LAT (e.g. by PCR) or of associated peptides (e.g. by serology) could unambiguously identify latently infected pigs carrying reactivable PRV strains or live vaccines.

Keywords:
LATENCY, PSEUDORABIES VIRUS, PIGS, LAT GENES, LIVE VACCINES

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BIOSAFETY OF GENETICALLY-MODIFIED BACULOVIRUSES FOR INSECT CONTROL

CONTRACTNUMBER: BIOT CT-910291
START: 1991-10-01 *DURATION*: 24 months
EC CONTRIBUTION: 358,600 ECU

Objectives:

The objective is the genetic engineering and field testing of biosafe baculovirus insecticides for control of insect pests in agriculture. This objective will be achieved by constructing viruses with increased virulence, but reduced persistence and survival in the environment.

Brief Description:

Baculoviruses are insect pathogens which are successfully used as biological control agents of insect pests in agriculture and forestry as alternatives to chemical insecticides. Safety testing has confirmed that these natural viruses are insect specific and cause no hazards to other animals and to plants. A major drawback to a much wider commercial application of these viruses is their slow speed of action, which is most relevant for crops With low damage thresholds. A number of potentially useful baculoviruses with enhanced insecticidal activity, have been engineered. Within the BAP program genetically-marked viruses were constructed to aid in ecological studies, such as on host-range and persistence of wild-type viruses. In addition, a microcosm has been designed and tested, and limited field releases using recombinant baculoviruses, notably *Autographa californica* nuclear polyhedrosis virus (AcNPV) have been executed.

The current research builds on this knowledge and expertise as well as on developments elsewhere and intends to evaluate the biosafety of engineered baculoviruses which are more effective, but also possess reduced capacity for survival in the field. In this project baculoviruses, in particular ACNPV and possibly other baculoviruses, will be marked and provided with a 'built-in' suicide mechanism. In addition, deletions will be introduced in the virus which reduce its persistence and survival in the environment. The recombinant viruses will undergo extensive laboratory and biosafety testing (host

range testing and microcosms evaluations) before, in a second phase, experimental releases in the environment are considered.

Ultimately, this project will increase our understanding of the ecology of genetically modified baculoviruses and also allow predictions to be made about their behaviour in the environment.

Keywords:
BIOSAFETY, BACULOVIRUSES, GENETIC MODIFICATION, INSECT CONTROL, BIOLOGICAL CONTAINMENT, SUICIDE MECHANISMS, MICROCOSM

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RISK EVALUATION FOR GENETICALLY MODIFIED SOIL MICROBIAL INOCULANTS

CONTRACTNUMBER: BIOT CT-910283
START: 1991-10-01 *DURATION*: 24 months
EC CONTRIBUTION: 460,000 ECU

Objectives:

(1) Development and evaluation of alternative vector systems without antibiotic resistance genes as selectable markers, and of suicide systems for the biological containment of soil microbial inoculants; (2) Risk assessment on the release of soil microorganisms altered in simbiotically relevant traits and evaluation of strains, recovered from inoculated fields, for genetic and phenotypic properties that may be altered since inoculation (computer assisted analysis).

Brief Description:

In order to be able to make an assessment of the risks involved in releasing genetically modified microorganisms (GMMs) into the environment, it is necessary to have information on the potential for such microorganisms to persist, multiply and spread. This project will continue work started in the BAP programme to study the population of (genetically modified) rhizobia released into soils in which they are not indigenous. By isolating rhizobia from such soils it is possible to determine population, and hence survival, persistence and spread in different soils. By analysing the chemistry of the soils, cropping history and other relevant characteristics, it is possible to correlate these with the potential for the (genetically modified) rhizobia to persist. This is being done by using various computer-based statistical packages. The different tasks of the project are distributed as follows among partners: (i) the group at the University College Cork (IE) will primarily deal with the development and evaluation of suicide systems for the biological containment of soil microbial inoculants altered in simbiotically relevant traits, and evaluation of alternative vectors without antibiotic resistance genes; (ii) the group at the University of Padova (IT) will focus on the development of vectors systems with highly expressed catabolic marker genes, without antibiotic resistance genes as selectable markers, and on the

risk assessment of the GMMs released in microcosm and in the field; (iii) the group at the HELIGENETICS (IT) will mainly deal with the baseline study on introduced non-modified and genetically modified soil microbial inoculants; (iv) the group at the University of Bristol (GB) will primarily focus on collection of data on the populations and trends in relevant computer-based retrieval and statistical packages - isolated strains will be checked for genetic and phenotypic properties that may be altered since inoculation.

Keywords:
RHIZOBIA, RISK ASSESSMENT, REPORTER GENES, SUICIDE SYSTEM.

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ASSESSMENT OF A METHODOLOGY FOR THE FAST DESIGN OF FUNGAL DNA PROBES AND PCR TAGS

CONTRACTNUMBER: BIOT CT-910301
START: 1991-12-01 *DURATION*: 24 months
EC CONTRIBUTION: 120,000 ECU

Objectives:

One key need of fungal biotechnology and molecular biology is the design of non ambiguous DNA probes and PCR markers. For patenting and quality control, molecular signatures or markers are useful for identifying natural organisms and their recombinant derivatives. For biosafety, similar non ambiguous markers are a prerequisite for regulatory procedures since EEC regulations request identification and detection tools for genetically modified organisms. The subject of this concerted action is the assessment of a methodology for the rapid design of fungal PCR tags, markers and DNA probes. Such methodology can be considered as a model applicable to other taxonomic groups.

Brief Description:

Two actions will be organized:

1) A bibliographic review of the last development in fungal molecular biology, biotechnology will be made with a particular attention on the identification problems in patenting and biosafety.

2) Coordinated scientific research applying the same methodology to a collection of selected fungal species and genera.

2.1 Methodology

Principle: The basic and simple idea is to amplify genomic regions of interest by PCR, to sequence and align the sequences, to design PCR tags and identify genera or species specific PCR markers. The validated PCR markers can then - outside the frame of this concerted action - be used for detection and identification in luminescence-based or similar methodologies.

Genomic regions of interest:

a) Predetermined regions
   - the 18s ribosomal subunit,
   - the 18s/28S ribosomal spacer region,
   - the adjacent sequences upstream of the 18S ribosomal subunit.

b) Undetermined regions

In this option, random DNA amplification fingerprints are computerized to produce genera/species-specific statistical 'envelope profiles'. Differences between envelope profiles identify those DNA sequences of potential use as DNA probes and/or PCR tags.

2.2 Coordinated assessment of the methodology in the case of fungal species

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 standardization of culture collection aspects,
   - molecular biology (including validation and sampling) and computerization of results.

The potential probes and PCR markers are validated by the different participants with the aim that they are useful for molecular identification of fungi, patent certification and environmental studies.

The collection of strains, their DNA, the sequence data and clones, the probes of PCR primers and the standardized protocols are the practical output to the Community.

Keywords:
IDENTIFICATION, PCR, PROBES, MARKERS, R-RNA, FUNGI, FINGERPRINTING, TAXONOMY

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BRIDGE

T-PROJECTS

T-PROJECT

YEAST GENOME SEQUENCING

SEQUENCING OF THE YEAST GENOME

CONTRACTNUMBER: BIOT CT-900167
START: 1991-01-01 *DURATION*: 36 months
EC CONTRIBUTION: 4,760,000 ECU

Objectives:

Determination of the entire nucleotide sequence of *S. cerevisiae* chromosomes II (840 Kb) and XI (680 Kb) by the end of 1993. Identification, thanks to this systematic sequencing exercise, of over 500 totally new genes never described before.

Brief Description:

As successfully implemented in the previous BAP project 'Sequencing of the yeast chromosome III 'the work is carried out by a highly integrated network of European laboratories. The two DNA coordinators have constructed and distributed ordered cosmidial libraries covering the entirety of chromosomes II and XI. Individual cosmids are allocated to each sequencing laboratory which is then responsible for the subcloning and sequencing of the chromosomal insert. As soon as the entire chromosomal fragment (on average: 35 Kb of DNA) has been fully sequenced a new cosmid is allocated to the sequencing laboratory. In order to ensure accuracy, complete sequencing is carried out on both the two complementary DNA strands and junctions between subclones are confirmed by extensive sequencing of overlaps. Downstream the data are collected and analysed in the informatics coordination centre. With the help of the DNA coordinators the different sequences contributed are progressively ordered and finally merged into a single chromosomal contig. The different Open Reading Frames (ORF) longer than 100 aa identified along the two chromosomes are routinely matched with all the sequences available in databanks. It can be predicted that for more than 500 ORF (out of the 800 expected to be present on chromosomes II and XI) no homologies will be detected, at protein level, to previously known genes from yeast and other organisms. The construction of ordered cosmidial libraries of yeast chromosomes VIII, XII and XIV is also carried out in the framework of this project so as to prepare the ground for the continuation of activities in future EC programmes.

Keywords:
SYSTEMATIC SEQUENCING, YEAST, GENOME, CHROMOSOME, *SACCHAROMYCES CEREVISIAE*

INFORMATICS NETWORK (SEQUENCING THE YEAST GENOME)

CONTRACTNUMBER: BIOT CT-900161
START: 1991-01-01 *DURATION*: 36 months
EC CONTRIBUTION: 300,000 ECU

Brief Description of this part of the T-project:

MIPS, acting as the informatics coordinator of the European Yeast Genome Sequencing Project is responsible for the collection, storage and analysis of all sequence data submitted by the laboratories involved. Submissions arrive by e-mail or on magnetic media. Preliminary sequence data are also sent to MIPS; they provide valuable information about clone orientation and overlapping regions and help in rapidly identifying known genes.
The processing scheme employed is generally outlined as follows:

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
   — 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, autonomously replicating sequences, introns, tRNA genes, other yeast-specific regulatory sequences, repeats

4. Extraction of open reading frames (ORFs). The locations of these ORFs are correlated to the positions of promotors and terminators in order to assess the probability of expression.

5. For the analysis of ORFs, the following steps are employed:
   — FASTA — rapid sequence comparison all known protein sequences available (approx. 40,000).
   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 that do not reflect unambiguous similarities, more sensitive comparison methods are applied.

- pattern search is then performed at the protein level using the ProSite Dictionary of Protein Sites and Patterns.
- 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 to the existing physical map of the chromosome. The new physical map is compared to the genetic map.

Furthermore, the yeast specific sequence databases have been created and are being maintained for the yeast project.

Finally, an on-line computing facility was established that enables participating laboratories to analyze their data and perform database queries.
Two mail servers to query all sequence databases available are open for public use.

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T-PROJECT

MOLECULAR IDENTIFICATION OF NEW PLANT GENES

DEVELOPMENT AND USE OF *ARABIDOPSIS THALIANA* AS A TOOL FOR ISOLATING GENES OF AGRONOMIC IMPORTANCE

CONTRACTNUMBER: BIOT CT-900207
START: 1991-02-01 *DURATION*: 24 months
EC CONTRIBUTION: 3,000,000 ECU

Objectives:

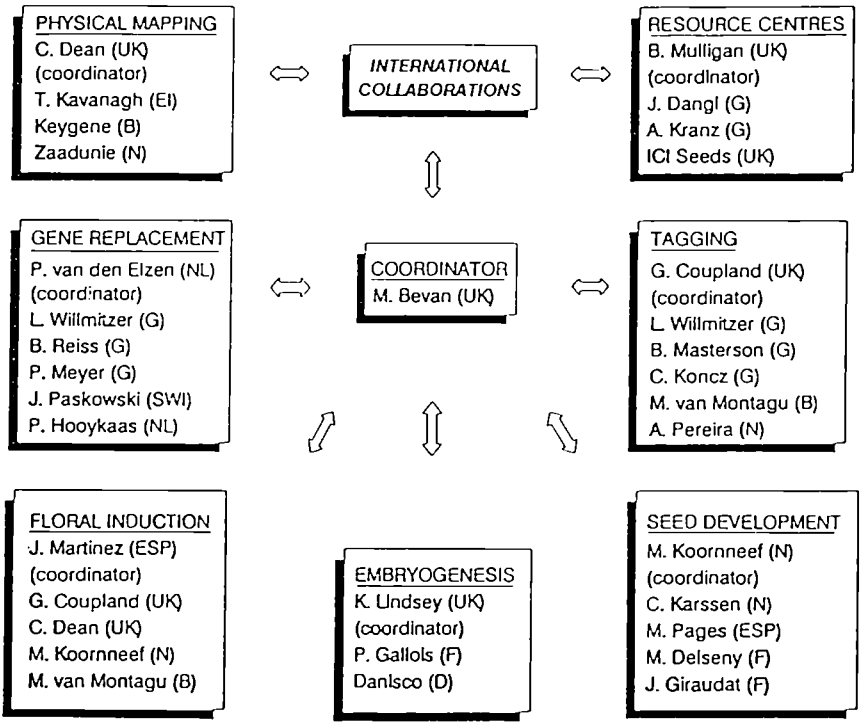
This 'T' project provides a framework that links scientists working on methods for gene identification to scientists dedicated to answering fundamental questions in plant biology using genetics and physiology as their disciplines. This linkage will accelerate the dissemination of techniques for gene search, and their applications to the molecular characterization of genes involved in flowering, seed development and embryogenesis.

Brief Description:

The small genome size, rapid life cycle and good genetic base in *Arabidopsis* makes it an ideal model plant. However, various molecular genetic methods used in other organisms need to be adapted for use in *Arabidopsis*. First, the programme is contributing to an international effort to establish a detailed physical map of the genome using cosmid and YAC libraries. This programme links with those using genome walking to isolate mutant loci conditioning e.g. flowering time and vernalisation requirement. Second, transposon tagging is being developed as an efficient way to generate mutations and to recover the mutated loci. Approaches include using modified versions of the maize elements *Ac* and *En*. An extensive collection of putative mutants caused by T-DNA insertion has been assembled in a stock centre. This is a joint activity with the AFRC (UK). Existing mutant and ecotype collections are being relocated and catalogued to provide a long-term resource for the *Arabidopsis* community. A DNA resource centre has been set up to provide a readily accessible store of recombinant libraries, RFLP probes, etc. for use by the participants in BRIDGE. Third, gene replacement is being studied with the aim of utilising this powerful technique for identifying gene function.

Floral induction is a process of great academic and commercial interest. Existing mutants are being characterised physiologically, mapped using RFLPs and walks to some of the loci have started. New combinations of mutants have been made to determine the pathways leading to floral induction. Seed development is another area of compelling agricultural importance. Mutants affecting seed maturation often involve abscisic acid and these are being mapped and isolated. The effect of these mutations on seed protein deposition and the activity of other genes is also being studied. Finally, a related programme aims to use a novel method called 'enhancer trapping 'to identify genes active in early stages of embryogenesis.

Keywords:
PLANT GENOME ANALYSIS, AGRICULTURAL TRAITS, *ARABIDOPSIS THALIANA*, SEED AND STOCK CENTRES



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T-PROJECT

LIPASES

CHARACTERISATION OF LIPASES FOR INDUSTRIAL APPLICATIONS: THREE-DIMENSIONAL STRUCTURE AND CATALYTIC MECHANISM

Objectives:

The structural and functional characterisation of 10-15 lipases. The aim of the project is to acquire so much new knowledge about a sufficient number of these enzymes that it will be possible to understand why they are lipases and how they function as such.

Brief Description:

To achieve the objectives of the T-project the following research is foreseen:

- the cloning, expression, sequencing, isolation and purification of the lipases to a level sufficient for their (kinetic) characterisation and crystallisation;
- the characterisation of the catalytic mechanism of these lipases;
- the crystallisation and three-dimensional structure determination of these lipases.

This research will be done by five teams of laboratories, each handling a series of specific lipases of microbial and mammalian origin.

To guarantee maximum transfer of information, guidelines have been established in which time periods are defined for the distribution of structural and functional data, obtained with the lipases studied in this project, between the various teams.

Keywords:
LIPASE, ENZYMES, THREE-DIMENSIONAL STRUCTURE, KINETICS, INTERFACE

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3-DIMENSIONAL STRUCTURE AND CATALYTIC MECHANISM OF 2-3 SELECTED LIPASES OF INDUSTRIAL RELEVANCE

CONTRACTNUMBER: BIOT CT-900181
START: 1991-02-01 *DURATION*: 36 months
EC CONTRIBUTION: 750,000 ECU

Brief Description of this part of the T-project:

The present study includes the determination of the 3-dimensional structure and catalytic mechanism of 2-3 triglyceride lipases of industrial relevance. The lipases studied are the fungal lipase from *Mucor miehei* and the mammalian lipase from guinea pig pancreas.

In the case of the *Mucor miehei* lipase, the 3-D structure has previously been determined to a resolution of 2.0 Å. A more detailed understanding of the catalytic mechanism and conformation changes needed for enzyme action will be achieved by co-crystallization of the lipase with inhibitors and substrate analogs and subsequent determination of 3-D structure of the lipase-inhibitor complex.

With respect to structural information, the guinea pig pancreatic triglyceride lipase is unknown in that neither the amino acid sequence nor the 3-D structure of this enzyme is known. This lipase is interesting from a structure/function point of view, due to its ability — in contrast to other lipases — to hydrolyse phospholipids as well as triglyceride. As the enzyme has not previously been studied in details, it will be necessary to isolate, clone and express a recombinant variant of guinea pig pancreatic lipase in order to obtain a sufficient amount of lipase for crystallization and 3-D structure determination. The overall aim is to generate the 3-D structure of guinea pig pancreatic lipase and to compare it with other mammalian lipases, e.g. the human pancreatic triglyceride lipase. In parallel with the structural studies, enzymatic studies on substrate specificity, interfacial kinetics and enzyme kinetics will be carried out with the overall aim of getting a detailed understanding of the mechanism of hydrolysis.

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CHARACTERIZATION OF LIPASES FOR INDUSTRIAL APPPLICATION

CONTRACTNUMBER: BIOT CT-900194
START: 1991-04-01 *DURATION*: 36 months
EC CONTRIBUTION: 586,260 ECU

Brief Description of this part of the T-project:

It is the objective of this project to obtain structural information of lipases from prokaryotic species, in particular from *Pseudomonad* species, by crystallographic methods. The diversity of primary structures of lipases in general points to a diversity of the 3- dimensional properties of lipases as well. Thus it is important to investigate those lipases that are good representatives of the family of lipases they belong to. Earlier studies have indicated that *Pseudomonas* lipases constitute such a distinct family, while these enzymes have excellent properties for application in areas ranging from foods to detergents.

The structural information obtained will be used again to gain understanding of lipolytic activity. Functional aspects will be studied using well-defined, stereochemically pure substrates and substrate analogs of e.g. triglyceride molecules containing amide and either linked fatty acids apart from the normal ester linkages. Kinetic studies in bulk and on those substrates that will form monolayers on water-air surfaces will provide detailed information concerning the mode of action of *Pseudomonas* lipases on organized lipid water interfaces.

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EXPLORING THE STRUCTURE-FUNCTION RELATIONSHIP OF *PSEUDOMONAS* AND *BACILLUS* LIPASES

CONTRACTNUMBER: BIOT CT-910272
START: 1991-08-01 *DURATION*: 36 months
EC CONTRIBUTION: 734,845 ECU

Brief Description of this part of the T-project:

The project aims at elucidating the 3 dimensional structure and the major kinetic properties of lipases from *Pseudomonas* and Bacillus species. The research involves collaboration between 4 laboratories in Germany (Prof. Winkler/Dr. Jäger — Ruhr Universität Bochum), Belgium (Prof. Colson — Université Catholique de Louvain-la-Neuve) and the Netherlands (Dr. Misset — Gist-brocades and Dr. B. Dijkstra — State University Groningen).
From culture filtrates of the mentioned microorganisms (supplied by Misset), the enzymes are purified and (kinetically) characterized (Winkler-*Pseudomonas* and Colson-*Bacillus*) and subsequently subjected to crystallization in order to be able to determine the 3-D structure by X-ray crystallography (Dijkstra). Furthermore, samples of the purified lipases will be kinetically studied by the group of Dr. Verger (Marseille) using monolayer and oil-droplet techniques.
Site directed mutagenesis will be used in order to identify amino acids which are important for the catalytic function of the lipases.

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TRIACYLGLYCEROL LIPASES AND COLIPASES OF THE DIGESTIVE TRACT AND EXOCELLULAR FUNGAL LIPASES: STRUCTURE, INTERFACIAL BINDING AND CATALYSIS

CONTRACTNUMBER: BIOT CT-910274
START: 1991-03-01 *DURATION*: 36 months
EC CONTRIBUTION: 1,030,000 ECU

Brief Description of this part of the T-project:

The aim of this part of the T-project is to elucidate the structure-function relationship of triacylglycerol lipases and colipases of the digestive tract and exocellular fungal lipases (PGS lipase) with special reference to the delineation of the particular domains involved in lipid-protein interaction and to the identification of residues essential for catalysis. The research will include crystallization studies and the preparation of heavy-atom derivatives for the X-ray diffraction analysis of several native and mutated gastric lipase as well as native and mutated PGS-lipases.

2-D and 3-D NMR spectroscopy will be used to elucidate the structure of pancreatic colipases in solution and to investigate the interactions of these colipases in solution with lipid micelles and pancreatic lipase. The kinetics of the lipase hydration state will also be studied using NMR spectroscopy. The topology of the binding domains of colipase will be approached using epitope mapping with monoclonal antibodies as well as site directed mutagenesis. Use of the monomolecular film technology to prove the pancreatic colipase epitopes involved in the lipid binding site and those responsible for the interfacial anchoring of pancreatic lipase. Reversed micellar systems will be used for kinetic and spectroscopic studies of pure lipases. The catalytic activity of lipase crystals will be monitored in solid-gas biphasic systems.

Development of the so-called 'oil drop method 'based on changes in interfacial tension of a triglyceride drop immersed in a lipase solution.

Studies on new lipase substrates and substrate analogs forming mixed monomolecular films. Studies of lipase stereoselectivity on chiral and prochiral substrate analogs.

Biochemical and kinetic investigations on native gastric lipases.

Various stages in the purification, crystallization and cloning of the selected lipases have already been reached by some of the partners. Our aim is either to create or reinforce close collaboration between the participants, whose fields of specialization are all complementary.

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MOLECULAR STRUCTURE AND SPECIFICITY RELATIONSHIP OF MICROBIAL TRIACYGLYCEROL LIPASES

CONTRACTNUMBER: BIOT CT-910258
START: 1991-06-01 *DURATION*: 36 months
EC CONTRIBUTION: 1,240,161 ECU

Brief Description of this part of the T-project:

The project aims at the elucidation of the structure of selected microbial triacylglycerol lipases, of different substrate specificity, using X-ray and high-resolution NMR analysis. This will allow formulation of the molecular basis of the enzymes' catalytic mechanism and specificity when combined with protein chemistry and site-directed mutagenesis.

As the enzymes of choice, lipase from *Geotrichum candidum* (specific towards unsaturated fatty acids), from *Rhizopus arrhizus* (1,3-regio-specific), from *Candida cylindracea* (non-specific) and from *Pseudomonas* and *Thermus* spec. (non-specific, thermostable) are proposed. Three distinctly different *Geotrichum* lipases will be investigated: Unilever '*Geotrichum* B '- which has a unique specificity for d9-unsaturated fatty acids, Unilever '*Geotrichum* A'- which has little specificity for this substrate, and *Geotrichum candidum* Amano ('*Geotrichum* C') which has moderate specificity.

The lipase from *Rhizopus arrhizus* will serve as example for a 1,3-regiospecific lipase with high enantioselectivity. The lipase from *Candida cylindracea* ATCC 14830, due to its wide substrate specificity, is an excellent candidate to study the structure-function relationship of a non-specific lipase. Thermostable enzymes offer interesting opportunities for industrial biotransformations. Thus, in addition, two thermostable non-specific lipases from *Pseudomonas* spec. ATCC 21808 and from *Thermus* spec. will be studied.

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T-PROJECT

BIOTECHNOLOGY OF LACTIC ACID BACTERIA

IMPROVEMENT AND EXPLOITATION OF LACTIC ACID BACTERIA FOR BIOTECHNOLOGY PURPOSES

CONTRACTNUMBER: BIOT CT-910263
START: 1991-03-01 *DURATION*: 36 months
EC CONTRIBUTION: 4,868.500 ECU

Objectives:

The objective on the T-Project on Lactic Acid Bacteria (LAB) is the development and advancement of the knowledge of genetics, molecular biology, physiology and biochemistry of LAB and the identification and/or construction of improved starter culture strains which meet the requirements of relevant industries that are of major economic importance in Europe.

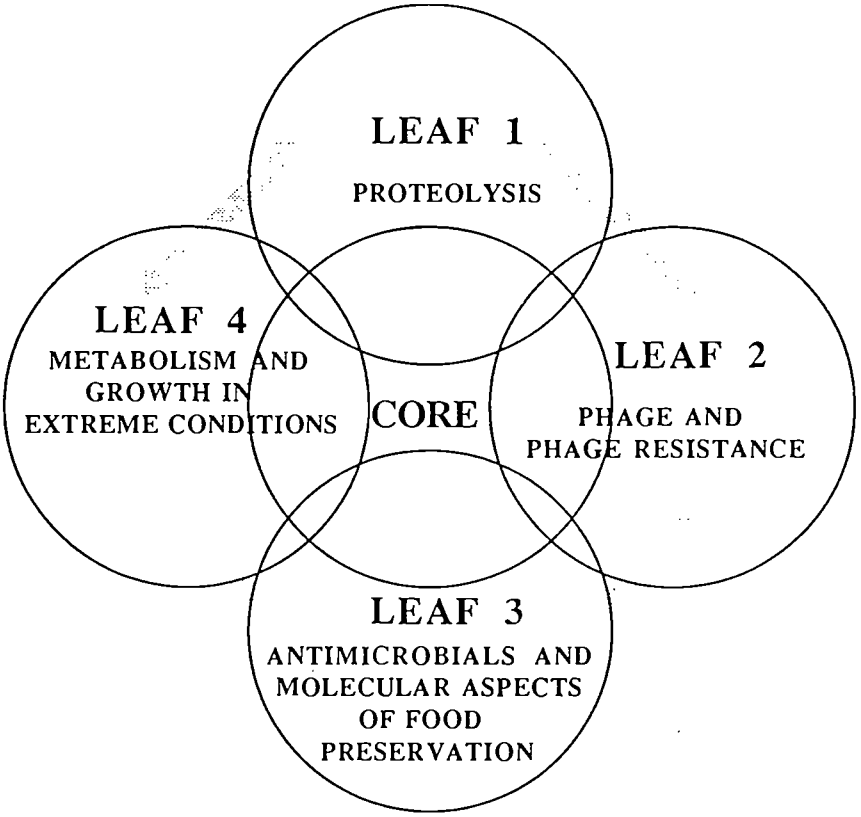
Brief Description:

The 34 participating groups are organised into **CORE** and **LEAF** activities to benefit collaboration and maximise the resource commitments in key areas of research on LAB important in food fermentations.
The programme of the **CORE** groups is intended to address the fundamental understanding and technology development in a range of LAB. This activity is of value in its own right but it also underpins the more applied targets of the **LEAF** programmes. The continued analysis of gene structure, expression and protein secretion in a variety of systems and the development of improved vector systems are prominent activities. Chromosome analysis including natural and engineered gene integration processes is targeted and provides one of a number of distinct approaches to the development of food compatible gene cloning strategies. Considerable effort is also devoted to the development and understanding of conjugative systems in LAB.
The groups in the **Antimicrobials LEAF** will exploit expertise in screening technologies, biochemistry and molecular biology to identify and characterise antimicrobial substances produced by LAB. This task is a prerequisite to the selection of strains for improved food preservation.
The research of the **Metabolism and Screening LEAF** will provide a detailed understanding of the molecular genetics and biochemistry of key metabolic functions of LAB important in dairy, meat, bread and wine fermentation. In addition, modern molecular techniques will be used to improve the screening and classification of LAB and enhance the understanding of their ecology.

The **Bacteriophage and Phage Resistance LEAF** will address the significant problem posed by phage mediated inhibition of starter cultures used food fermentation processes. The dual approach involves the study of the molecular basis of the phage/host interactions and the isolation and characterization of natural phage defense mechanisms from LAB.
The groups participating in the **Proteolysis LEAF** will focus on the increased understanding of the proteolytic system of LAB by genetic and biochemical studies of proteinases, peptidases and transport systems.

Keywords:
LACTIC ACID BACTERIA, GENE TECHNOLOGY, METABOLISM, BACTERIOPHAGE, ANTIMICROBIALS, SCREENING

CORE-LEAF INTERACTIONS IN THE T-PROJECT ON LACTIC ACID BACTERIA



CORE ACTIVITIES:

CHROMOSOME ANALYSIS
REGULATION OF GENE EXPRESSION
CONJUGATION SYSTEMS

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T-PROJECT

HIGH RESOLUTION AUTOMATED MICROBIAL IDENTIFICATION (HRAMI)

HIGH RESOLUTION AUTOMATED MICROBIAL IDENTIFICATION: IMPROVEMENT OF NUCLEIC ACID PROBE TECHNIQUES

CONTRACTNUMBER: BIOT CT-910294
START: 1991-10-01 *DURATION*: 24 months
EC CONTRIBUTION: 2,109,000 ECU

Objectives:

The major objectives of the proposed research are: i) to develop high resolution molecular methods for the rapid identification of microorganisms, ii) to assess the utility and to compare the efficiency of the different methods developed, iii) to exploit them to expand our microbial taxonomy base, in particular to microorganisms from natural environments, and iv) to automate the developed methods.

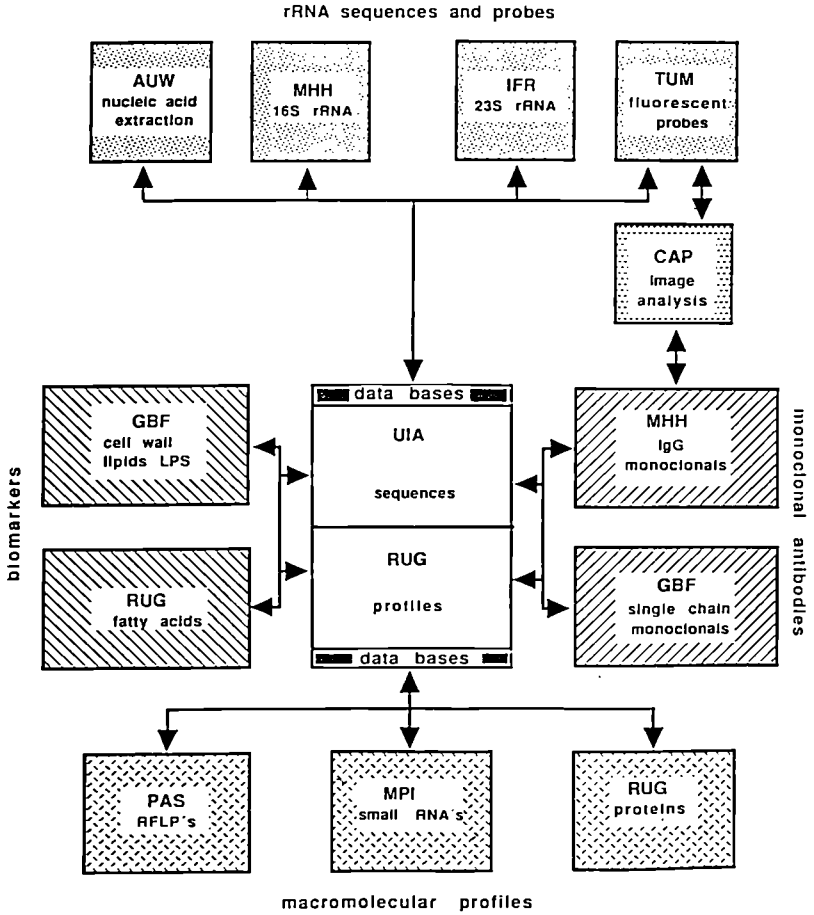
Brief Description:

A multidisciplinary research consortium (9 research laboratories and 1 software company) will develop **technology** in the fields of molecular sequence analysis, immunology, analytical chemistry, instrumentation and separation science, and apply this technology to the rapid and accurate identification of microorganisms. In a first step, ribosomal RNA sequences, chemical 'biomarkers', macromolecular 'profiles 'and stable antigens, will be used to characterize a standard set of commercially and environmentally relevant groups of microorganisms. Within these different analytical approaches determination and alignment of rRNA sequences (16S and 23S) enable definition of higher taxonomic relationships (genus-level and above) and thereby generate the backbone of a microbial taxonomy based on the phylogeny of microorganisms. Analysis of specific rRNA sequences will allow construction of species-specific oligonucleotide probes for bulk and single cell detection in environmental samples. The chemotaxonomic approach will emphasise the search for qualitatively new microbial compounds (biomarkers) using high resolution analytical instrumentation like pyrolysis mass spectrometry and tandem mass spectrometry on whole microbial cells. All major macromolecules of the microbial cell (DNA, RNA and proteins) will be used to obtain fingerprints (profiles) by high resolution elec-

trophoresis that enable a rapid overview of large sets of individual strains for their taxonomic grouping. The study of stable antigens will lead to strain-specific monoclonal antibodies used in automated bulk detection and single cell detection via epifluorescence microscopy. In a second step, the results of this polyphasic approach will be compared and evaluated with respect to identification utility and analytical facility. Those analytical systems found to be useful will be used to expand taxonomic data bases (i.e. MINE) and will be evaluated in terms of their applicability to the identification, quantitation, and sensitive detection of microbes in environmental samples. Additionally, the probes (nucleic acids, antibodies, etc.) generated for rapid identification and taxonomic purposes will be used for the development of highly sensitive procedures for the detection of specific organisms in environmental samples either *in situ* or following extraction from complex environmental matrices. Finally, these new methods and probes are to be automated for the analysis of large numbers of environmental samples in order to study pressing environmental issues.

Keywords:
MICROBIAL TAXONOMY, RIBOSOMAL RNA SEQUENCES, IMMUNOCHEMISTRY, BIOMARKERS, MACROMOLECULAR PROFILES, MOLECULAR PROBES, CHEMOTAXONOMY, MOLECULAR MICROBIAL ECOLOGY

HRAMI Organization and Information Flow



GBF = *Gesellschaft für Biotechnologische Forschung*
AUW = *Agricultural University Wageningen*
MHH = *Medizinische Hochschule Hannover*
IFR = *Institute of Food Research (AFRC)*
TUM = *Technische Universität München*
PAS = *Institut Pasteur*
RUF = *Rijks Universiteit Gent*
UIA = *Universitaire Instelling Antwerpen*
CAP = *Computer Applied Technologies Ltd (CAPTEC)*

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T-PROJECT

ANIMAL CELL BIOTECHNOLOGY

T-PROJECT ANIMAL CELL BIOTECHNOLOGY

Objectives:

Many products of major therapeutic value can be obtained only from animal cells grown in culture. The aim of this project is to improve the processes currently employed in this sector of biotechnology with special emphasis on the vectors and promoters used and on the quality of post translational modification.

Brief Description:

There are many products currently obtained from animal cell culture including TPA (for treatment of Heart Attacks) E.P.O. (for treatment of anaemia) Interleukins and other cytokines (for treatment of cancers) and monoclonal antibodies (for diagnostic uses and for imaging and targeted delivery strategies as well as for immunosuppressive treatment of transplanted patients). However much progress remains to be made. In this T project research will focus on the types of vectors that are used. Specifically it will be an aim to test the value of targeted integration into a preselected locus of the chromosome and of using artificial chromosomes based either on a collage of elements from different systems or on an anti tumourogenic linear virus. In all these instances the result should be a predictable location of the DNA within the cell. The promoter systems currently in use will also be extended by the isolation of promoters from CHO and by the transfer of well characterised procaryotic promoter systems to the animal cells. Finally the essential abilities of animal cells to process and modify the protein when it is synthesised will be studies in detail both in terms of glycosylation and specific endo proteolytic activities. All of the above activities will be carried out in conjunction with cell culture studies and including media composition, and in one case reactor design with the targeted disruption of some of the intracellular processes to provide information on the consequences for cell physiology.

Keywords:
GENETIC ENGINEERING, ANIMAL CELL CULTURE, VECTORS, PROMOTERS, GLYCOSYLATION, TARGETED INTEGRATION, ENDOPROTEINASES

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CONTROL OF RECOMBINANT PROTEIN GLYCOSYLATION UNDER DEFINED CULTIVATION CONDITIONS

CONTRACTNUMBER: BIOT CT-920304
START: *CONTRACT UNDER NEGOTIATION*
DURATION: 24 months
EC CONTRIBUTION: 260,000 ECU

Brief Description of this part of the T-project:

The posttranslational modification of biotechnologically produced therapeutic/diagnostic glycoproteins will be studied and compared by applying different fermentation techniques for recombinant mammalian cell lines (BHK-21 cells). Cultivation systems used in animal cell culture technology will be evaluated including: perfused stirred tank and airlift reactor, immurement and entrapment techniques; influence of high density cultures, coated microcarriers; effect of energy sources as well as presence/absence of FCS/protein additives will be controlled. Secreted products will be purified and the respective carbohydrate structures will be elucidated in detail. At the cellular level, studies will be carried out on the control of cellular nucleotide sugar level, formation of lipid intermediates as well as efficiency of initiation of N-and O-glycosylation. In addition, determination of the activity of cellular glycosyltransferases will allow a detailed description of the effect of different cell culture conditions on the cellular glycosylation machinery which determines the final carbohydrate structure of biotechnologically prepared glycoproteins. Modelling of culture conditions will be performed leading to controlled posttranslational modification of products in long term culture. A procedure will be established enabling rapid batch analysis of purified glycoproteins with respect to their carbohydrate status ('oligosaccharide-mapping ') at the sub-milligram level.

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OPTIMALIZATION AND VALIDATION OF VIRUS LINEAR VECTORS: INNOVATIVE TOOLS FOR THE CONTROL OF ANIMAL DISEASES

CONTRACTNUMBER: BIOT CT-920305
START: 1992-01-01 *DURATION*: 24 months
EC CONTRIBUTION: 260,000 ECU

Brief Description of this part of the T-project:

This work concerns the optimalization of linear, parvovirus-based vectors for the transfer and expression of foreign genes in mammalian cells. Existing molecular clones of parvovirus MVM will be modified by site directed mutagenesis of the P4 promoter and/or downstream regulatory sequences, in order to boost expression from this promoter.

A packaging cell line, constitutively expressing capsid proteins, will be constructed for the obtaining stocks of recombinant parvoviruses.

In order to extend the range of systems allowing the sustained propagation of replication-competent MVM-based vectors, new parvoviral and cellular variants will be isolated that produce non-cytotoxic NS proteins or are resistant to the wild-type protein, respectively. The hyperactivity of parvoviral promoters in a number of transformed cells will be exploited to achieve targeted killing of these cells by the products of appropriate genes places under parvoviral control. These can either be toxins (HSV-1 thymidine kinase in combination with acyclovir) or products that stimulate the immune system (cytokines). The interference of parvovirus driven interleukin genes with the formation of tumours in experimental animals will be assessed.

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IMPROVEMENT OF PRODUCTION OF BIOACTIVE PROTEINS BY GENETICALLY ENGINEERED ANIMAL CELLS USING THE NOVEL FURIN-CLASS OF MAMMALIAN PROTEOLYTIC PROCESSING ENZYMES

CONTRACTNUMBER: BIOT CT-910302
START: 1992-04-01 *DURATION*: 24 months
EC CONTRIBUTION: 810,000 ECU

Brief Description of this part of the T-project:

A large variety of bioactive mammalian proteins is known to have such valuable properties that great scientific effort is invested to find ways for their controlled production in animal cells. Modern cellular and molecular technologies have enabled identification and isolation of the genes that encode such proteins and the corresponding genetic sequences constitute the basic material to genetically engineer animal cells to manipulate production. A number of events are or importance here. For efficient production, proper sorting within a secretory pathway, transport and release into the extracellular environment is required. In many instances, the primary translation product is a precursor protein that has to be specifically cleaved into a mature bioactive product. Furthermore, post-translational modification (glycosylation, phosphorylation, acetylation, amidation, sulfation, methylation) is often involved in the maturation process of these components. Finally, to ensure high levels of controlled expression in genetically engineered animal cells, appropriate vector systems are to be used.

Our research program in this T-project contains the following projects:

— Proprotein processing using the novel furin-class of processing enzymes;

— Expression of active forms of the furin-like prohormone converting endopeptidases;

— Identification of genes involved in sorting, post-translational modification and secretion of peptide hormones;

— Development of extrachromosomal vectors for introduction and amplification of foreign genes in animal cells;

— Expression of cloned genes into mammalian cells using retroviral- and EBV-derived vectors;

— Production of bioactive proteins by animal cells in large scale cell culture;

— Processing of precursor proteins for blood clotting factors.

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TRANSGENIC ANTIBODIES: DEVELOPMENT OF AN INTEGRATED VECTOR SYSTEM FOR THE EXPRESSION OF IMMUNOGLOBULINS IN DIFFERENT CELLULAR COMPARTMENTS OF MAMMALIAN CELLS

CONTRACTNUMBER: BIOT CT-920306
START: *CONTRACT UNDER NEGOTIATION*
DURATION: 24 months
EC CONTRIBUTION: 390,000 ECU

Brief Description of this part of the T-project:

It is anticipated that in the future monoclonal antibodies will be routinely isolated from phage libraries. Similarly, the expression of binding domains derived from antibodies within different cellular compartments of mammalian cells is likely to become a widely used technique to inhibit the function of recognised molecules. An important feature of the phage antibody technology is that the process of antibody selection is simultaneous with that of the cloning of the corresponding encoding sequences. It would, therefore, be highly desirable that vectors for the expression of antibody domains within mammalian cells should be compatible with the phage vectors used to select antibody specificities. The development of such an integrated vector system for the expression of immunoglobulin domains within different cellular compartments of mammalian cells after they have been selected from phage antibody libraries is the objective of this project.

By the end of this project we would expect a researcher to be able to screen a phage library of diversity large enough to allow the selection of a monoclonal phage antibody (as ScFv of FAb) which recognises the antigen used for screening at a useful affinity. The cloning of the antibody binding specificity into an appropriate vector using convenient and consistent sites would allow expression of the selected antibody in the most suitable form (as ScFv, FAb, or complete antibody) within the particular cellular compartment of interest of a mammalian cell (intracytoplasmic, nuclear, secretory, endoplasmic reticulum), by the incorporation of appropriate protein tags at N or C terminal ends. The use of different promoters (tissue specific, inducible, constitutive) and vectors (plasmid, retroviral) will allow further refinement of expression. By this means the expression of targeted molecules is expected to be inhibited in situ.

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CONSTRUCTION OF PERMANENTLY TRANSFECTED CELLS EXPRESSING STEROID HORMONE RECEPTORS

CONTRACTNUMBER: BIOT CT-920308
START: *CONTRACT UNDER NEGOTIATION*
DURATION: 24 months
EC CONTRIBUTION: 280,000 ECU

Brief Description of this part of the T-project:

Steroid hormone receptors are known to be transcriptional activators. They therefore carry the potential to control the expression of target genes. In nature they are expressed at very low levels. In this project a novel approach will be used to increase the level of their expression and indeed the expression of other proteins of interest. The methods used will involve the transfer to animal cells of the complete transcription mechanism from prokaryotic systems using the lac operon and repressor, the T3 promoter and the T3 RNA polymerase as the expression cassette. In this way the information which is available from prokaryotics on the control of expression and the achievement of high levels of expression will be transferred in one unit into the animal cells. In an extension of this project the cell lines which are developed which over express the steroid hormone receptor will be useful to screen for analogues of steroid hormones which may have therapeutic benefits.

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TARGETTED INDUCIBLE AMPLIFIED HOMOLOGOUS EXPRESSION SYSTEM FOR QUALITY PRODUCTS FROM ANIMAL CELLS IN CULTURE (ANIMAL CELL EXPRESSION SYSTEM — A.C.E.S.)

CONTRACTNUMBER: BIOT CT-920307
START: *CONTRACT UNDER NEGOTIATION*
DURATION: 24 months
EC CONTRIBUTION: 519,500 ECU

Brief Description of this part of the T-project:

The aim of this project is to:

(i) isolate promoters from CHO which are strong and/or inducible by alterations in the culture conditions;

(ii) achieve targeted integration of DNA into a region of the CHO genome which may be amplified; and

(iii) monitor the glycosylation profile of a protein made in CHO under these different conditions. At all stages comparisons will be made with the vector host systems currently in use. The project involves both genetic and cell culture methods to achieve the goal of improved productivity from animal cells.

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T-PROJECT

FACTORS REGULATING PLANT CELL GROWTH AND DIFFERENTIATION

THE MOLECULAR ANALYSIS OF HIGHER PLANT EMBRYOGENESIS

CONTRACTNUMBER: BIOT CT-900206
START: 1991-01-01 *DURATION*: 36 months
EC CONTRIBUTION: 28,000 ECU

Objectives:

The primary objective is to produce basic knowledge on the mechanisms by which a variety of signals control growth and cell differentiation. The secondary objective will be to develop tools at cellular and molecular levels to render morphogenetic events accessible to scientific analysis.

Brief Description:

The T-project will contribute to the elucidation of the mechanisms of action of plant signals, mostly how growth factors are perceived and transduced, and how individual cells are committed to differentiate. Five sub-groups integrate a variety of interests and expertise including molecular and cellular biology, biochemistry, plant physiology, cytology, etc. All five give a marked attention to the regulation of the early steps of key morphogenetic events, with the following specific attributions:

1. Perception, Interaction and Response of Plant Growth Regulators (900158)*

2. Molecular Analysis of Auxin-Specific Signal Transduction in Plant Cell Communication (900178)*

3. rol Genes as Privileged Tools to Study Plant Morphogenesis (900179)*

4. Molecular Analysis of Higher Plant Embryogenesis (900177)*

5. Regulation of the Induction of Microspore Embryogenesis (900160)*.

Interactions between the subgroups are based on common interests in the mechanisms of action of different growth factors, exchanges of

methodologies and analytical tools, transfer of knowledge from gene expression studies to the field of hormone perception, similarity of communication pathways which determine the fate of various developmental processes, cross-links between auxin responses and somatic embryogenesis.

*: contract numbers as reported on the following pages.

Keywords:
RECEPTORS, PLANT HORMONES, AUXINS, ETHYLENE, FUSICOCCIN, ANTIBODIES, AGROBACTERIUM, DIFFERENTIATION, HAIRY ROOT, EMBRYOGENESIS, EXTRACELLULAR GLYCOPROTEINS, CELL WALL PROTEOGLYCANS, OLIGOSACCHARINS, REGENERATION

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REGULATION OF THE INDUCTIVE PHASE OF MICROSPORE EMBRYOGENESIS

CONTRACTNUMBER: BIOT CT-900160
START: 1991-01-01 *DURATION*: 36 months
EC CONTRIBUTION: 540,000 ECU

Brief Description of this part of the T-project:

Many plant species, including economically important crops, cannot be regenerated from single cells *in vitro*. Consequently, such plant species cannot be exploited to their full potential by plant biotechnology companies. As our contribution to solving this problem, we are investigating the mechanism controlling the induction of cell proliferation *in vitro*. We are using *Brassica napus* microspores as a model system since freshly isolated microspores can be redirected to an embryo formation pathway at frequencies of up to 70%. Heat treatment (32°C) of an 8 hour duration is sufficient to initiate and sustain the microspore embryogenic process. The *B. napus* microspore culture system has a number of other characteristics well suited for such studies. The investigation concentrates on the first 8 hours of microspore culture initiation, a time period which precedes the first embryogenic nuclear division.

The specific objectives of the project are as follows:

(a) Isolation and characterization of genes unique to the inductive phase of microspore embryogenesis.

(b) Characterization of modulation and regulation of the cytoskeleton using light and electron microscopy; special attention is also being paid to *in situ* localization of newly isolated gene products associated with induction of microspore embryogenesis.

(c) Isolation of cytoskeleton-associated proteins phosphorylated during the inductive process.

(d) Determination of the inheritance of the embryo induction characteristic in a plant population. Genetic analysis is being carried out on crosses between plants containing microspores differing in their ability to be induced to undergo embryogenesis subsequent to heat treatment.

(e) Adaption of the results of this project to the needs of the plant breeding programs in industry. To this end, comparisons are being made between the embryogenic potentials of *Brassica napus* and *Hordeum vulgare* microspore cultures to identify common molecular features of microspore embryogenesis induction in dicots and monocots.

The results of this project will help us to understand why microspores of some economically important plant species cannot be induced to undergo embryogenic development *in vitro* and open the possibility to manipulate and transfer genes regulating induction of microspore (cell) proliferation to tissue culture non-responsive plant species.

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PLANT GROWTH REGULATORS: PERCEPTION, INTERACTION AND RESPONSE

CONTRACTNUMBER: BIOT CT-900158
START: 1991-01-01 *DURATION*: 36 months
EC CONTRIBUTION: 713,000 ECU

Brief Description of this part of the T-project:

Four complementary, integrated approaches will be used in the project as follows:

1. The isolation of genes for the components of perception and transduction chains for auxin, ethylene and fusicoccin, to examine the expression of such genes during development, to manipulate them and to introduce (e.g. antisense) constructs into appropriate host plants (*Arabidopsis*, tobacco, tomato) in order to probe for function.
2. Using *in vitro* reconstitution systems to demonstrate the functionality of identified components of perception and transduction chains and to determine whether interactions occur between components.
3. To use protoplasts to study the functionality of binding proteins for auxin, ethylene and fusicoccin and to determine whether interactions are observable, and through the use of impermeant analogues and specific anti-receptor antibodies to determine whether surface or intracellular receptors control similar or separate pathways.
4. To use affinity columns constructed from putative receptors to separate, purify and identify components of transduction chains..

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THE MOLECULAR ANALYSIS OF HIGHER PLANT EMBRYOGENESIS

CONTRACTNUMBER: BIOT CT-900177
START: 1991-01-01 *DURATION*: 36 months
EC CONTRIBUTION: 946,300 ECU

Brief Description of this part of the T-project:

In this project we will first focus on the functional analysis of a set of molecular markers that are specific for defined stages in the development of somatic embryos in carrot (Daucus carota L.). These markers are monoclonal antibodies that recognize cell-surface proteoglycans, cDNA clones encoding secreted glycoproteins and purified secreted glycoproteins that affect embryo development. Based on one of the monoclonal antibodies the pathway leading to cells that have acquired embryogenic competence will be explored. The secreted glycoproteins will be analysed by molecular techniques and for their potential enzymatic activity on cell walls.

Secondly, we will relate the markers obtained to the development of zygotic embryos. This approach has already shown itself to be highly profitable, since most of the molecular markers described in this proposal were originally obtained from *in vitro* grown cells and were subsequently shown to be highly specific for various stages of somatic as well as zygotic embryo development. Taken together, we can now begin to answer the question whether parallel molecular events occur during the reprogramming of explant cells finally leading to somatic embryos in tissue culture and during the reprogramming of cells embarking on the gametophytic pathway.

Thirdly, the application of the markers obtained to somatic and zygotic embryogenesis in other plant species will be investigated.

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THE ROL GENES AS PRIVILEGED TOOL TO STUDY PLANT MORPHOGENESIS

CONTRACTNUMBER: BIOT CT-900179
START: 1991-03-01 *DURATION*: 36 months
EC CONTRIBUTION: 846,800 ECU

Brief Description of this part of the T-project:

The research projects of the different subgroups of group 3 within the T-Project 'Regeneration 'concern various aspects and levels of the morphogenesis triggered by the T-DNA genes of *Agrobacterium rhizogenes* in plants. Thus the University of Rome (DGBM) concentrates on the regulation of *rolB*, whose promoter has been shown to be under hormonal, developmental and tissue-specific regulation. The role of *rolB* in triggering rooting is being studied at the Max-Planck-Institut (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. At MPI also *rolC* is being studied and a conjugated cytokinin hydrolase activity assigned to its gene product. The Institut des Sciences Végétales in Gif (ISV) is mainly concerned with ORFs 13 and 14, the characterization of their promoters and the identification of their gene products. The University of Paris (CEMV) is characterizing at the histological and ultrastructural level the developmental and morphological modifications induced by the *rol* genes *A*, *B* and *C* in collaboration with MPI and the University of Antwerp (UIA) where various advanced microanalytical techniques are being utilized to measure hormone levels in even small districts of plant tissues transformed by the *rol* genes. At the MPI-associated laboratory of Moet-Hennessy (LVMH), *Agrobacterium*-based transformation techniques are being developed for woody roses in order to exploit the developmental modifications induced by *rol* genes. 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 in Montelibretti (IREV).

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MOLECULAR ANALYSIS OF AUXIN-SPECFIC SIGNAL TRANSDUCTION IN PLANT COMMUNICATION

CONTRACTNUMBER: BIOT CT-900178
START: 1991-01-01 *DURATION*: 36 months
EC CONTRIBUTION: 837,000 ECU

Brief Description of this part of the T-project:

Auxins evoke a remarkable diversity of growth responses, including differentiation and morphogenesis, control of apical dominance and the stimulation of root growth. The advent of the powerful techniques for genetic engineering plant transformation and electrophysiological analysis offers a unique opportunity to experimentally investigate the molecular mechanisms of auxin action. Detailed knowledge in plant signal transduction, cell division and its hormonal control will be essential to finally control plant regeneration.

Aims of research

- to identify and characterize genes encoding putative receptors for the phytohormone auxin with a view to understanding at the molecular level the primary physiological and morphogenic consequences of auxin action;
- to investigate properties of intracellular signalling chains including genes encoding GTP-binding proteins or genes controlling plant cell division;
- to use transgenic plants to assign functions to these genes and determine directly their influence on plant growth control and morphogenesis.

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Summary

The Commission of the European Communities is implementing several priority actions specifically designed for improving the competitiveness of European biotechnology. One of these actions aims at the establishment of a Community network for training and research and has been executed from 1982 to 1989 in the framework of two successive Community programmes: the Biomolecular Engineering Programme (BEP; 15 million Ecu; April 1982-March 1986) and the Biotechnology Action Programme (BAP; 75 million Ecu; 1985-1989). These programmes aimed therefore at establishing a supportive infrastructure for biotechnology research in Europe and the elimination of bottlenecks which prevent such exploitations. The current programme BRIDGE (*B*iotechnology for *I*nnovation, *D*evelopment and *G*rowth in *E*urope) covers the period 1990-1993, with a budget of 100 million Ecus. The research activities in the 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. These encompass 579 participating organizations (388 for the N-projects and 191 for the T-projects) from 11 Member States and 5 EFTA countries. The N-projects and T-projects are described in two different parts of this catalogue of contracts. The catalogue gives for each of these projects a title, the contract number, a summary of the objectives, as well as a brief description and a number of keywords. This is followed by a list of names and addresses of each of the participants in the projects. Indexes of participants, contract numbers and keywords are given at the end.

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