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PROCEEDINGS OF THE 4th INVESTIGATORS' MEETING FOR EPA'S BIOTECHNOLOGY-BIOLOGICAL CONTROL AGENT RISK ASSESSMENT RESEARCH PROGRAM APRIL 8-12, 1991

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PREFACE

The Office of Research and Development (ORD) in the U.S. Environmental Protection Agency is responsible for providing EPA program offices with scientific and technical information on biotechnology products intended for environmental release. This includes research on genetically engineered microorganisms (GEMs) and biological control agents (BCAs). To accomplish this, ORD created a Matrix-managed program for Biotechnology/BCA Risk Assessment and designated the Gulf Breeze Environmental Research Laboratory as the lead laboratory. To assure coordination and promote multi-disciplinary research approaches, the Matrix manager holds periodic meetings attended by laboratory and headquarters scientists and cooperative agreement and contract researchers. This document is a compilation of extended abstracts of the research presented at the 1991 All-Investigators meeting, the fourth meeting of this type. The meeting was held at the Holiday Inn Crowne Plaza, Crystal City, Virginia, April 8-12. Extended abstracts submitted from specially invited guests representing biotechnology efforts in Canada and several European countries reflect the international collaboration theme of the meeting. These invited abstracts are followed by scientific presentations from EPA laboratory, cooperative agreement and contract personnel separated into the three program element research areas: exposure, effects and risk control.

SECTION 1
INTRODUCTION

During the last two decades, sophisticated scientific advances in molecular biology have increased our ability to change or combine the inherited characteristics of microorganisms. The power of recombinant DNA techniques to make extremely precise alterations in the genetic character (genotype) and expression (phenotype) of organisms has created both the promise of enormously useful products as well as concern over potential environmental release.

Among the potential industrial products and applications of this technology are microorganisms designed to degrade toxic pollutants, leach minerals, enhance oil recovery, produce industrial chemicals, and act as pesticides. These activities fall within EPA's regulatory purview under the Toxic Substances Control Act (TSCA) and, for pesticidal applications, the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) as established by the Coordinated Framework for Regulation of Biotechnology (EPA, 1986). These regulations were intended to protect the environment from perturbation by industrial products regardless of the scientific process of their manufacture, and are therefore applicable to the products of biotechnology.

EPA regulators rely on the results of research and development programs to provide the scientific and technical knowledge required for successful regulatory policies. Risk assessment methods for predicting the risk posed by some chemical pesticides and toxic industrial chemicals, including fate and effects data, are reasonably well developed and have been incorporated into regulatory policies.

The more dynamic properties presented by microorganisms, including replication, mutation and gene transfer, coupled with the complexity of molecular biological techniques create a high degree of uncertainty when risk assessment methods developed for chemicals are applied to genetically engineered microorganisms (GEMs) or biological control agents (BCAs). BCAs include microbial pest control agents i.e. *Bacillus thuringiensis* derivatives, and biochemical pest control agents, such as insect growth regulators. Concern over the potential risks introduced by the environmental release of biotechnological products and the need to reduce uncertainty in assessing risk led the Agency to establish the Biotechnology/Biological Control Agent Risk Assessment Research Program in 1985.

Participating ORD research laboratories and their expertise areas are listed below:

Gulf Breeze, FL., Environmental Research Laboratory (GBERL), Lead Laboratory, Fate and Effects of GEMs and BCAs in Marine and Estuarine Ecosystems.

Corvallis, OR., Environmental Research Laboratory (CERL), Fate, Effects, and Transport of GEMs and BCAs in Terrestrial Ecosystems.

Duluth, MN., Environmental Research Laboratory (DERL), Fate and Effects of MPCAs in Freshwater Environments.

RTP, NC., Health Effects Research Laboratory (HERL), Human Health Effects from GEMs and MPCAs.

Cincinnati, OH., Risk Reduction Engineering Laboratory (RREL), Control of Risk through Containment and Decontamination.

Las Vegas, NV., Environmental Monitoring Systems Laboratory (EMSL), Aerosol Dispersal of Biologicals, Model Development.

Also, the Office of Environmental Processes and Effects Research in EPA Headquarters at Washington, DC., serves as a liaison between ORD and Program Offices.

ORD scientists have developed a complementary extramural research program fostering close interaction with academic scientists in the fields of genetics, biochemistry, ecology and molecular biology. This cooperative agreement process promotes collaboration between diverse disciplines to solve research problems often resulting in scientific advancement. Over twenty-five universities in the U.S. and abroad have contributed to EPA's program and to scientific information exchange. Scientists at EPA laboratories and under EPA cooperative agreements have further facilitated scientific exchange by participating in national and international scientific meetings, publishing journal articles, and participating in research coordination workshops.

The knowledge base for characterizing the risk posed by GEMs/BCAs continues to improve and has potential to estimate *and* to reduce those risks. The use of information developed for assessing risk to create predictive models and to develop control strategies represents a shift in our capabilities towards risk reduction. Research designed to improve our understanding of problems and to identify promising solutions to those problems are important and recognized tools for reducing environmental risk. EPA funded research provides an important basis for continued development of methods and techniques needed for reliably evaluating the behavior of microorganisms and microbial communities.

The ecological processes mediated by microorganisms are crucial to the operation and health of ecosystems. If these processes are affected by environmental stress resulting from the introduction of novel microorganisms or their toxic by-products, then the potential for affecting the rest of the ecosystem is significant. Continued development of methods for evaluating and improving the quality of ecosystems is necessary if strategies for assessing and reducing deleterious changes in ecosystems health are to be effective, timely, and relevant. The research program's mission is to develop these methods and techniques and to provide guidance in their appropriate application for characterizing and reducing risk to the environment.

Extended scientific abstracts describing EPA funded research comprise most of this report, the fourth in a continuing proceedings series. The abstracts are grouped by broad research areas of environmental exposure, environmental and human health effects, and control of risk through mitigation and containment strategies. These abstracts demonstrate the scientific direction and accomplishments of EPA's Biotechnology/Biological Control Agent Risk Assessment Program.

SECTION 2
INVITED SPECIAL PRESENTATIONS

Information dissemination and technical information transfer are important facets of EPA's Biotechnology Risk Assessment Program. To improve international understanding and foster collaborative research efforts, representatives from several European countries, Canada, USDA, and EPA were invited to present an overview of their biotechnology efforts. Extended abstracts of those presentations comprise this section.

M. Hoefle, National Research Center for Biotechnology, Braunschweig, Germany

P. Kearns, European Organization for Economic Cooperation and Development, Paris, France

M. Reuss, Ministry of the Environment, Charlottenlund, Denmark

T. McIntyre, Environment Canada, Hull, Quebec, Canada

D. MacKenzie, National Biological Impact Assessment Program, U.S. Department of Agriculture, Washington, D.C.

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W. Schneider, Office of Pesticides Programs, U.S. Environmental Protection Agency, Washington, D.C.

OVERVIEW OF GESELLSCHAFT FUR BIOTECHNOLGISCHE FORSCHUNG mbH/NATIONAL RESEARCH CENTER FOR BIOTECHNOLOGY (GBF)

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The GBF (Gesellschaft fur Biotechnolgische Forschung mbH/ Research Institute for Biotechnology had its origins in the "Institute for Molecular Biological Research (GMBS)" started and funded by the Volkswagen Foundation. Since the inception of GBF, it has been financed by the Federal Government, represented by the Federal Ministry of Research and Technology (BMFT) on the one hand, and the State of Lower Saxony on the other, to a ratio of 90:10. The articles of the company incorporated in 1976 reinforce the structure of GBF as a research-oriented national research centre. The statutes governing the GBF were amended in 1984. The GBF is a member of the AGF (Association of National Research Centres), and employed a total of 590 staff at the end of 1989.

The objective of the company is the operation of a multidisciplinary research centre in the area of biotechnology with the aim of contributing to the solution of public duties and tasks by means of comprehensive biotechnological research and development work. The research and development activities at the GBF are basically located within the framework of the Federal Government's "Applied Biology and Biotechnology" programme. The GBF pursues exclusively peaceful purposes, publishing the results of scientific work.

The structure and operation of the bio-pilot plant based on interdisciplinary cooperation, facilitates the work on research projects within the GBF as well as in the form of cooperation in working groups involving industry, universities and other institutions. Accordingly the GBF is devoted above all to the following tasks:

* applications-oriented basic research in the areas of microbiology, cell biology, molecular biology, genetics, biochemistry, natural product chemistry, enzyme technology and bioengineering,

* the development of new pharmacological and technically significant natural products and research on their mode of action,

* development of new biotechnological processes as a contribution to maintaining an assured supply of basic products for the chemicals, pharmaceutical and foodstuff sectors, as well as processes for the reduction of environmental pollution,

* the transfer from the biotechnological laboratory process to the semi-industrial scale as a precondition for the development of tried industrial processes,

* the support of external research groups in the sectors of biology, chemistry and medicine by the provision of natural products from enzymes and biomasses, which are not available commercially,

* use of the scientific-technical equipment and installations at the GBF as a service to projects performed by external research groups and industry,

* participation in joint projects, in particular within the framework of the biotechnology programme of the Federal Ministry of Research and Technology (BMFT); and in addition, participation in the main areas of research of bioengineering together with the universities of Braunschweig, Hannover and Gottingen,

* enhanced interdisciplinary training of scientists, engineers and technicians in the framework of further training courses on a national and international basis, and

* strengthening of the national and European biotechnology infrastructure by the provision and performance of scientific services such as informatics (data bases), or the analysis of biological molecules.

THE MAIN AREAS OF RESEARCH AND PROJECTS OF THE GBF

In 1989 the research activities at the GBF were undertaken under the framework of the following four main strategic research areas:

1. Biosynthesis and biocatalytics
2. Biomolecules and molecular design
3. Environmental biotechnology
4. Biochemical engineering

These involve the screening for new materials as well as method-oriented developments. A balance between target-oriented basic research and industrial development work is sought.

The projects were essentially performed in the framework of interdisciplinary cooperation by numerous departments/researchgroups at the GBF. The central facilities of the bio pilot plant service unit and the central computer group provided support to these activities. Project participation is clearly presented in the matrix below. The scientific organization of the GBF is detailed in the organigram at the end of this abstract.

Scientific Organization of GBF

■ Priority
▒ Essential Work
□ Secondary Activities

Main Areas of Research	Projects	Microbiology			Cell Biology Genetics					Enzyme Technology Natural Product Chemistry				Biochemical Engineering					
Divisions		Microbiology			Genetics					Enzyme Technology				Biochemical Engineering					Industrial Partners
Departments Research Groups		Microbiology	Microbial Secondary Metabolites	Microbial Transformations	Genetics	Genetic of Eukaryotes	DNA-Synthesis	Molecular Immunology	Immune Biology	Enzyme Technology	Natural Product Chemistry	Enzymology	Molecular Structure Research	Biochemical Engineering	Cell Culture Technique	Downstream Processing	Material Science	Fermentation Service Unit	
Biosynthesis Biocatalysis	Biologically Active Secondary Metabolites from Microorganisms	▒	■	▒	▒	▒	▒	▒	▒	▒	■	▒	▒	▒	▒	▒	□	■	●●
	Biotransformation of Natural Compounds, especially Terpenoids			■									▒					□	●●
	Enzymatic Reactions									■									●●
	Biosensors									■									●●
	Enzymes from Higher Cells											■							
Biomolecules Molecular Design	Protein Design	□			■	□	■			▒			■	▒		□	□		●●
	Immuno Modulators	■			▒	□		■	■										●
	Regulations of Eukaryotic Genes				□	■													●●
	Cell Cycle Dependent Gene Expression				▒														
	Bone Growth Factors				■														
Environmental Biotechnology	Biological Detoxification of Environmental Pollutants	■			▒	▒	▒	▒	▒	▒			■	■				□	●●
	Biological Safety in Handling Mammalian Cells					▒													
	Inactivation of Recombinant DNA													▒					
	Behavior of Recombinant Micro-organisms in Model Ecosystems	■			□														
Biochemical Engineering	Animal Cell Culture Techniques					■				▒					■	▒			●●
	Optimization and Modeling of Fermentation Processes													■		□	□		●●
	Down Stream Processes													□		■	□	□	●●
	Computer Applications and Control Techniques													■	▒			▒	●
	Materials for Biotechnology						□								□		■		●●

The main research areas and projects of the GBF 1989/90 and the participation of the departments and research groups in the projects. The first projects of each research area is the central project in this field. (oo means cooperation with two or more industrial partners.)

THE WORK OF OECD RELEVANT TO THE ENVIRONMENTAL IMPLICATIONS OF BIOTECHNOLOGY

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THE OECD IN BRIEF

The Organization for Economic Co-operation and Development (OECD) is an inter-governmental organization which is part of the system of Western international institutions developed after World War II.

OECD replaced the Organization for European Economic Cooperation (OEEC) which was founded in 1948 to administer the Marshall Plan of the United States.

There are 24-member countries which are the free-market democracies of North America (USA & Canada), Western Europe (e.g. the EC & EFTA nations, Switzerland, etc.) and the Pacific (Australia, New Zealand & Japan). The Commission of the European Communities also participates in OECD work, as does Yugoslavia as a "special status" country.

It is worth nothing that these account for only 16% of the world's population but produce two-thirds of its goods and services, three-fifths of its exports, and generate four-fifths of economic aid to developing countries.

The basic aims of the OECD, as described its founding Convention, are:

1) to achieve the highest sustainable economic growth and employment,

2) to promote economic and social welfare throughout the OECD area by co-ordinating the policies of its Member countries, and

3) to stimulate and harmonize its Members' efforts in favor of developing countries.

For those of you with an interest in history, you might like to know that following the signing of the Convention on 30 September 1960, OECD was established on 30 September, 1961. This year is the 30th anniversary.

HOW DOES THE OECD WORK?

Each of the Member countries maintains a permanent delegation of OECD which is headed by an ambassador. The ambassador represents his country on the Council, the supreme body of the OECD which normally meets about once a week. The Chairman of the Council is the Secretary-General of the OECD.

Once a year, the Council meets at Ministerial level under the Chairmanship of one of the national Ministers.

The Council, which operates on the principle of consensus, produces *Decisions*, which are legally binding on Member countries. The Council also makes *Recommendations* which are more an expression of political will than something binding.

The Council also approves the work of numerous specialized committees,

expert groups and working parties which carry out the major part of OECD's work.

To give you a flavour of the scope of the work, a few of the main Committees are:

Economic Policy,
Trade,
Financial Markets,
Energy Policy,
Scientific and Technological Policy,
Environment,
Agriculture,
and Management Committee of the Special Programme on the Control of Chemicals.

The common approach of public policy of the OECD countries means that discussions on a given issue usually reach consensus. It is then possible to establish "rules of the game" or codes of behavior, for a given topic, to which the participants can bind themselves. The OECD has given rise to several such agreements, for example, an agreement on the movement of hazardous wastes over borders.

OECD WORK ON BIOTECHNOLOGY

OECD work on Biotechnology really began in 1982 with the recognition, by the OECD Committee for Science and Technology Policy, that Biotechnology was an major emerging field from which various policy issues would arise with wide scientific and economic impacts.

Consequently, a group of national experts was convened to consider future OECD work in this area. Their report on "Biotechnology: International Trends and Perspectives (OECD, 1982)" is still, in many ways, a forward-looking study of many of the issues associated with use of biotechnology.

The authors recognized the need for a unique multidisciplinary approach integrating the development and implementation of policies and technical instruments in the areas of science and technology research, industry, agriculture, education, environment, and health and worker safety.

As a follow-up to this activity, a study on "Safety and Regulations in Biotechnology" was undertaken. That study led to a *Recommendation* of the OECD Council concerning Safety Considerations for Applications of Recombinant DNA Organisms in Industry, Agriculture and the Environment which called for further research to improve the prediction, evaluation and monitoring the outcome of application of recombinant DNA organisms. This work also led to the publication of "Recombinant DNA Safety Considerations (OECD, 1986)".

A new programme of work, began in 1988, is being carried out by the OECD Directorate for Science, Technology and Industry (DSTI) in co-operation with the Environment Directorate, under the supervision of a group of National Experts on Safety in Biotechnology.

The Group of National Experts decided that part of the programme should be the development of general principles which would identify a generic approach and guidelines for the design of small-scale field research with genetically modified plants and micro-organisms. As this work evolved, the Group of National Experts recognized

the need for developing criteria and test methods for the environmental monitoring of genetically modified organisms in "Good Developmental Practices for Small Scale Field Research with Genetically Modified Plants and Microorganisms: A Discussion Document (OECD, 1990)".

The OECD Secretariat was called on to analyze monitoring methods and data requirements with a view of identifying the most promising approaches for conducting and evaluating the deliberate release of genetically engineered organism to the environment.

As part of subsequent efforts to prepare a publication, an "International Survey on Biotechnology Use and Regulations (OECD/ Environmental monograph no. 991, 1990)", information on monitoring programmes for the environmental release of GMOs in the Member countries was requested. While this information was not included in detail in the survey report, some information was annexed.

Additional information is summarized in BIOTRACK which is OECD's computerized Pointer System on the Use of Genetically Modified Organisms. BIOTRACK contains summarized details of over 260 releases of genetically modified organisms in Member countries.

As a further step on the OECD work on monitoring releases of genetically modified organisms , a workshop was held in Copenhagen the first week of December, 1990. This workshop was an opportunity for experts from a variety of member countries to exchange views and experiences. The report of the workshop is currently in preparation and will include two main parts. The first, "Principal Findings of the Workshop", is comprised of three sections: General Approaches to Monitoring; Plants - Monitoring Approaches and Methods; and Microorganisms - Monitoring Approaches and Methods. The second part is suggestions for future work and priorities.

I have stressed our interest in monitoring because that is the aspect of the work that I feel is most relevant to a meeting like this.

However, there are other relevant biotechnology activities in the OECD programme; for example, there is work underway to elaborate Good Industrial Large Scale Practices, and this work is likely to result in a publication before much longer. There is also work underway on Food Safety and Biotechnology.

NATIONAL ENVIRONMENTAL RESEARCH INSTITUTE
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PERSPECTIVES AND EXPECTATIONS OF BIOTECHNOLOGY

Since genetic engineering started accelerating in the mid 1970s there have been great expectations of this new technology. Both industry and agriculture interests hoped that biotechnology would soon be able to solve a number of problems. However, we now must acknowledge that the speed of the development has not been quite what we expected.

In spite of this, several genetically engineered microorganisms have been developed and are now exploited commercially. So far, it has mostly been industry that has benefitted from biotechnology and the new organisms have been well-known strains of microorganisms, which have been used in fermentation industry for many years. The microbes have been engineered to produce new or modified products in fermentation. Several Danish industries have been able to implement the new technology.

LEGISLATION

In June, 1986, the Danish parliament, passed the worlds first comprehensive act regulating genetic engineering. The act introduced tight regulation from the start, partly on the basis of previous experience with the regulation of pollutants and partly because this was a new area involving possible risks. The act will be revised as we acquire more insight into the problems resulting from discharge and release of genetically engineered microorganisms.

In connection with the administration of the act, the National Environmental Research Institute's (NERI) job is to provide the necessary professional expertise needed by the National Environmental Protection Agency in order to evaluate the risks and decide on the rules in this area.

NERI has participated in a group working with the realization of the OECD concept GILSP (Good Industrial Large-Scale Practice). The idea of GILSP is that certain organisms may be categorized as low risk organisms and used in ordinary production systems where the discharge of organisms is limited as much as possible. In 1990, two directives were introduced on genetically modified organisms which are now being incorporated in Danish legislation. So far, only contained use of genetically engineered microorganisms has been approved in Denmark. Approval has been given for the use of the organisms in fermentation plants' production and restrictions have been imposed on the number of

living cells in the effluents.

The organisms used so far have been strains that do not survive in the environment. They have a very reduced frequency of gene transfer compared to the parental strains and they do not produce substances that constitute any environmental risks.

In the future however, an increasing number of genetically engineered microorganisms will be deliberately released into the environment. These organisms will be designed to carry out various tasks in the environment, e.g. biodegradation of recalcitrant organic pollutants or biological pest control. These organisms will have a much larger potential of survival than the organisms used for industrial production. They will be able to persist in the environment at least while they perform the function for which they were designed. Because of their new traits some will interfere with ecological processes such as mineralization and nutrient cycling. Viewed in an ecological perspective this type of genetically engineered microorganisms poses a risk of unintended ecological impact on the environment. We have no experience in assessing this kind of risk as the release of genetically modified organisms is a completely new activity with no previous examples that can help in identifying the potential dangers.

The research at NERI has been focused on the ecology of microbes and their influence on ecological processes. By experimenting in contained ecological models (microcosms) researchers have tried to elucidate what happens to microorganisms released into nature.

DETECTION OF GENETICALLY ENGINEERED MICROORGANISMS

To be able to study the fate of genetically modified microorganisms in the environment or in experiments in the laboratory sensitive detection techniques are needed.

The classical microbiological methods are all based on identification and enumeration of the organisms on selective media. By composing media with a very special content of essential nutrients it is possible to select the type of organisms growing on the media.

Very specialized media have been made for detection of specific microorganisms as well as general media for the enumeration of total number of bacteria in environmental samples.

Genetically engineered micro-organisms have very often been endowed with antibiotic resistance that can be used for detection. These organisms can be grown in the presence of concentrations of antibiotics that inhibit the indigenous microflora and when samples are plated on media containing one or more antibiotics only resistant organisms, i.e. the genetically engineered strains, form colonies.

Other traits such as special temperature requirements or the ability to degrade toxic organic compounds may be useful for detection.

Plating on selective media is still the most frequently used method of detection and the one that new techniques are compared with.

However, selective plating has several drawbacks. The plating and the colony counting are very labor-intensive. Many microorganisms grow very slowly on the plates, and often the results are only obtained after several days.

The most serious drawback of selective plating is, however, that not all bacteria from environmental samples are able to form colonies. Bacteria which are stressed, e.g. when released into waste water or into the environment, are able to change into a dormant state. Bacteria in this state (viable-but-nonculturable) are not dead as they may be brought back to a culturable form under certain circumstances. They are however, unable to grow on the selective media they would otherwise grow on. The phenomenon has first been described for pathogenic bacterial strains that are stressed and enter the viable-but-nonculturable state when they are released into the environment, but it seems now to be a very general bacterial characteristic.

It is possible to reinduce the culturable state for pathogenics again through injection into a host-organism, but the mechanisms governing this are unknown.

When organisms are in a nonculturable state they may be present in the environment but undetectable when using selective plating. Because of the general problems concerning the selective plating method and because the development of biotechnology has made specific detection of genetically engineered bacterial strains necessary, research has been directed towards the development of alternative detection methods. What these methods all have in common is that they are direct, meaning they do not imply cultivation of the organism in the laboratory. Direct detection methods are based on immunological and molecular biological principles. At NERI selective plating as well as direct methods have been implemented in the detection of microbes.

Previously, an ELISA (Enzyme - Linked Immunosorbant Assay) has been implemented for the detection of bakers yeast producing insulin in industrial waste water. This method has been used in monitoring the survival of yeast in the field.

For the validation of terrestrial microcosms (see below) a method for detection of *Enterobacter cloacae* by immunofluorescence has been developed. In this analysis, specific polyclonal antibodies react with cells of *E. cloacae*. After treatment with fluorochromconjugated secondary antibodies cells can be counted with the aid of an epifluorescence microscope only.

Detection of microorganisms based on molecular biological methods has focused on DNA hybridization. By the use of a specific DNA probe for a 2,4-D degrading *Alcaligenes eutrophus* strain, it has been possible to study the survival of the bacteria in soil microcosm and water samples.

It is a necessary step for several molecular biological approaches to the study of microbial communities and for the detection of specific microorganisms in nature to be able to extract purified DNA from environmental samples.

Extraction and purification of DNA from environmental samples is not an easy task. Lysis has to be effective on

all bacteria present in the sample and once extracted, the DNA is contaminated by various organic substances. At NERI a great deal of work has been put into the implementation and the improvement of methods for recovering high quality DNA from complex environmental samples.

MICROCOSMS

It is internationally agreed that microcosms are an essential experimental tool for research in microbial ecology and for the risk assessment of genetically engineered microorganisms.

At the microbiological department of NERI aquatic and terrestrial microcosm (ecological models) have been constructed. The models have been designed to contain parts of natural ecosystems in such a way that physical, chemical and biological conditions are kept as natural as possible.

The philosophy behind the structure of the models is that because microorganisms in nature are controlled by a complex set of determinants, complex ecosystem models are essential to studies of the ecology of genetically engineered microorganisms and their possible ecological effects.

Only little is known of the factors controlling microbes. Recreating the natural ecosystems in the laboratory as far as is possible is the best way to ensure that all decisive factors are included in the experiment.

But experiments in microcosms cannot stand alone. Experiments designed to uncover causalities on a more detailed level are needed.

The context for the design of the microcosms has been to create an as natural as possible model system in the laboratory and it has therefore, been important to investigate how well the models simulate nature. Extensive validations of the models have been carried out.

The aquatic models have been used to simulate the eutrophic Lake Bagsvaerd and validation has been made by comparing important ecological variables measured in the models to similar measurements in the field. Microcosms have been run for up to 4 months and an array of variables have been measured: total bacterial numbers determined by microscope and by colony forming units on plates; the numbers of fluorescent Pseudomonads; changes of bacterial community structure; bacterial production; primary production; algal biomass and the concentrations of inorganic nutrients.

The overall conclusion is that with the actual microcosm design it is possible to simulate important features of aquatic ecosystems.

At the bacterial level there is a very high concordance between measurements made in the models and in the lake. This is important as the models are intended to be used for studies of bacterial ecology.

The microcosm validations also reveal that the model is able to function satisfactorily through long experimental periods (months) and that replication between uniformly treated microcosms is very high. Therefore, it will be possible to study microbial populations throughout many generations in the models and to discover long term

ecological effects of the organisms' presence and activities in the environment.

The terrestrial microcosms have been validated by conducting the same experiment in the field and in the microcosms. A non-genetically engineered strain of *Enterobacter cloacae* was sprayed on barley and bean plants growing in the field and in microcosms.

Survival of the bacteria was followed for 7 weeks on the leaves of the plants and in the soil. *E. cloacae* survived equally well in the two systems and at the end of the experiments the numbers of organisms present in the field and in the models was the same.

In the field experiment the numbers of *E. cloacae* that could be detected by selective plating decreased dramatically immediately after spraying, while the numbers detected by immunofluorescence were unchanged. The field release took place during a spell of drought and the discrepancy between the two detection methods may be explained by stress suffered by the bacteria that were sprayed into the dry environment. This stress may have induced a non-culturable state. After a couple of weeks the number of culturable cells increased and at the end of the experiment were indistinguishable from the numbers determined by the direct method. In the microcosms none of this was seen, probably because the models did not simulate the dry period and the rain. The conclusion about the behavior of the terrestrial microcosms is that they are able to simulate ecological conditions that are of importance to bacterial survival and activity, but that extreme climatic conditions cannot be simulated.

CONCLUSION

Through the extensive validation of both types of microcosms we have obtained important knowledge of some of the factors determining the extent to which ecological models resemble natural ecosystems.

Experiments with genetically engineered microorganisms which will be carried out in the microcosms will benefit from the thorough evaluations of ecological parameters, by making extrapolations to nature possible from results obtained in the models. Extrapolations will be meaningful because the model behavior and the model limitations are well known.

In the near future the models will be used in studies of the fate and the possible ecological effects of genetically engineered microorganisms in the environment. This work will be done in collaboration with international scientific groups which work on the same topics.

The molecular biological approaches to detection will be an important and integrated part of the future work and one of the techniques that are going to be implemented is the polymerase chain reaction (PCR). By PCR it is possible to improve the detection limit for DNA hybridization to 1-10 cells per gram of soil or millilitre of water.

Extensive research will be put into the study of possible ecological effects of the introduction of genetically engineered microorganisms into the environment.

DEVELOPMENT OF REGULATORY AND ENVIRONMENTAL OVERSIGHT FOR BIOTECHNOLOGY IN CANADA UNDER THE CANADIAN ENVIRONMENTAL PROTECTION ACT (PRESENTATION TRANSCRIPT)

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INTRODUCTION

Environment Canada is currently in the process of finalizing draft regulations for biotechnology products under the Canadian Environmental Protection Act. This Act, passed in 1988, provides for a comprehensive regulatory scheme to control toxic substances at each stage in their life cycles: from development and manu-facture; through transport, distribution, use and storage; to their ultimate safe disposal as wastes. The purpose of my presentation this morning is threefold:

i) Provide an update on the extent of organizations currently carrying out biotechnology R&D activities in Canada, (Figure 1);

ii) Share the proposed methodological approach for the regulation of biotechnology products by Environment Canada under the Canadian Environmental Protection Act; and finally

iii) Highlight ongoing in- house research and development activities in support of the regulatory oversight initiatives of Environment Canada.

In discussing the environmental components of the CEPA biotechnology regulations this morning, I will briefly examine three forces that greatly influenced the structure and methodological approach proposed in our draft regulations.

1) CEPA AND THE LEGISLATIVE FRAMEWORK FOR ENVIRONMENTAL PROTECTION

Our proposed biotechnology regulations are based on a number of elements underlying the legislative framework for CEPA that features:

CEPA AND THE LEGISLATIVE FRAMEWORK FOR ENVIRONMENTAL PROTECTION

* Strong prevention focus
* Life cycle approach to management of toxic substances (cradle to grave)
* Shift in onus of proof to manufacturer/ importer
* Integration of environmental and health reporting
* An ecosystem approach to environmental protection

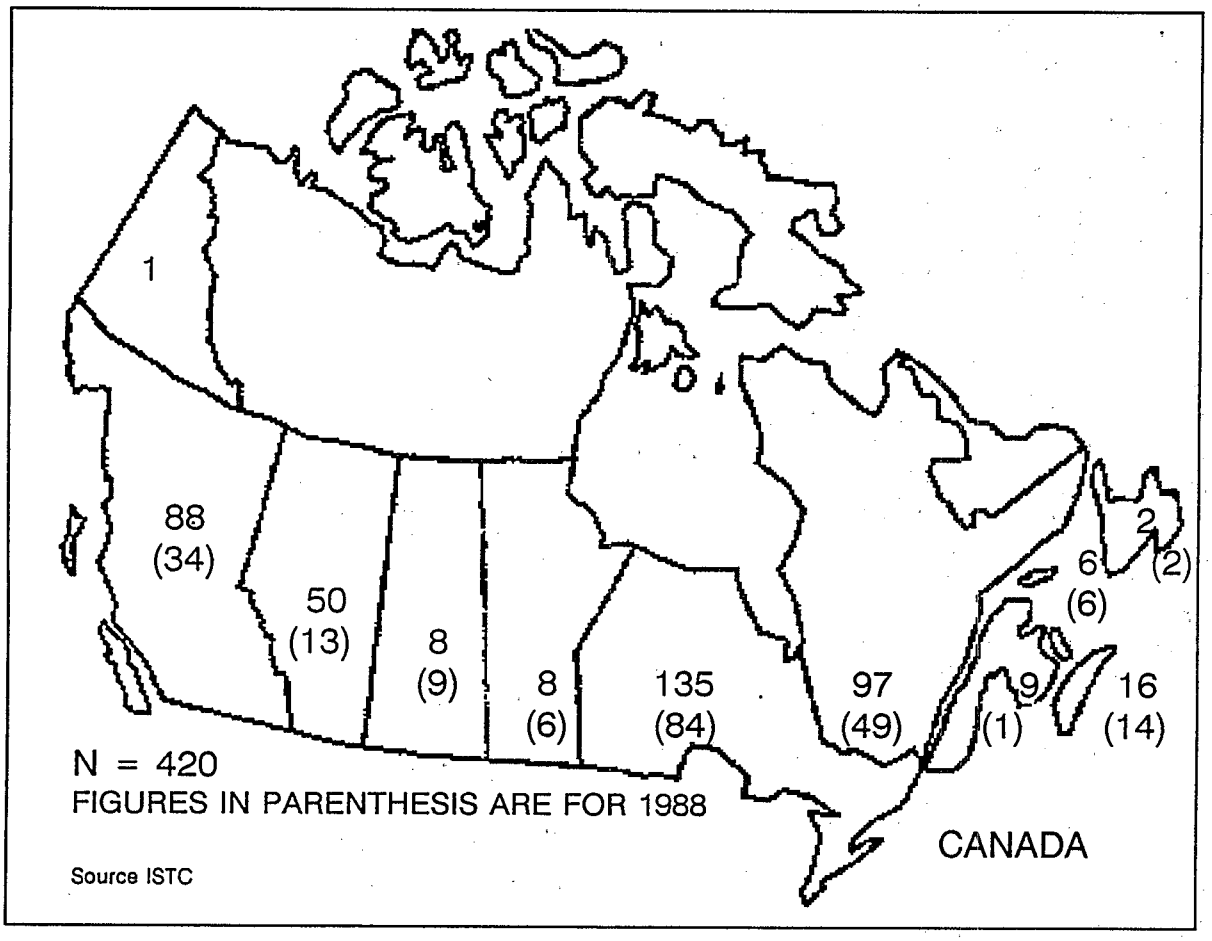

Figure 1. Geographical distribution of organizations carrying out biotechnology R&D activities in canada 1990.

CEPA AND THE LEGISLATIVE FRAMEWORK FOR ENVIRONMENTAL PROTECTION (CONT.)

* National Inspection plan and enforcement policy based in criminal law
* Extensive public role in the administration of the act
* Innovative mechanisms for federal-provincial partnerships
* Enforceable standards for federal lands, works and initiatives
* Environment economy linkages and environmental quality objectives

2) Our draft regulations acknowledge criteria developed at the national level as essential components of a regulatory framework for biotechnology. Some of these include:

CRITERIA CONSIDERED AS ESSENTIAL COMPONENTS OF A REGULATORY FRAMEWORK FOR BIOTECHNOLOGY

* Engenders public confidence
* Makes economic sense
* Transparent to all affected stakeholders
* Facilitates industrial planning for development
* Compatible with international approaches
* Flexible and accommodating of new approaches
* Clarifies jurisdictional responsibilities
* Draws upon independent scientific advice

3) Our regulations acknowledge certain working principles that have evolved in the international community for the regulation of biotechnology products that include:

FEDERAL GOVERNMENT WORKING PRINCIPLES FOR THE REGULATION OF BIOTECHNOLOGY PRODUCTS IN CANADA

* Use of a standard definition of biotechnology
* Build upon existing legislation where ever possible
* Regulate product as opposed to process
* Assess biotechnology products on a case by case basis
* Build upon internationally developed guidelines and harmonization whenever possible at both federal and international levels

PART II Methodological Approach

General provisions of the biotechnology regulations under the Canadian Environmental Protection Act include:

* Products assessed under the proposed Regulations include micro-organisms to be used in a variety of applications such as, but not limited to, bioremediation, mineral leaching, degradation of chemicals, mining, waste treatment, waste disposal, chemical production, lignin degradation, microbial enhanced oil recovery, biosensors, and energy production.

* An interactive and staged approach to notification and provision of information is used, based on the stages of development of the product towards commercial exploitation. The corresponding information requirements and assessment periods build on those of previous stages of notification.

* Notification is required prior to manufacture or importation for contained uses beyond the scale of laboratory research.

* Information will be assessed within prescribed assessment periods.

* CEPA contains explicit provisions for protecting confidential business information.

* The waiver provisions of CEPA provide flexibility for dealing on a case-by-case basis with specific products where the progression through the stages is either not appropriate or necessary, or for waiving information requirements that may not be applicable for the product.

* A mechanism is proposed for listing specific uses of microorganisms for which notification would not be required (Schedule XVI).

Categories of Notification

Five stages of development are defined for notification.

For contained use, notification is required:

* prior to research and development beyond laboratory scale (Schedule XI)

* prior to commercial manufacture (Schedule XII)

For environmental introduction, notification is required:

* prior to small-scale field trials (Schedule XIII)

* prior to large scale field trials (Schedule XIV)

* prior to commercial production (Schedule XV)

Information Requirements

Each of the five information schedules specify the application information requirements. Categories of information to be submitted include:

* identification and characterization of the microorganism(s)

* data on environmental fate and effects on nontarget substances in the environment

* human health safety testing data

* description of intended uses, manufacturing methods and quality control and quality assurance procedures

* specifics on field trials including location and procedures to be used during the trial, where applicable

PART III Biotechnology Research

In development of the CEPA biotechnology regulations, we recognized that a properly drafted and administered regulatory framework can be a catalyst and not an impediment to technology development.

We recognize the need for flexibility in addressing the needs of the dynamic and expanding biotechnology industry in Canada. The flexibility we are building into the regulations is linked to and dependent upon information concerning both human health and the environment. Environment Canada is funding and continues to fund basic research in microbial ecology addressing such issues as:

* mechanisms for gene exchange in the environment
* development of methods for detection, monitoring and determination of environmental fate of microorganisms,
* microbial ecological profiles on specific genus of organisms targeted for environmental release,
* environmental codes of good practice for contained research and development involving biotechnology
* characterization and quantification of emission streams from bioprocessing facilities, and
* identification of factors/characteristics that affect survival, persistence, and dissemination of microorganisms in the environment.

The data generated by our research programs will not only assist us in our regulatory program but will be a source of valuable scientific information on the behavior of microorganisms in the environment for industry, academia, and other government departments.

We constantly seek to promote national and international cooperation and information exchange in biotechnology through our involvement in a number of interdepartmental committees under the National Biotechnology Strategy, the OCED, federal provincial regulatory consultations, and more recently, under a memorandum of understanding (MOU) we have with the Environmental Protection Agency in United States.

We believe that we are establishing a sound and responsive health and environmental oversight structure for biotechnology in Canada under the Canadian Environmental Protection Act.

Conclusion

The design of a regulatory oversight mechanism for any activity, particularly one as novel and exciting as biotechnology will not be an easy task. We are however, guided in our endeavours by previous experiences in the regulatory arena that have demonstrated unequivically , that a consensus building process involving all stakeholders can be a powerful and effective tool for the establishment of an appropriate regulatory framework.

USDA'S BIOTECHNOLOGY RISK ASSESSMENT RESEARCH GRANTS PROGRAM

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Section 1668 of the 1990 Food Security Act (a.k.a., Farm Bill) directs the Secretary of Agriculture to establish a grants program in biotechnology risk assessment research. The program is to be funded as a 1% set-aside of the Department's biotechnology research expenditures. This Section also specifies necessary coordination with EPA and consultation with Department agencies in establishing the direction and activities of the grants program.

The responsibilities for administering the grants program have been assigned to the National Biological Impact Assessment Program. At the time of this writing, discussions are underway on the dimensions of the program, and the mechanisms for funding. A meeting with representatives from the appropriate federal agencies and interested public groups provided a starting point for discussions on how the program could best meet the spirit of Section 1668.

One of the obvious intentions of the program is to meet the needs of federal regulatory agencies for sufficient information to make informed, science-based decisions on regulatory permits for field testing with genetically modified organisms. It has thus been proposed that an advisory committee solicit "wish lists" each year of researchable questions that could be incorporated into the annual request for proposals. The received grant proposals would then be "flagged" for interest by the regulatory and research agencies of the USDA. Subsequently, a panel of independent peer scientists would rank the proposals for scientific merit. These rankings would then be reviewed by the regulatory and research agency representatives for interest and appropriateness. The scientifically meritorious, high-priority proposals would then be recommended for funding.

Through these mechanisms, a new set of questions could be asked each year, but the duration of the research undertaken to answer those questions would likely extend for several years to obtain the necessary answers. For instance, a research proposal might look at seed dormancy factors of genetically engineered canola. It might be necessary to study the seed buried in different types of soil over a period of years. This reality would not limit the program from asking new questions each year, and then funding them for a period of time appropriate to the question.

Another recommendation from the first meeting was that an annual conference should be held with the scientists receiving funding from the program. This conference would be a forum for the exchange of information, and for annually reporting results to all

interested parties. It has been proposed that this annual meeting of the USDA Biotechnology Risk Assessment Research Grants recipients be linked to the EPA's Biotechnology Risk Assessment Research annual meeting, for mutual benefit. This proposal is now under discussion between the two agencies.

What has not been decided as of this writing is the definition of biotechnology, which will be critically important for determining the amount of funds available to support the grants program in risk assessment research. A very broad definition of biotechnology for the Department of Agriculture would circumscribe about $140,000,000, yielding at 1% $1.4 million for risk assessment research. A very narrow definition of biotechnology research being conducted in the field by the U.S. Department of Agriculture would yield $60,000 - a rather insignificant sum.

Whatever the final outcome of the mechanisms used to select and support biotechnology risk assessment research, there is a clear and evident commitment by the Department of Agriculture to launch this program as a responsible complement to its ongoing investment in biotechnology research. The solicitation for proposals should be issued early summer 1991 for funding in early Fiscal Year 1992 (begins October 1, 1991).

THE EPA PERSPECTIVE ON BIOTECHNOLOGY

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For centuries, people have used living organisms to produce useful substances such as bread, cheese, beer, and wine, and more recently to perform useful functions such as the control of harmful insects. Initially, use of the organisms was empirical; however, over the centuries people have developed techniques to manipulate the genetic materials of useful organisms to select and enhance desired characteristics. Recently, powerful new techniques in molecular biology have been developed that permit the manipulation of the genetic materials of organisms in ways that heretofore were not possible, such as moving genetic material between organisms that would not normally exchange such material. Public awareness and concern that use of these new technologies may be associated with dramatic consequences have resulted in a call for some type of oversight to ensure adequate protection of human health and the environment.

FEDERAL OVERSIGHT

The United States Environmental Protection Agency (EPA) is one of several United States agencies involved in the regulation of biotechnology products as part of a coordinated federal effort. During the past several years, EPA has been developing the specifics of its regulatory approach to these products. In order to put EPA's approach into perspective, it is helpful to have an overview of the federal concept encompassed within a coordinated federal effort. In 1984, recognizing its responsibilities to address issues raised by the use of biotechnology, the United States government formed an interagency working group under the White House Cabinet Council on Natural Resources and the Environment. This group examined existing laws and concluded that for the most part, these laws would adequately address regulatory needs for biotechnology. A regulatory matrix describing applicable laws and responsible agencies was published in the Federal Register on November 14, 1985 (50 Fed. Reg. 47174).

Subsequently, the "Coordinated Framework for Regulation of Biotechnology" was published on June 26, 1986 (51 Fed. Reg. 23302). As used in the Coordinated Framework, the term biotechnology is broadly defined as "the application of biological systems and organisms to technical and industrial processes" (see "Proposal for a Coordinated Framework for Regulation of Biotechnology," 49 Fed. Reg. 50856, December 31, 1984). The Coordinated Framework spells out the basic federal philosophy for regulating products of biotechnology, details the network of agency jurisdiction over both research and commercial products, and includes statements of regulatory policy from the agencies principally responsible for such regulation. The

United States approach to regulating biotechnology is based on the recognition that biotechnology encompasses a large and varied collection of techniques and activities, and that the potential products resulting from this technology will cover a wide spectrum of uses. The Coordinated Framework provides that biotechnology products will be regulated in the United States as are products of other technologies -- that is, by the various regulatory agencies on the basis of *use*. Thus, many agricultural uses of microorganisms, plants, and animals are regulated by the Department of Agriculture; foods and drugs are regulated by the Food and Drug Administration; microorganisms used as pesticides are regulated by EPA under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), 7 U.S.C. §136 et seq.; commercial uses of microorganisms not covered by other existing authorities are regulated by EPA under the Toxic Substances Control Act (TSCA), 15 U.S.C. §2601 et seq. Examples of uses covered by TSCA include microorganisms used in metal mining, degrading wastes, conversion of biomass for energy, production of proteins and enzymes for non-pharmaceutical purposes, and non-pesticidal agricultural applications such as nitrogen fixation.

EPA REGULATION

As indicated above, an essential characteristic of any regulatory scheme for biotechnology is that is promotes public confidence that the oversight process will protect human health and the environment. Similarly, the scientific and regulated communities must believe that the process is credible, equitable, and sound. At the same time, it is important that the development and commercialization of biotechnology products be allowed to proceed in a reasonable and timely manner. Accordingly, EPA's goals in developing a regulatory scheme for products of biotechnology are to (1) protect human health and the environment, (2) meet the public's need to be assured that use of living products does not present unacceptable risk, (3) expedite the regulatory process, (4) maintain flexibility in order to respond to rapidly developing knowledge and technology, and (5) to accomplish this end without stifling innovation and the development of valuable new products.

At this time, EPA's regulatory effort involving these products is, in large part, pursuant to its authority under FIFRA and TSCA. FIFRA creates a statutory framework under which EPA, through a registration process, regulates the development, sale, distribution, and use of pesticides, regardless of how these pesticides are made or their mode of action. FIFRA, therefore, covers natural and genetically altered microbes that are used for pesticide purposes. EPA has routinely reviewed and registered natural microbial pesticides, such as *Bacillus thuringiensis*, for years.

A pesticide can be registered for use only if the pesticide will not cause unreasonable adverse effects to humans or the environment. The "unreasonable adverse effects" test involves a weighing of the risks and benefits of use of the pesticide. In

order to demonstrate that a pesticide will not cause unreasonable adverse effects, an applicant seeking to register the product must submit or cite data on subjects such as product composition, toxicity, environmental fate, and effects on nontarget organisms.

Some of the information needed to support registration must be developed under actual field conditions, and such preregistration field testing is authorized under FIFRA through the experimental use permit process. The results of such early testing are a critical element in determining when and under what circumstances a full registration may be issued.

TSCA gives EPA jurisdiction over the manufacturing,processing,importation, distribution, use, and disposal of all chemicals in commerce (or intended for entry into commerce) that are not specifically covered by other regulatory authorities (e.g., substances other than foods, drugs, cosmetics, and pesticides). TSCA authorized EPA, through a "screening" process, to acquire information on chemical substances and mixtures of chemical substances in order to identify and regulate potential hazards and substantial exposures. Under TSCA, EPA can require testing of any chemical substance that may present an unreasonable risk to human health or the environment or which is produced in substantial quantities and may result in substantial environmental release or substantial human exposure.

TSCA's applicability to the regulation of microbial biotechnology products is based on the interpretation that living organisms are chemical substances under TSCA -- an interpretation that was embodied in the development of the initial chemical inventory in 1979. The basis for this interpretation is that all substances, living and nonliving, have a chemical foundation at the most fundamental molecular level. As a result of this interpretation, microorganisms (except for those in excluded use categories) are subject to all provisions of TSCA.

The premanufacturing notice (PMN) process implements TSCA's goal of screening new substances before they enter commerce in order to identify those that pose potential hazards. Manufacturers and importers of "new" chemical substances are required to submit data and other information to EPA that will allow the Agency to evaluate the potential risks of the product. If a potentially unreasonable risk is identified during the PMN screening process, EPA is authorized to [exert] comprehensive oversight of the commercialization of the affected product.

Because microorganisms introduced into the environment have the ability to replicate, increase in number, and disseminate from the test site, EPA feels that it is prudent to address certain questions before some of these microorganisms are released to the environment. Accordingly, EPA's regulatory approach encompasses procedures designed to provide sufficient oversight of the initial stages of environmental testing of certain microbial products of biotechnology. In its section of the Coordinated Framework, EPA indicated that, under FIFRA and TSCA, it intended to focus regulatory emphasis on three categories of microorganisms:

1. microorganisms with "new" characteristics or that are new to the environment in which they are intended to be used (and whose behavior is therefore less predictable),

2. microorganisms that have hazardous components or characteristics (and therefore a recognized potential to cause adverse effects in other organisms), and

3. microorganisms that are used in the environment (and therefore have the potential for widespread exposure).

Through its statement in the Coordinated Framework, EPA made certain provisions immediately effective and indicated that rulemaking would be required before other provisions could be formally implemented. Where provisions were not immediately effective, EPA invited voluntary compliance in the interim.

One of the most complex (and contentious) issues for the EPA biotechnology rulemaking process has been identification of the scope of organisms to be subject to regulatory oversight. Because this is a cross-cutting issue for all the agencies included in the Coordinated Framework, development of a "scope definition" was undertaken as a joint effort. The result of this effort, "Principles for Federal Oversight of Biotechnology: Planned Introduction Into the Environment of Organisms With Modified Hereditary Traits", was published on July 1, 1990, and serves as guidance for EPA and the other agencies during their rulemaking processes.

In developing the Principles for Scope, the agencies relied upon "familiarity" as the basis for determining whether oversight is appropriate, and emphasized the role of information in the determination of "familiarity". (The appropriateness of using familiarity based on solid information as the basis for a flexible regulatory process is clearly supported by the 1989 National Academy of Sciences report, "Field Testing Genetically Modified Organisms".) Specifically, the Principles for Scope set forth the general directive that "to the extent permitted by law, planned introductions into the environment of organisms with deliberately modified hereditary traits should not be subject to oversight... unless information concerning the risk posed by the introduction indicates that oversight is necessary." Also included are examples of organisms that would "generally be excluded from oversight because their introduction is considered to be similar to previous safe introductions, or because other information available regarding the risk posed by such introductions (including knowledge that existing practices or regulations adequately address possible risk posed by the introduction) makes such oversight unnecessary." With the Principles for Scope as guidance, and utilizing provisions available under both TSCA and FIFRA that allow for flexibility in the regulatory process, EPA is [well on its way to establishing the regulatory program promised in the Coordinated Framework]. The Agency

is currently nearing completion of the drafting process for its biotechnology regulations under both TSCA and FIFRA, and it is anticipated that the proposed rules will be published for comment in the near future.

EPA REVIEW PROCESS AND EXPERIENCE

Since 1984, EPA has reviewed several small-scale tests using products of biotechnology, many of which involved release to the environment. A scientific review process for such tests has been developed that EPA believes is credible, sound, and respected by the scientific community and which allows the public to feel confident that potential human or environmental impacts have been addressed. To date, the review procedures, which are briefly described below, have involved case-by-case evaluation.

When a submission for small-scale testing is received, TSCA or FIFRA staff groups evaluate the submission and develop a coordinated scientific position. During this process, hazard and exposure are addressed, potential problems, issues, or significant unanswered questions are identified, and the likelihood of significant risk from the proposed test is assessed.

Under both TSCA and FIFRA, intro-agency workgroups then comment on the positions developed by staff. If appropriate, the submission and EPA's scientific position are sent to other federal agencies for comment. Appropriate state regulatory agencies are also contacted in order to alert them to the submission, to discuss EPA's assessment, and to ensure that the federal and state positions are as consistent as possible. For some submissions, visits to the test sites are conducted in order to evaluate actual field conditions.

In order to obtain an independent peer review of EPA's scientific position, to address specific scientific questions raised by staff, or to identify any additional data that may be needed to complete the risk assessment, the submission and the EPA's scientific evaluation may be sent to a group of independent scientists. EPA has established a specific advisory committee, the Biotechnology Science Advisory Panel (SAP) for these independent peer reviews. Several proposed field trails have been reviewed by specially convened BSAC, SAP, or joint BSAC/SAP subcommittees, and these groups have consistently endorsed the EPA assessments.

Public comment is considered an important aspect of the reviews, and for many proposals the public is provided several opportunities to comment during the review process.

In some cases, microorganisms that are subject to TSCA or FIFRA are also subject to the Federal Plant Pest Act or other statutes administered by the Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture. In such situations, APHIS and EPA conduct coordinated reviews. The two agencies cooperate closely, alerting each other to submissions, and sharing expertise and information. This close cooperation benefits both submitters and the agencies.

At the conclusion of a review, EPA determines whether the microorganism

may be released into the environment. Under both TSCA and FIFRA, this decision is based on an assessment of both the potential risks and the potential benefits of the proposed use of the microorganism. Should the analysis indicate that use of the microorganism may pose unreasonable risks, EPA has authority under either statute to impose restrictions on its use.

Between the two programs, TSCA and FIFRA, EPA has reviewed approximately 75 submissions, some of which have been "second-generation" variations of previously approved tests. While it is still too early to speak in general terms or draw definitive conclusions about the reviews, certain observations can be made:

1. EPA's review process has been effective in identifying and addressing risk concerns.

2. Evaluation of the proposed tests often requires different types of background studies, data bases, and scientific expertise than are required for review of traditional chemical products. Similarly, monitoring for living microorganisms once they have been released to the environment is frequently more complicated.

3. The issues giving rise to the greatest concerns are often not related to whether the microorganism performs its intended function, but rather to other, nonintended impacts that might occur.

4. Many (if not most) of the risk concerns about these microorganisms have been related to their potential impacts on the environment and nonhuman, nontarget species; *to date*, potential impacts on human health have not been a significant concern.

5. Monitoring data from some of the tests have confirmed that under actual use conditions, physical containment of the test microorganisms may not be feasible or possible. Accordingly, issues related to spread of the test organisms from the test site are appropriately considered in most risk assessments.

6. The small-scale tests reviewed thus far have been found to pose no significant risks. However, large-scale or commercial use of some of these microorganisms may raise more significant concerns, particularly regarding environmental and ecological impacts, than those addressed for small-scale tests.

As to nonscientific observations, it has become increasingly clear that public involvement is an important element of most test programs involving environmental release. Without such involvement, public fear of the unknown could derail the whole process and prolong the testing through extensive administrative and judicial maneuvers. The impact of such administrative and judicial activity can be seen by contrasting the first and a more recent EUP review of two genetically engineered microbial

pesticides. In the first, the time elapsed between receipt of the EUP application and the test was almost two years. In between were two lawsuits brought in federal and state courts to stop the test, and numerous state and local administrative proceedings. The more recent EUP application, submitted after more extensive preliminary public outreach and presumably after the public had gained more confidence in EPA's review process, moved from submission to field testing approximately five months. There were no lawsuits or supplemental administrative proceedings involved with this review. It appears that the more the public knows and feels a part of and comfortable with the process, the less likely it is to obstruct the test. Hence, many submitters have elected to shield from public scrutiny only the barest essentials about their microorganisms and proposed tests.

EPA is using the experience gained from its early test reviews, along with public comments and advice from our BSAC, in fashioning its proposed regulations. Based on these sources, EPA has bolstered confidence that its basic approach as set out in the Coordinated Framework and procedures developed for individual test reviews are adequate to accomplish the goals for its biotechnology regulatory program. Therefore, in many areas, EPA's proposed regulations are envisioned in much the same light as its policy statement in the Coordinated Framework.

To summarize, EPA's experiences over the last few years with the approach for regulating products of biotechnology first articulated in 1984 and later included in the 1986 Coordinated Framework, demonstrate that the process has met its major goals:

* utilization of a systematic approach for identifying and assessing potential human or environmental risks,

* incorporation of a peer review mechanism that draws upon independent scientists expert in diverse areas of relevant knowledge,

* establishment of an open process that allows for and invites public participation, and

* approval of field tests, the results of which confirm EPA's original judgment of insignificant risk.

Notwithstanding these successes, EPA recognizes that certain modifications to the current process are probably appropriate in order to accommodate in a timely manner the number of tests anticipated for review in the future. EPA is currently developing proposed regulations that will implement its modified approach. However, based on its past experiences, EPA is confident that its ultimate regulatory program will protect the public health and the environment from unreasonable risks while allowing the public to reap the benefits of scientific and technological innovations.

OFFICE OF TOXIC SUBSTANCES PURVIEWS AND RESEARCH NEEDS

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The Office of Toxic Substances (OTS) implements the Toxic Substances Control Act (TSCA), which applies to commercial microbial products. The microbial products reviewed under TSCA are those that are specifically excluded by other federal statutes, e.g., pesticides (which are regulated by the EPA's Office of Pesticide Programs (OPP) under FIFRA), foods, drugs, and cosmetics which fall under the purview of the Food and Drug Administration, and nuclear materials regulated by the Nuclear Regulatory Commission. The products reviewed thus far by OTS fall into two categories: (1) fermentation applications for production of cellular components such as enzymes to be used in the production of ethanol, detergents, and culture media; (2) microbial products intended for intentional environmental release such as recombinant rhizobia for enhanced nitrogen fixation in legumes. Other applications which are subject to TSCA include for example those for degradation of toxic wastes, metal mining, desulfurization of coal, enhanced oil recovery, and biomass conversion (for reduction of solid waste). Products for bioremediation of toxic compounds such as organic solvents, crude and refined petroleum wastes, and creosote may account for a significant portion of future submissions to OTS.

There are several differences in the research needs of OTS and OPP, which naturally follow from the current interpretation of TSCA as it applies to microbial products. First, a potentially diverse group of microorganisms and uses are subject to TSCA. Second, the microorganisms reviewed by OTS are not intended to be toxic or pathogenic, as are microbial pesticides. Third, OTS examines submissions for fermentation applications of microorganisms. OPP does not, although FIFRA provides for ensuring quality control in the manufacturing process. Fourth, the OTS risk assessment process places more emphasis on microbial fate analysis, in addition to examining human health and ecological effects concerns. Concerns for microbial fate necessitate research which leads to better detection of microorganisms, and better estimation of microbial dispersal as a result of fermentation releases and intentional environmental releases. Although interest in research on microbial fate will continue, the research program is now shifting to place a stronger emphasis on human and ecological effects.

Currently, OTS is in the process of developing tiered testing schemes for both ecological effects and human health. In order to implement these testing schemes, additional protocols beyond those developed for OPP, will be needed. OTS has begun a joint project with ORD to develop simple screening-level tests to determine the survival and competitiveness of genetically engineered microorganisms

in various environmental media. Further, since exposure to OTS-reviewed products often includes areas beyond the agricultural environment, protocols for effects testing should be developed for different species of organisms that are not only important ecologically but are commonly found in the general environment (such as some ant species). Protocols may also need to be developed which can discriminate between effects of a microorganism and its metabolites to address both human health and ecological concerns; such protocols are particularly relevant to the evaluation of microorganisms used for bioremediation of toxic pollutants or recalcitrant compounds. Due to the fact that many microbial products reviewed under TSCA are not designed to be toxic/pathogenic but may play a role in other critical interactions (e.g., mineral cycling), OTS will continue its interests in test systems such as microcosms which examine a broader range of effects concerns. Finally, OTS will continue to seek support as it develops computerized risk assessment tools such as those for gene sequence analysis, microbial taxonomy, and microbial fate modelling.

THE OFFICE OF PESTICIDE PROGRAMS: REGULATION OF MICROBIAL PESTICIDES AND RESEARCH NEEDS

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The Office of Pesticide Programs (OPP) is responsible for regulating all pesticides under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and the Federal Food, Drug, and Cosmetic Act (FFDCA). FIFRA defines pesticides as any substance, or mixture of substances, intended for preventing, destroying, repelling, or mitigating any pest, and specifically includes plant regulators, defoliants, or desiccants. Before the Agency can register a pesticide, FIFRA requires the Agency to have sufficient data to determine that the pesticide, when used in accordance with widespread and commonly recognized practice, will not cause (or significantly increase risk of) unreasonable adverse effects to humans of the environment.

The microbial pesticides include bacteria, viruses, fungi, protozoa, and algae. The first microbial pesticide was registered in 1948 (*Bacillus popilliae*) and 20 others have been registered to date. Of those, 8 have been registered in the last 2 years. Over one third of the new pesticides registered recently have been microbial pesticides. This is indicative of the overall trend for industry to turn to biological pesticides to address the agricultural problems of the 90's: insect resistance to chemical pesticide and the need for safer pesticides. These biologically derived pesticides include the microbial pesticides, non-toxic biochemicals such as pheromones, and the newest and largest group: plants that are genetically engineered to product pesticidal chemicals. Although our regulatory system for transgenic plants is not yet in place, we have reviewed over 50 small scale field tests through a memorandum of understanding with the U.S. Department of Agriculture Animal and Plant Health Inspection Service.

Part 158 of 40 CFR 158 specifies the data and information that must be submitted to EPA to support registration of pesticides. Subdivision M of the Pesticide Testing Guidelines (NTIS # PB89-211676, July, 1989) provides information relating to the data requirements listed in 40 CFR 158.690 and 158.740, including conditions under which each data requirement is applicable, standards for acceptable testing, information that should be included in a test report, guidance on evaluation and reporting of data, and examples of protocols. In addition, scientific publications and ORD laboratory reports are cited in the guidelines as useful information for designing test protocols. OPP must be able to evaluate these protocols since 40 CFR 160.120, the regulation for Good Laboratory Practice Standards, specifies that "each study shall have an

approved written protocol....".

In addition to registering microbial pesticides, the Agency must perform risk assessments to evaluate applications for Experimental Use Permits (EUPs) as described in 40 CFR 172. Microbial pesticides must have EUPs before being tested on cumulative total of not more than 10 acres of land or not more than one surface-acre of water, or if to be used for food or feed uses, or on water containing animals or plants that my be used for food or feed. In addition, some microbial pesticides are subject to an interim policy, published in the June 26, 1986 FEDERAL REGISTER (51 FR 23302). This policy essentially states that the Agency must be notified prior to conducting small scale field testing of genetically altered or non-indigenous microbial pesticides in order to allow the Agency to determine if an EUP should be obtained. Under this policy, OPP has reviewed a number of interesting constructs including ice enucleating gene deletions in *Pseudomonas,* and *Bacillus thuringiensis* toxin genes in *Pseudomonas* and in *Clavibacter xyli*. Indications are that most microbial pesticide genetic engineering work in the immediate future will involve toxin genes inserted into baculoviruses. In reviewing these notifications, we have found that the majority of the risk issues are the same as for the wild-type, naturally occurring microbial pesticides.

OPP uses a risk assessment framework for microbial pesticides that was derived from traditional chemical assessment methods, i.e. the overall risk is a function of both exposure and hazard (the effects on non-target species, including human). In order to minimize the data requirements, OPP has elected to require environmental exposure data only if unacceptable hazards are observed in non-target species or short-term mammalian tests.

Although we have recommended specific protocols for many tests in Subdivision M, relatively few have been performed and some tests have been difficult to perform and evaluate. Accordingly, OPP has identified Subdivision M protocol development and evaluation as its primary research need. Some problems with the ecological effects testing are: (1) We generally test adult honeybees since larvae are difficult to keep alive, yet larvae are the most likely to be susceptible to biological insecticidal toxins. (2) It has been difficult to maintain *Daphnia* for a sufficient time to evaluate potential pathogenicity. (3) Very few species of beneficial insects are available for testing. (4) Which aquatic species are most at risk and are we adequately protecting them? (5) Do the protocols adequately reflect a maximum hazard approach?

In addition to the actual testing protocol development, there is a need for information that allows us to evaluate the study results. In many cases, we do not have much information on the role of pesticidal gene products in the environment, even for the most prevalent *Bacillus thuringiensis* toxins. *Bacillus thuringiensis* is ubiquitous in soil but not at levels that appear to affect insects. Microcosm, mesocosm, and/or field studies could be utilized to study the relationship of *Bacillus thuringiensis* and/or its many toxins to the soil and aquatic eco-

systems. Are there alternative soil hosts? Do the toxins give *Bacillus thuringiensis* a competitive advantage over other soil microorganisms?

Potential human health effects of pulmonary exposure to microbial pesticides are one of our primary concerns, since bacterial, fungal, and viral pesticides are frequently broadcast over large areas by aerial applications. These extensive uses present possibilities for human epidemiology studies and for relating experimental test results with actual effects.

Health research has centered on the tier 1 acute pulmonary exposure tests for Subdivision M of the Pesticide Assessment Guidelines. These tests were developed, standardized and evaluated for the assessment of the *infectivity* primarily of naturally-occurring agents. In these studies, intratracheal and intranasal challenges were demonstrated to be equivalent to, if not better than, aerosol inhalation for the assessment of pulmonary exposure to infectious bacterial and viral agents. In addition, results from EPA-sponsored studies have shown that pulmonary exposure of rodents to *Bacillus thuringiensis* have shown adverse toxicological effects at sufficiently high dose levels. The extent of toxicity observed appears related to differences in the *Bacillus thuringiensis* strain administered. While this is a significant first step in methodology and protocol development for testing guidelines, relevant new technologies and innovative methods must be incorporated to increase the breadth of coverage of these tests for a wider range of agents in the future. This can be attained by obtaining, through research, a better understanding of the basis of these empirical tests. Areas of additional inquiry should address the microbial agent/cellular interactions, factors influencing the reisolation and identification of the microbial pesticides, the utility of these tests for repeated pulmonary exposures, the equivalence of intratracheal and intranasal challenge results with aerosol inhalation results when *toxicity* instead of infectivity is the endpoint, and the scientific underpinnings of new approaches so appropriate tests can be developed to address problems such as assessment of the potential for transfer of deleterious genes to mammalian tissues and cells by more innovative and complex genetically altered agents which can be expected to be developed for such problems as assessment of the potential for transfer of deleterious genes to mammalian tissues and cells by more innovative and complex genetically altered agents which can be expected to be developed in the future. Microbial agents of greater anticipated use should be identified and studied to better understand any adverse cellular responses or effects that might be produced in the pulmonary tests. In particular, *Bacillus thuringiensis*, baculoviruses, and *Beauvaria bassianna* have high pulmonary exposure potential. Additionally, as successful agents are more utilized and exposure becomes more frequent, components of microbial pesticides of minor relevance to the human health effects must be reassessed.

SESSION I
ENVIRONMENTAL EXPOSURE RESEARCH

Detection and Enumeration

Scientists are using serological, biochemical, physiological, and genetic methods for improving detection and enumeration of microorganisms under laboratory, microcosm, and field conditions. Methods are being developed and refined for their sensitivity, specificity, and reliability in a variety of terrestrial, aquatic, and air habitats. The following extended abstracts summarize continuing research in detection and enumeration of microorganisms of special interest.

Dispersal and Transport

Developing a better understanding of the movement of microorganisms through the environment, either by the natural elements, or through associations with higher organisms is crucial. The mechanisms and dynamics of transport among and within various environmental components (air, soil, groundwater, plants, insects, and other animals) are being studied. This research includes development of mathematical models and other aids for predicting transport and exposure. The following extended abstracts summarize continuing research in the potential of microorganisms of special interest to disperse or be transported from the site of release.

Survival and Colonization

Understanding the factors that affect the ability of microorganisms to survive and colonize habitats under a variety of conditions is central to this research element. Cellular, molecular, and environmental factors influencing survival or actual multiplication are being identified and described.

Gene Transfer

This research provides test methods that describe conditions for determining the frequency and probability of genetic exchange between microorganisms released to the environment and their indigenous counterparts. Factors that affect and control gene stability and rates of transfer in the environment are being evaluated.

MOLECULAR STUDIES OF MICROBIAL ECOSYSTEM PERTURBATIONS

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INTRODUCTION

A central question in environmental microbiology is: how do the activities of microbial communities correlate with the diversity of populations responsible for those activities? This question remains essentially unanswered because of well recognized limitations of pure culture isolation and existing determinative schemes of classification. Pure culture isolation and phenotypic characterization have not served to adequately describe environmental diversity. However, the need for pure culture isolation is no longer a prerequisite. Techniques of molecular biology (including recombinant DNA, nucleic acid hybridization, molecular systematics, comparative sequencing and the polymerase chain reaction (PCR)) now provide additional tools for characterizing natural populations. In particular, comparative sequencing of the ribosomal RNAs is serving to unify microbial systematics and has provided the foundation for the explicit characterization of natural microbial diversity.

We have developed a variety of techniques using comparative rRNA sequencing and hybridization for use in the characterization of microbial populations in natural communities. These techniques and a phylogenetic framework have served our ongoing studies of microbial population ecology; addressing questions of community stability, competitive exclusion, succession and synergism, community response to perturbation, and the relationship between community structure and community function. These questions are central to microbial ecology. From a more practical standpoint, the rational manipulation of the activity of environmental populations [e.g. via the introduction of exogenous organisms (or selected DNA encoded traits)] should be based on an understanding of population ecology. Similarly, rational risk assessment is dependent upon this understanding.

Our research with the EPA has focused upon two "model" communities to further develop comparative molecular methodologies and establish basic paradigms of microbial ecology. These communities are the bovine ruminal microbial community and those represented by marine anaerobic sediments. Studies of the ruminal community have focused on the principal fiber-digesting populations whereas sediment studies have examined the diversity and distribution of sulfate-reducing bacteria in relationship to their activity. This abstract addresses only the ruminal studies. Studies of the environmental diversity and activities of sulfate-reducing bacteria will be addressed by Dr. R. Devereux and M. Winfrey, whose work builds upon our earlier studies of the sulfate-reducing bacteria.

METHODS

Comparative 16S rRNA Sequencing and Oligonucleotide Probe Design

The determination of partial or near-complete 16S rRNA sequences has been the foundation for the population studies. Initially we focused on the now-recognized major fiber-digesting populations of the rumen (the anaerobic ruminal fungi, *Butyrivibrio fibrisolvens*, species of *Ruminococcus* and *Fibrobacter*). These studies emphasized the above stated limitations of the existing taxonomy; organisms classified as strains of *Fibrobacter succinogenes* and *F. intestinalis* (previously *Bacteroides succinogenes*) and *Butyrivibrio fibrisolvens* were demonstrated to encompass diversity at the levels of genus or greater. This is an observation common to other natural communities as well. Population diversity (genetic diversity) has generally been greatly under-estimated. Thus, past studies of environmental abundance and distribution have most often obscured the true population/genetic diversity.

Comparative 16S rRNA sequencing has served both to reveal genetic diversity and to provide sequence information to design oligonucleotide probes for use in environmental studies. Probes are either labeled with $_{32}P$ or a fluorescent dye. Radioactive probes have been used for determining population abundance, either of specific species (or subspecies) and of larger, phylogenetically coherent, groups (e.g. eukaryotes or archaea). Fluorescent probes have been used to identify single cells, either directly in environmental samples or in pure culture.

The Fibrobacter Paradigm

The genus Fibrobacter provides the paradigm for our general experimental approach to defining the environ-mental diversity of functionally comparable populations of micro-organisms. Our work with both the sulfate-reducing bacteria and the fiber-digesting populations of the bovine rumen has shown that many functionally comparable groups are phylogenetically coherent. In general, a collection of microorganisms that is phylogenetically coherent can also be circumscribed by regions of rRNA sequence (signature sequences) common to, and unique to, the group (a group could consist of a subspecies, species, genus or larger more inclusive assemblage). These signature regions serve as target sites either for oligonucleotide probes (used for identification and quantification) or PCR primers (for selective sequencing). Thus, it has been possible to fabricate probes (and primer sets) to specific functional target groups.

We have outlined the genetic diversity within the genus *Fibrobacter* by several genetic criteria, including comparative 16S rRNA sequencing, DNA similarity, restriction fragment length polymorphism and oligo-nucleotide probe hybridization to both pure culture isolates and total rumen contents. The now recognized diversity within the genus as inferred by comparative 16S rRNA sequencing and DNA similarity is displayed in Figure 1. Oligonucleotide probes specific for the genus, species and subspecies have

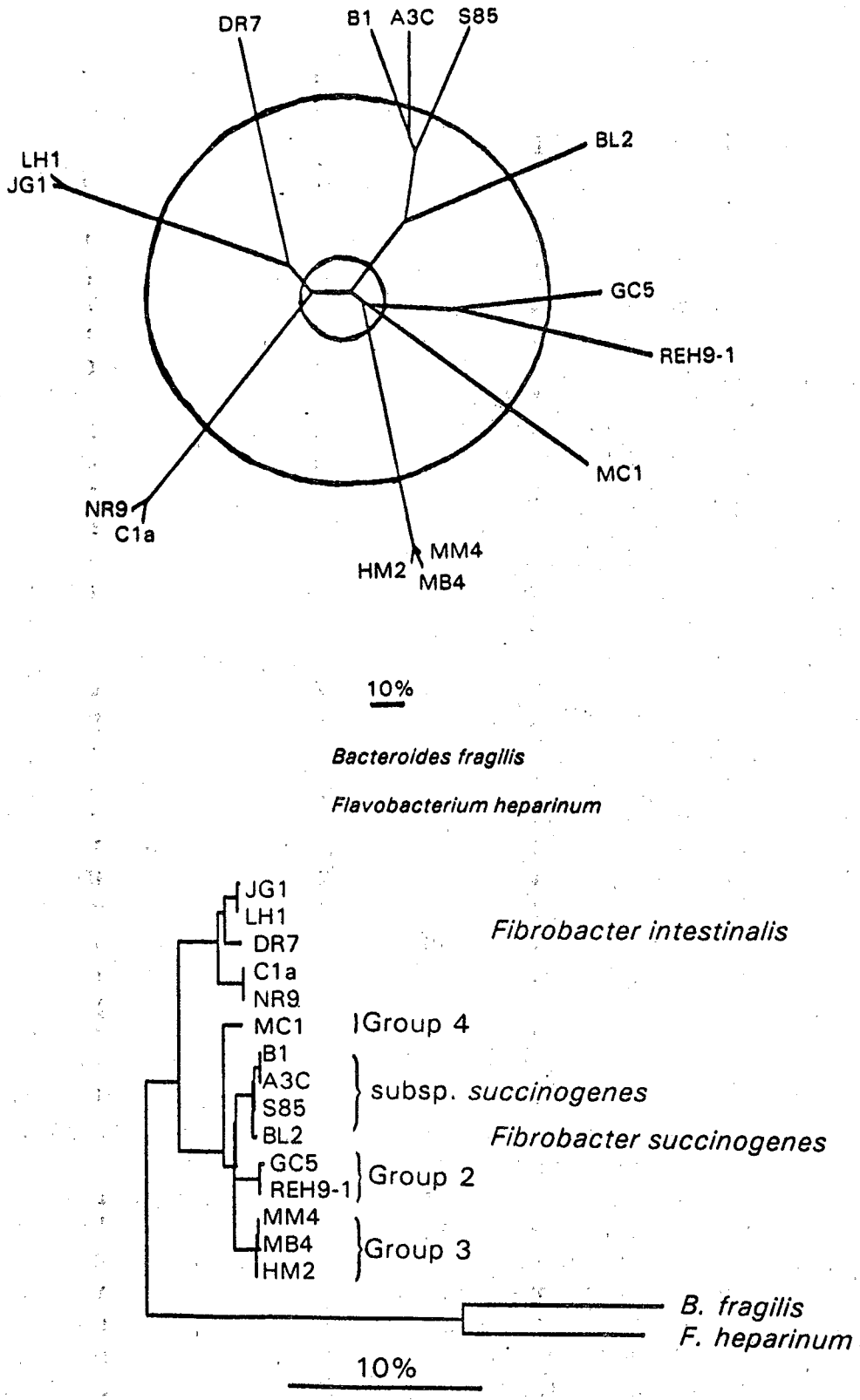

Figure 1. Comparison of genetic relationships among *Fibrobacter* as inferred by comparative 16S rRNA sequencing (bottom) and DNA similarity (top).

been used both for classification and natural population studies (below).

Assurance that natural population diversity has been reasonably-well circumscribed comes from consistency of quantification between the general and specific probes: the sum of groups quantified by hybridization to specific probes (e.g. species and subspecies) should be comparable to the value determined by use of a more general probe (e.g. genus). In addition, we have recently constructed a "global" panel of 66 reference organism nucleic acids (representing the diversity of archaea, bacteria and eukaryotes) against which the specificity of each probe will be evaluated.

RESULTS AND DISCUSSION

Community Stability and Community Response to Perturbation

The overall goals of the research are to define the major ruminal populations, their constancy (between animals and over time) and associations (succession, competitive exclusion, synergism). Only with appreciation of general community structure and population associations can more specific questions be addressed. We are establishing the necessary overview of community structure via two interrelated studies. First, we are establishing normal variation among major ruminal populations and second, we are using this baseline of normal variation to evaluate changes in abundance of specific ruminal populations following perturbation (altered diet) and with normal diet variation.

Long Term Population Stability

Studies of population abundance and distribution among subspecies of Fibrobacter have revealed unexpected population stability. Of animals so far inspected (five steer), only one subspecies of *Fibrobacter* predominates in a single animal at a given time. Among the five steer examined over extended time periods (months), either the group 2 subspecies or *F. succinogenes subsp. succinogenes* predominated. Initial population studies of a single animal demonstrated that *F. succinogenes subsp. succinogenes* predominated in that animal. A more recent study of four animals (Figure 2) demonstrated a different subspecies (group 2) to be stably associated with each of these animals. Although representatives of other species and subspecies of *Fibrobacter* were present, their numbers remained low throughout the study period (2 months).

Diel Population Variation

Another approach we are using to identify population associations and population successions is to follow population changes through time following feeding. A study set of four animals was fed once daily at 11:00 AM and samples were taken at four hour intervals over a three day period. Initial populations studies examined the diversity and abundance of different subspecies of *Fibrobacter*. *Fibrobacter* numbers were depressed immediately following feeding and reached their greatest numbers almost 20 hours after feeding (as measured by total rRNA

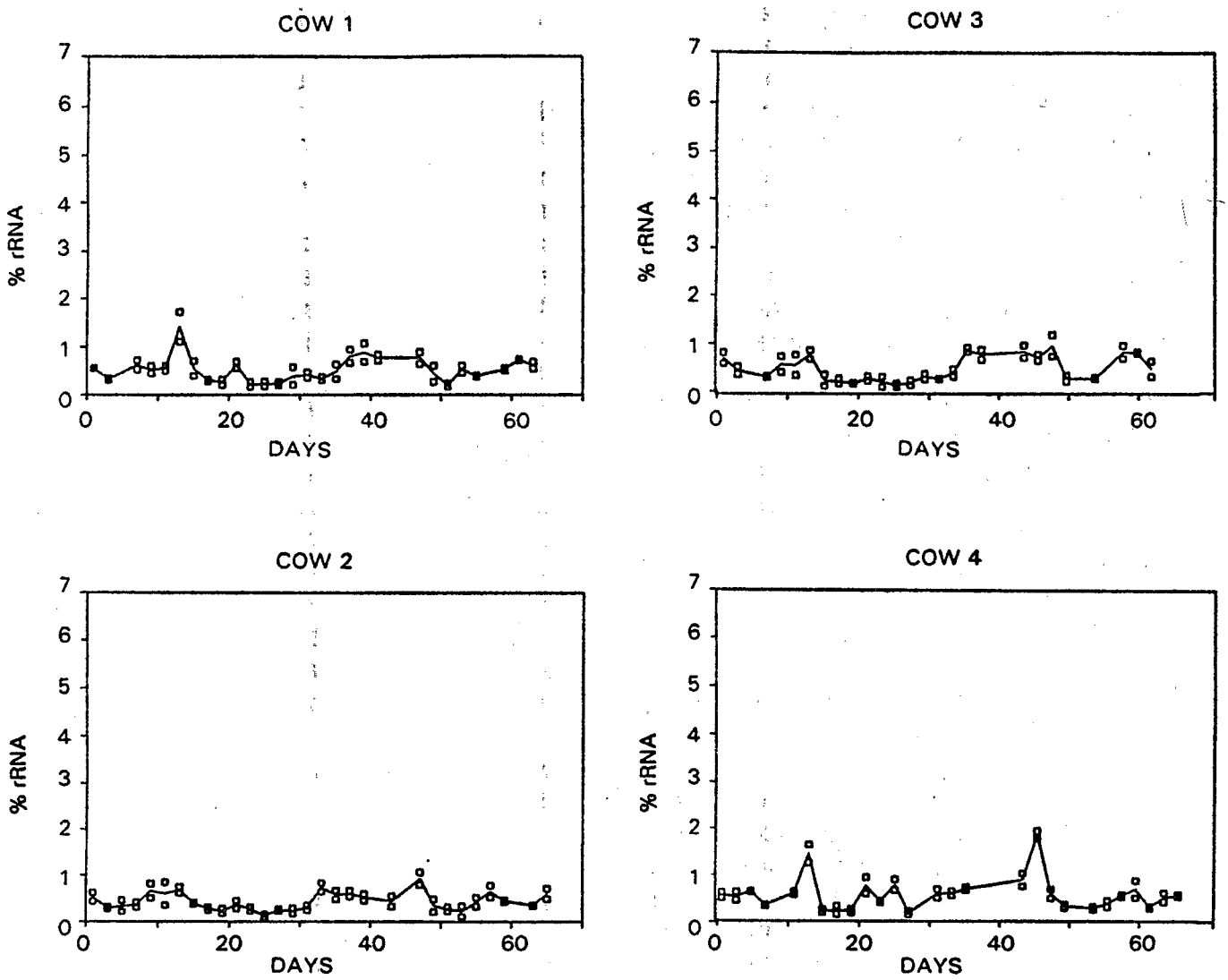

Figure 2. Long term ruminal population stability study demonstrating the relative constancy and stability of populations of *Fibrobacter succinogenes* among a study set of four cows. The species-specific probe and universal probes were used to quantify relative abundance of this species. Hybridization with the different subspecies-specific probes has demonstrated that the group 2 subspecies (Figure 1) accounts for most of the genus-specific hybridization throughout the study period.

abundance or fraction of total ruminal rRNA). This is consistent with this group functioning to digest more refractory plant cell wall polymers (and retained with larger feed particles). Continued studies will examine the relationship of this population to other major fiber-digesting populations (*e.g.* ruminococci and the anaerobic ruminal fungi) during the diel cycle. This should point up synergism, competition and succession among populations serving a similar function role in the ruminal community (fiber digestion).

Autecological Studies

We earlier demonstrated the use of fluorescent oligonucleotide probes in combination with fluorescence microscopy to visualize individual bacterial cells. Fluorescent probes have been used to identify species and subspecies of *Fibrobacter*, both in pure culture and within rat cecal contents. Continued studies will use fluorescent probes in combination (each labeled with a different fluorescent dye and targeting *Fibrobacter* subspecies and other fiber-digesting populations) to examine the distribution of individual fiber-digesting bacteria on the surfaces of plant material. This analysis should also serve to address issues of niche, competition and synergism.

Genetic Isolation of Catabolic Traits

An outstanding question in environmental microbiology is the fluidity of gene transfer among populations. The ribosomal RNA offers an historical framework for evaluating the stability of specific genes within microbial populations. Specifically, if a gene is stably associated with a given population (i.e. not subject to lateral transfer), then the genealogy of that gene should correspond to the genealogy of the microbial populations that it is associated with.

In general, catabolic genes appear to be more readily transferred between populations. The catabolic genes of greatest importance to the ruminal microbial community are those that participate in the hydrolysis of plant cell wall material (e.g. polysaccharidases). We initially evaluated the potential lateral transfer of two polysaccharidases (an endo-glucanase and a xylanase) among subspecies of *Fibrobacter*. Both genes (as assessed by heterologous hybridization) were found to be widely distributed among representatives of the genus. This raised the question of whether this distribution reflected lateral transfer or conservation of structure. To address this question, we sequenced the homologous endoglucanse from three *Fibrobacter* isolates (representing the diversity of the genus).

The endoglucanse genes are highly conserved relative to total genomic similarity. Excluding the third codon position from sequence comparisons, the more highly conserved C-terminus (ca. 900 nucleotides out of 2000) shares approximately 75% sequence similarity with the most distantly related strains (S85 and DR7) and approximately 92% with the closest (A3C and S85). This compares with total DNA similarity values of 5-7% and 65% (Figure 1). Conservation does not appear to reflect lateral transfer of these catabolic genes among sub-

species. The relationships of the different subspecies, as inferred by sequence divergence of both the endoglucanses and the 16S rRNAs are consistent (Figure 3). This data strongly suggests that this endoglucanase is a stable component of the cell and also is a strong comparative argument that the enzymes participating in fiber digestion are closely integrated. More generally, the retrospective approach outlined here could serve for the assessment of past gene transfer events among genetically distinct populations.

FUTURE WORK

Continued studies will continue to use the nucleic acid samples obtained from the study set of five animals to further characterize ruminal population diversity, stability, population associations and response to perturbation. Initially, major fiber-digesting populations will be examined in greater detail. The long term goal is to establish the basis for stable community structure (reflected by long term association of specific populations over time and following community perturbation). Given that the study animals all received the same diet, an initial hypothesis is that specific populations (each representing a subset of a larger functionally comparable collection of populations) are stabilized by overall community architecture. If so, this should be reflected by specific associations between populations, for example, between genetically distinct populations of fibrobacter and ruminococci.

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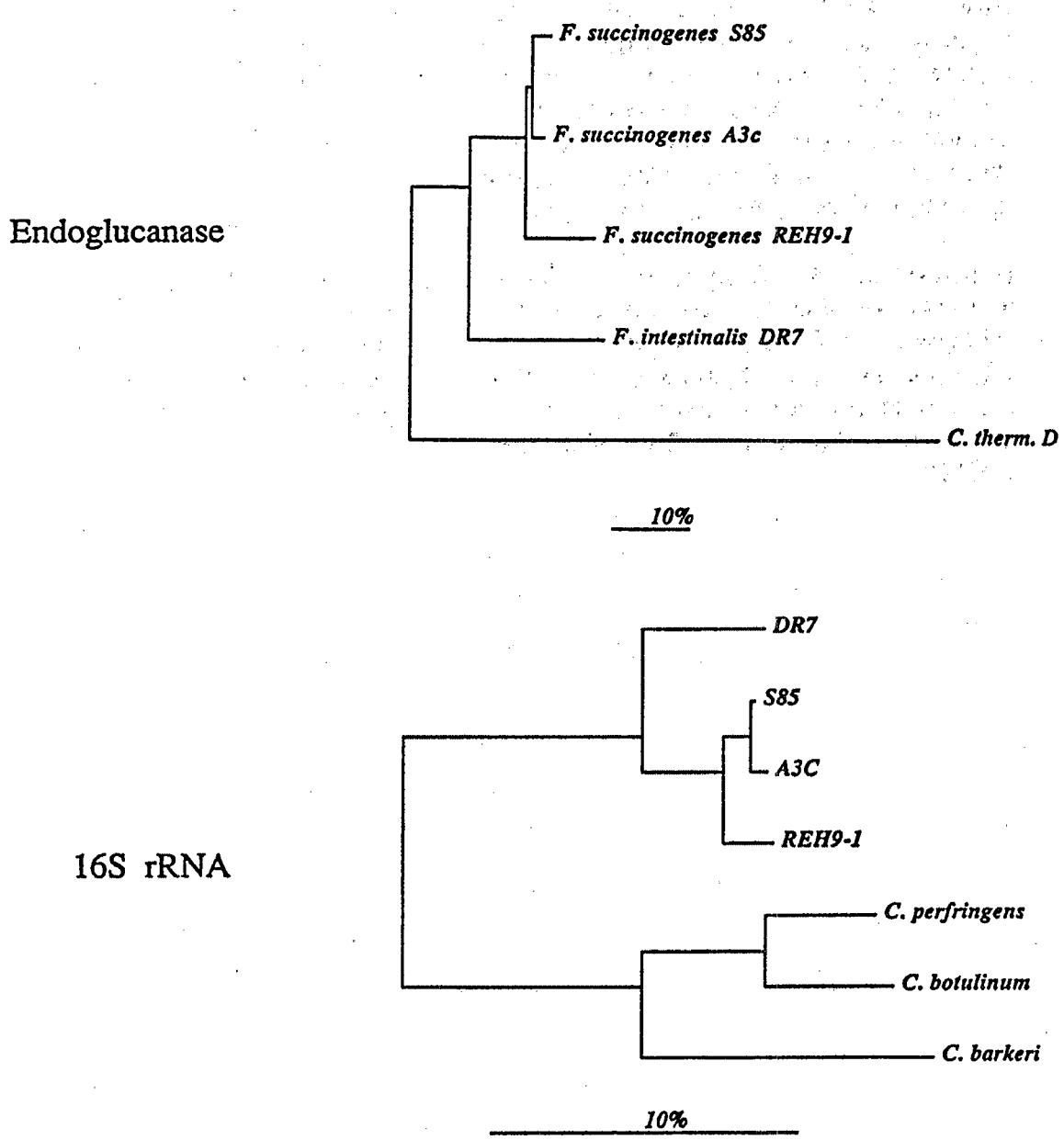

Figure 3. Comparison of endoglucanase and 16S rRNA sequence relationships among *Fibrobacter*.

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USE OF 16S rRNA PROBES TO CORRELATE SULFATE REDUCER COMMUNITY STRUCTURE WITH MERCURY METHYLATION

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INTRODUCTION

In order to assess the effect of releasing genetically engineered microorganisms (GEMs) in the environment on native microbial communities, it is necessary to be able to detect changes in the microbial community. A potential method to evaluate microbial ecosystem changes is to quantify changes in microbial activities and changes in microbial community structure in response to a perturbation. Although methods are available to quantify most microbial activities, severe limitations exist in the ability of microbiologists to characterize microbial communities. Traditional enumeration and identification techniques require cultivation of microorganisms, yet many native microorganisms are difficult or impossible to cultivate. This dilemma has precluded any accurate definition of microbial community structure in natural habitats.

The use of 16S rRNA-targeted probes to detect microbial groups offers a method to overcome this limitation in microbial ecology. Since the probes are used on total RNA extracted from a natural sample, this method eliminates the need to cultivate the microorganisms and has the potential to accurately define microbial community structure.

Because of the wide diversity of microbial populations in natural communities, the use of this technique as a risk assessment tool should target microbial groups that play a vital role in the community. In addition, an ideal target group would change in abundance and/or diversity in response to any changes in the microbial community. The sulfate-reducing bacteria (SRB) represent such a microbial group because they act as terminal organisms in anaerobic food chains and are essential to efficient anaerobic decomposition in habitats containing sulfate. By consuming products of bacterial fermentations in higher trophic levels, they allow thermodynamically unfavorable fermentations to occur and "pull" the entire anaerobic food chain. Thus, a change in the SRB population would be detrimental to carbon decomposition in the environment they inhabit, and changes in other microbial groups that provide the substrates for the SRB may also alter the SRB population.

We used 16S rRNA-targeted probes to groups of SRB to determine their community structure in sediment from northern Wisconsin lakes and correlated these communities with mercury methylation and lake pH. Since SRB are known agents of mercury methylation and methylmercury bioavailability is enhanced in low pH

lakes, it is possible that sulfate loading due to acid-rain may play a role in the enhanced methylmercury production by stimulating methylation by sulfate reducers. Thus, acidified lakes provide an excellent system to evaluate the use of rRNA-targeted probes to examine changes in community structure in response to an ecosystem perturbation.

METHODS

Sample Collection

Lake sediment and water samples were collected from selected lakes (ranging in pH from 4.6 to 7.1) in the Northern Highlands Lake district of Wisconsin. One of the lakes, Little Rock Lake, is particularly useful in studying SRB community structure because it has been divided and one basin is being artificially acidified with sulfuric acid. Sediment samples (upper 5 cm.) were either hand collected by a diver (for littoral sediments) or with a peristaltic pump (for flocculent profundal sediments). All sediments were placed immediately on ice after collection and either frozen or extracted within 24 hours of collection. Methylation assays were set up within 24 hours of collection. Water samples were collected using a peristaltic pump. A Pellicon tangential flow filter system (Millipore) fitted with four 0.45 μm filter cassettes was used on site to concentrate water down to 1 liter. Lake water and concentrates were kept on ice to minimize RNase activity. Water concentrates were transported to the lab on ice, centrifuged, and the pellets frozen. Processing of water concentrates was done within 24 hours of collection. Strict anaerobic technique was used during collection and storage of anoxic sediment and water samples.

Methylation Assays

Mercury methylation was measured by spiking water or sediment samples with $^{203}Hg(II)$ (1.0 μg as $^{203}HgCl_2$) and incubating 24 hours at *in situ* temperature. Methylation was quantified by extracting the radioactive methylmercury produced followed by scintillation counting.

RNA Extraction from Environmental Samples

RNA in sediment samples was initially extracted by placing 1 ml of sediment in a 2-ml bead beating vial containing 1.2 g baked zirconium beads and 0.2 g acid washed polyvinyl-polypyrrolidone (PVPP). Vials were then filled with 50 mM sodium acetate/10 mM EDTA buffer (pH 5.2) containing β-mercaptoethanoland SDS. The vial was shaken at high speed on a Mini Beadbeater for six 1-min intervals with a two minute incubation on ice between each beating cycle. The aqueous phase was separated by centrifugation and extracted once with an equal volume of phenol (equilibrated with 50 mM sodium acetate/10 mM EDTA (pH 5.2), once with phenol: chloroform and twice with chloroform. RNA was ethanol precipitated, resuspended in RNase-free water, and stored at -20°C. All extracts were quantified by absorbance at 260 nm and the purity was evaluated by 260/280 and 234/260 ratios.

In order to quantify the amount of RNA per volume of environmental sample, we modified the procedure so that the percentage of RNA recovered in the extraction was quantitatively known. To increase the aqueous volume in the extraction vials, we decreased the amount of PVPP by half (to 0.1 g/vial) and decreased the amount of zirconium beads from 0.5 ml (1.2 g) to 0.5 g. The total aqueous volume of an extraction mixture in a 2-ml bead beating vial was calculated by subtracting the volume of non aqueous materials (PVPP, zirconium beads, and sediment dry weight) from the total volume of liquids in each tube. After bead beating and centrifugation a constant volume (900 μl) of aqueous supernatant was removed. After solvent extraction, we recovered a constant volume (500 μl) of the aqueous extract. From the initial and final aqueous volumes we calculated the percent recovery of the RNA in the sample.

Nucleic Acid Hybridizations and Detection

RNA extracted from environmental samples and reference standard RNAs (from pure cultures of SRB) were denatured for 10 minutes in 1.5% glutaraldehyde at 30°C and applied to nylon membrane with a slot blotter. Amounts applied ranged from 1 to 1000 ng (based on A260) for environmental samples and from 0.01 to 10 ng for reference standards. Membranes were baked at 80°C for 1 hour, then prehybridized at 40°C for 4 hours in a hybridization bag containing 0.1 ml hybridization buffer per cm^2 of membrane. Universal (1400) or SRB-specific oligonucleotide probes were end labelled with polynucleotide kinase and (gamma-^{32}P)-ATP. SRB specific probes used were: 687 (for *Desulfovibrio*), 660 (for *Desulfobulbus*), 129 (for *Desulfobacter*), BTM (for *Desulfobacterium*), 813 (for *Desulfosarcina*, and *Desulfococcus*), and 804 (which detects all organisms detected by the 129, BTM and 813 probes). Probes were added to prehybridized membranes at a concentration of 2 X 10^6 cpm/ml hybridization buffer. Each membrane was hybridized at an optimized probe-specific temperature for 16 to 18 hours. Membranes were washed 30 minutes at room temperature and 30 minutes at an optimized probe-specific wash temperature in a 1% SDS, 1X SSC wash buffer. Air dried membranes were placed in X-ray cassettes against preflashed Kodak X-AR film, exposed at -70°C (or room temperature for the universal probe), and developed to visualize hybridization signal. Hybridization signal was quantified by scanning densitometry coupled with a peak integration software package. Hybridization signals of environmental samples were converted to ng RNA based on the signals from hybridized reference standards.

Quality Assurance

We initiated several quality assurance protocols to evaluate factors that may affect the amount of hybridization signal produced from RNA extracted from environmental samples. These procedures included: (a) evaluating the effect of varying degrees of

glutaraldehyde denaturation on the hybridization signal from environmental RNA, (b) testing the hybridization of SRB-specific probes to non SRB negative controls containing RNA equivalent to the amount of total RNA present in environmental samples, and (c) evaluating the presence of components in environmental extracts that alter the hybridization signal by performing standard addition experiments.

To evaluate the effect of glutaraldehyde denaturation on the hybridization signal, environmental RNA samples were denatured in 1.5, 3.0 or 4.5% glutaraldehyde and incubated in a 30°C heat block for intervals from 0 to 60 minutes. These samples were applied to nylon membranes and hybridized with the 1400 (universal) probe and the 687 (*Desulfovibrio spp.*) probe.

The amount of non-specific hybridization of SRB-specific probes to environmental RNA was determined by preparing negative control RNA extracted from grass, yellow perch fillets, *Clostridium perfringens*, and *Pseudomonas aeruginosa*. Extracts from each species were combined in equal amounts (based on A260) and the mixture applied to membranes in amounts equivalent to the amounts of environmental RNA applied.

Matrix effects in environmental extracts were examined by the addition of reference standards to sediment and water extract, or to extracts that had been treated with RNase or with RNase plus DNase. Samples were hybridized with the universal probe and the slope of standard curve in the standard addition experiments was compared to the slope obtained with reference standards alone.

RESULTS AND DISCUSSION

Methods Development and Quality Assurance

We modified the extraction procedure to allow quantitative calculation of the total amount of RNA extracted from a given volume of environmental sample. Decreasing the amount of zirconium beads or PVPP had no effect on the recovery (based on A260) or purity (based on A260/A280) of the extracted RNA and allowed us to routinely recover 29% of the aqueous extract in sediment samples. This will allow accurate calculation of the absolute amount of SRB-specific 16S rRNA in addition to the relative abundance (percent of the total populations accounted for by SRB). Absolute amount of 16S rRNA is a better parameter to describe community structure since the total microbial population varies significantly among different habitats. One potential limitation of the current extraction procedure is that the effectiveness of bead beating on quantitatively disrupting all cells in the sample population is unknown. This will affect the accuracy of quantifying microbial communities using 16S rRNA probes and should be investigated in the future.

Quality assurance protocols are important in any quantitative assay and should be developed and incorporated into rRNA hybridization studies if the results are to be used quantitatively or semiquantitatively. We have begun to

develop procedures to quantify factors that may interfere with the hybridization signal of SRB-specific probes. Since glutaraldehyde denatures by covalently bonding to the RNA molecule, the degree of exposure to glutaraldehyde could conceivably affect the resultant hybridization signal. However, varying the time and concentration of glutaraldehyde used to denature RNA showed no significant effect on the hybridization signal obtained from sediment extract. Only the elimination of glutaraldehyde resulted in a lower hybridization signal.

Matrix interferences were observed in standard addition experiments when sediment extract was added to reference standards. The addition of 10 and 50 ng (based on A260) of sediment RNA to reference standards decreased the slope of standard addition curves (compared to curves prepared with reference standards) by about 24%. This indicates that the sediment extract contains material that interferes with the detection of 16S rRNA, but the matrix effect can be corrected for with traditional standard addition procedures. When RNase treated or RNase and DNase treated sediment extract was added to standards, the slope was not significantly different from the slope of the standards alone. This suggests that the interfering matrix in the extract was RNA.

We observed significant non-specific hybridization with SRB-specific probes when amounts of negative control RNA comparable to amounts of environmental sample were applied to membranes. Since the hybridization signal obtained from environmental extracts is often low, correcting for non specific hybridization by negative controls is important if quantitative estimates of SRB populations is to be obtained.

SRB Community Structure in Wisconsin Lakes

The relative abundance of the SRB groups examined was similar in all lakes and *Desulfovibrio spp.* comprised greater than 66% of the total SRB population in each lake. The total 16S rRNA (based on the 1400 probe) was positively correlated with lake pH suggesting that total sediment biomass decreases at low pH. Methylation was active in all lake samples and was negatively correlated with lake pH. The relative abundance of all SRB groups detected was negatively correlated with lake pH although the correlation was only significant ($P < 0.10$) with two of the probes. This suggests that sulfate reducers make up a larger portion of the microbial population in low pH lakes, but should be further evaluated with larger sample sizes. Since SRB are known to be active methylators of mercury, this may account for the inverse correlation observed between sediment methylation and lake pH.

Although the use of 16S rRNA-targeted probes is in its infancy in microbial ecology, our results indicate that they show much promise in defining microbial community structure in natural habitats. Further work needs to be done before truly quantitative estimates of bacterial community structure are available, but the use of SRB-specific probes provide information on the community structure of these

key microorganisms in anaerobic habitats that was previously unobtainable. Thus, determination of SRB community structure based on 16S rRNA probes provides a useful risk assessment tool to evaluate changes in microbial communities.

FUTURE WORK

In the remainder of the current funding period we will characterize the SRB communities in the anoxic hypolimnion of Little Rock lake. Samples for this study were collected in the summer of 1990 to follow the development of SRB communities as the anoxic hypolimnion built up, and in the fall to monitor persistence of SRB after the fall turnover when the water column became aerobic. We will also be sampling sediments from the acidified and reference basin of Little Rock Lake in a random design to allow us to show any statistical differences in SRB community structure and mercury methylation between basins.

Future areas of work involving the use of 16S rRNA-targeted probes to determine microbial community structure should focus on further methods improvement to allow more quantitative measurements to be made, and further evaluation of changes in SRB community structure and activity in response to environmental perturbations.

PUBLICATIONS (abstract)

Winfrey, J., R. Devereux, and M. R. Winfrey. 1991. Use of 16S rRNA-targeted Probes to Correlate Community Structure of Sulfate-reducing Bacteria with Mercury Methylation in Freshwater Sediments. American Society for Microbiology Annual Meeting, Dallas, TX, May 5-9.

APPLICATION OF DNA HYBRIDIZATION TO COMPARE BULK DNA'S FROM DIFFERENT SOILS

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INTRODUCTION

The distribution and persistence of recombinant DNA in the terrestrial environment has brought molecular ecologists to develop techniques for extracting DNA from soil samples. One approach relies on recovery of intact bacteria from soil before the DNA is extracted. Another approach involves direct- extraction of DNA without prior removal of the cells from soil. Based on these procedures, we developed a new direct-extraction method that is small-scale, rapid, and simple, yielding up to 20 μg of DNA from a gram of soil. Furthermore, we have applied DNA hybridization of RFLP's and dot blots to demonstrate differences between DNA's extracted from different soils.

METHODS

Soil samples were collected near Corvallis, Oregon: under an Oak tree, in a sheep pen, from the rhizosphere under grass. One gram of soil in a 50 ml plastic Oakridge tube was combined with six ml of mixing buffer (0.5 M D-sorbitol, 15% PEG 4000, 2% diethyl-dithiocarbamic acid, 100 mM EDTA, and 50 mM Tris-Cl, pH 8.0) and vortex-mixed for one minute. Then, 500 mg of polyvinylpolypyrrolidone was added. Lysis of bacteria was promoted with 100 μl lysozyme solution (50 mg/ml) and fungal cell walls were degraded with 120 μl Novozym 234 solution (50 mg/ml). The sample was vortex-mixed for 15 sec and incubated on ice for 1-2 h. Next, 3.8 ml lysis buffer (4% SDS, 100 mM EDTA, proteinase K at 500 μg/ml, and 50 mM Tris-Cl, pH 8.0) were added. After mixing the contents, the tube was returned to ice for 1-16 h. The extract was then centrifuged at 5000 x g for 5 min. at 4°C and placed in a sterile Oakridge tube on ice. To recover additional DNA, the pellet was resuspended in 3 ml wash buffer (100 mM EDTA, 50 mM Tris-Cl, pH 8.0), vortex-mixed, and centrifuged. The previous step with wash buffer was repeated and both supernatant liquids were combined. Then, 5 M potassium acetate was added to a final concentration of 0.5 M. After 1-2 h on ice, the sample was centrifuged at 4°C for 10 min. at 15,000 x g. The supernatant liquid was mixed with two volumes of 95% ethanol and centrifuged at 10-15°C for 10 min. at 15,000 x g. The pellet was dried and suspended in 1 ml buffer (0.01 M Tris-Cl, pH 8.0, and 0.001 M EDTA; TE).

Ten μl of each DNA extract was electrophoresed for 1.5 h at 125 V in 0.7% agarose using a buffer consisting of 0.04 M Tris-acetate (pH 8.0) and 0.001 EDTA (TAE), and stained for 30 min. in TAE containing 0.4 μg ethidium bromide per milliliter. Gels were

photographed over UV light. DNA extracts adjusted to a density of about 1.58 g/ml were purified by gradient centrifugation using standard methods. Extracts were further purified with Geneclean Glassmilk (BIO 101, Inc.) according to manufacturer's directions.

Restriction enzyme digests were performed with approximately 0.5 μg DNA and 2 μl of endonuclease in 20 μl buffer. To determine if impurities in DNA extracts inhibited restriction enzyme activity, 0.2 μg of pBR322 was added to some reaction mixtures. After incubating for 16-24 h at 37°C, more enzyme was added for another hour. DNA samples were electrophoresed, transferred to a nylon membrane (procedure by Schleicher and Schuell), and hybridized with the following ^{32}P-labeled probes: nick-translated pBR322 DNA or random-primed labeled chromosomal DNA from *Pseudomonas putida*, *Enterobacter cloacae* and *Bacillus cereus*, and the rRNA genes of *Escherichia coli* (Bam H1 insert of plasmid pKK3535). The final post-hybridization washes for pBR322-hybridized filters were performed at 56°C using 0.1X SSPE (18 mM NaCl, 1 mM $NaPO_4$, pH 7.7, and 0.1 mM EDTA) and 0.5% SDS. Southern transfers of RFLP's hybridized with the random-prime labeled probes were washed in 2X SSC (0.3 M NaCl and 30 mM sodium citrate, pH 7) and 0.5% SDS at 65°C. Autoradiograms were prepared by standard methods.

Dot blots were prepared on nylon membranes (Schleicher and Schuell) according to the manufacturer's instructions. Concentrations of serial dilutions applied through a filtration manifold (Bethesda Research Labs.) included: 500 to 3.9 ng/dot for soil DNA's; 10 to 0.08 ng/dot for chromosomal DNA's of *P. putida, E. cloacae,* and *B. cereus*; 1 to 0.008 ng/dot for salmon DNA and *E. coli* rRNA genes. Membranes were hybridized according to methods of Boehringer Mannheim with random-primed, digoxigenin labeled DNA probes (*P. putida, E. cloacae, B. cereus,* and *E. coli* rRNA genes). Final post-hybridization washes were performed under highly stringent conditions using 0.1X SSC and 0.5% SDS at 68°C. Autoradiograms were produced using the chemiluminescent substrate, Lumi-Phos 530 (Boehringer Mannheim).

RESULTS AND DISCUSSION

Soils from different sources were used to demonstrate the effectiveness of the DNA extraction method. The method reported here yielded up to 20 μg DNA from one gram of various agricultural soils. In some cases (e.g., from the soil of a sheep pen), we recovered up to 35 μg DNA in one gram. Determinations were measured by spectrophotometric absorbance (wavelength, 260 nm). Accurate readings depended on the complete elimination of NaI and glassmilk following the Geneclean purification step. Due to the possible selectivity of the extraction process, all of the DNAs from a sample may not be retrieved. For example, some species of DNA may be firmly bound to soil components.

The degree of purity and restrictability that resulted from the use of this method was measured on agarose gels. High molecular weight DNA, 20-25 kb in size, was routinely observed

following the extraction procedure. This DNA was digested by restriction enzymes so that the entire DNA sample was fragmented into smaller pieces. This observation suggested that the DNA lacked residual, soil constituents that inhibit endonuclease activity. This was further demonstrated by the appearance of completely restricted pBR322 DNA that was added to digests containing purified soil DNAs. The resulting one band at 4.4 kb clearly demonstrated complete restriction of the plasmid to the linear form. Southern transfers of these agarose gels were probed with pBR322 in the more sensitive method of DNA hybridization. The autoradiograms verified complete digestion with Eco RI, Sal I, and Bam HI (Bethesda Research Labs.). These findings emphasized the reliability of the method as an effective means to purify DNA from different types of soils.

Bulk DNA extracts from soils were examined by RFLP analysis to determine differences in the observed fragmented band patterns between samples. Distinguishable, isolated bands were not detected in any of the soil extract samples analyzed. The completely fragmented DNA appeared as a wide distribution of molecules ranging from less than 0.125 kb to approximately 23 kb. We suggest that the lack of discernible bands is indicative of the presence of a wide variety of DNAs. The work of other researchers confirms the diversity of microorganisms found in the soil and the resulting high heterogeneity of their DNAs. Discernible restriction fragments would be seen if the DNA extract was relatively homogeneous, *e.g.*, a sample containing DNA from a predominant microorganism in large numbers.

Hybridization of electrophoresed soil DNAs was observed when DNA extracts were digested with SalI and NotI (Promega) and probed with the *E. coli* rRNA genes. In contrast, little or no hybridization was observed when the chromosomal DNA's of *P. putida*, *E. cloacae*, and *B. cereus* were used as probes. As with visual analysis of the RFLP's, discernable bands were not seen following DNA hybridization with any of the bulk DNA extracts examined. Due to the lack of specific bands, dot blots were utilized to determine whether quantitative differences between samples were evident. Using post-hybridization washes under conditions of high stringency, differences were detected. Different samples demonstrated different degrees of homology to a probe encoding the *E. coli* rRNA genes. The extract from the soil of the rhizosphere of grass contained 8 pg of hybridizable DNA and that from the sheep pen had 62.5 pg, i.e., 0.0016% and 0.0125% of the total DNA, respectively. When using the *P. putida* chromosomal DNA probe, differences were also detected between the two DNA extracts. Related DNA recovered from the rhizosphere of grass and the soil from a sheep pen represented 0.06% and 0.25% of the total DNA. Hybrids were not detected when *E. cloacae* and *B. cereus* chromosomal DNA's were used as probes. Based on the sensitivity of the method, we estimated that both extracts contained <0.06% of DNA related to *E. cloacae* DNA and <0.12% of DNA related to *B. cereus* DNA, if present.

In summary, we describe a bulk soil DNA extraction method that is rapid and small-scale. The DNA extracts are pure enough to be restricted and used in hybridization experiments. Coupled with the use of dot blot techniques, specific DNA populations in soil DNA extracts can be compared and quantified.

FUTURE WORK

Plans for this project include: a) use various probes to identify DNAs from various "functional groups" of microorganisms, b) enhance sensitivity of detection with PCR methods, c) assess efficiency of method for recovery of fungal, protozoan, plant rhizosphere, and "microfauna" DNAs, d) retrieve recombinant DNA after introducing a specific GEM into a terrestrial microcosm, e) develop methods to recover bulk DNA from leaf litter of tilled soil containing transgenic plants, and f) demonstrate effects of a GEM on population of DNA molecules indigenous to soil.

PUBLICATION

Porteous, L. A., and J. L. Armstrong. 1991. Recovery of bulk DNA from soil using a rapid, small-scale extraction method. Current Microbiol. (accepted for publication).

COMPARISON OF *xy*/E GENE ACTIVITY IN DIFFERENT MOLECULAR CONSTRUCTS

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INTRODUCTION

The activity of the *xy*/E gene product, 2,3-catechol dioxygenase, is a useful colorimetric tool for autecological investigations. The yellow color (2-hydroxymuconic semialdehyde) produced due to the enzymatic cleavage of catechol, a colorless substrate, has been exploited to enumerate target microorganisms released into soil, lake water or sprayed onto plant leaves. Thus far however, the application of the *xy*/E marker gene has been confined to plating and spectrophotometric assays of sonicated filter concentrated cells.

The objectives of the current study were: a) to assess the stability and level of expression of a plasmid-borne versus chromosomally inserted *xy*/E marker gene, b) to examine the effect of cell density and growth phase on catechol 2,3-dioxygenase activity, c) to determine the efficacy of *xy*/E gene expression for direct determination of cell numbers via a spectrophotometric analysis of 2-hydroxymuconic semialdehyde, and d) to establish the sensitivity of the technique in detecting target microbes by HPLC analysis.

MATERIALS AND METHODS

Cultures and media

Luria-Bertani (LB) agar was used for plate counts. Liquid cultures were grown in LB broth. All cultures were incubated at 30°C. Broth cultures were maintained on a rotary shaker at 200 rpm. Appropriate antibiotics were added when selective media were needed.

Construction of *xy*/E Gene Marked Strains

Most molecular biological techniques were performed according to standard procedures. A typical approach used was similar to that for inserting the *xy*/E gene into the iceC structural gene. Plasmid pICE1.2, which contains the iceC gene, was subjected to partial digestion with *sal*1 (Boehringer Mannheim), followed by dephosphorylation with calf intestinal phosphatase (Boehringer Mannheim).

Plasmid pR01733 was restricted with *xho*I (Boehringer Mannheim) and electrophoresed in low melting temperature agarose (International Biotechnologies, Inc.). The 2.3 kb band containing the *xy*lE structural gene was excised and extracted. The 2.3 kb *xho*I fragment from pR01733 was ligated with the partially digested PICE1.2 DNA, and transformed into *E. coli* DH5. Colonies resistant to 50 μg ml-1 ampicillin were sprayed with 0.1M catechol, and those colonies which turned yellow were isolated. One of the *xy*lE+ colonies that exhibited an Ice- phenotype was selected and the recovered plasmid was designated pSEL2.

Marker Exchange

Selection for integration of non-replicating but mobilizable plasmid. Plasmid pSEL2-4 was mobilized into *P. syringae* Cit7 which contains the iceC gene using pRK2013 as a helper plasmid. Colonies which fluoresced under UV were selected, and tested for *xy*lE activity by spraying with catechol. Single *xy*lE+ Flu+ (colony growing on King's B agar plates fluoresce upon exposure to UV light) colonies were restreaked onto KB amended with 100 μg ml^{-1} rifampicin, 30 μg ml^{-1} kanamycin, and 100 μg ml^{-1} ampicillin, and tested for ice nucleation activity to confirm the Ice+ phenotype and thus ensure that plasmid integration had occurred but a second recombination event had not. Strains designated *mer* 3, 12, 15, and 17 were used for subsequent marker exchange steps.

Identification of Second Recombination Events

Four different *xy*lE+ Flu+ Ice+ colonies were inoculated into KB amended with 100 μg ml^{-1} rifampicin to 10^7 cells ml^{-1}, and cultured at 28°C for about 12 hours to a cell density of over 10^9 cells ml^{-1}. Cultures were diluted 1:50 into fresh medium, and regrown to 10^9 cells ml^{-1}. This cycle of dilution and regrowth was repeated 10 times. Dilution series of the final culture were plated onto KB amended with 100 μg ml^{-1} rifampicin and grown 2 days at 28°C.

Approximately 10^4 colonies were replica-plated onto KB containing 100 μg ml^{-1} rifampicin, 30 μg ml^{-1} kanamycin, and 100 μg ml^{-1} ampicillin. About 1% of these colonies did not grow in the presence of kanamycin and ampicillin and were thus hypothesized to have undergone a second recombination event in iceC distal relative to *xy*lE from the first recombinational event. Of the kanamycin- and/or ampicillin-sensitive colonies, about 9% were also *xy*lE+ and Ice-, suggesting that excision for vector-derived sequences and recombinational placement of the native iceC gene with the *xy*lE-iceC fusion construct had occurred. Five such strains, designated *hap* 18, 45, 49, 69, and 72 were obtained.

Stability and Expression of Genetic Markers

Antibiotic and *xy*lE gene expression and stability were assayed by spread

plating onto selective and nonselective media. All cultures except *P. syringae* Cit7 #69 colonies, were assayed for expression of catechol 2,3-dioxygenase activity after 48 hours of incubations at 30°C. *P. syringae* Cit7 #69 was assayed after 72 hours of incubation. White colonies, on selective and non-selective agar plates, were enumerated before they were sprayed with a 1% aqueous catechol solution for counting *xyl*E gene harboring constructs.

Determination of Bacterial Growth

Cell density and bacterial growth phases were determined by plate counts and optical density measurements. A 300 ml LB broth amended with an appropriate antibiotic(s) was inoculated, from an over night culture, to a final cell concentration of less than 10^6 cells/ml and incubated at 30°C. Samples were withdrawn for plating and optical density measurements (spectronic 21 Milton Roy Co., Rochester, NY set to a wave length of 600nm) at intervals 0, 15, 24, 39, 48, 63, 72 and 87 hours.

Spectrophotometric Enzyme Assays

Cells were washed twice with 10 ml 20 mM phosphate buffer, pH 7.2. The pellet from the second wash was resuspended in 100 mM phosphate buffer, pH 7.5. One milliliter of this cell suspension was then treated with catechol solution to a final concentration of 0.05% and incubated for 30 to 60 minutes at room temperature. Catechol 2,3-dioxygenase activity was then determined spectrophotometrically (Beckman DU-B8) by reading the increase in absorbance at 375 nm. Where catechol 2,3-dioxygenase activity resulted in an intense yellow coloration (high 2-hydroxymuconic semialdehyde production), serial dilutions of the product was made to accommodate ranges of spectrophotometric specificity and sensitivity.

HPLC Enzyme Assays

For HPLC analysis, cells were washed twice with 20 mM phosphate buffer, pH 7.2 and resuspended in the same buffer. Final catechol concentration and incubation time was 0.05% and 30 to 60 minutes, respectively. Following incubation however, the samples were pelleted at 13000 rpm for 10 minutes at 4°C using a microfuge (Beckman Microfuge 11). The supernatant was transferred into LC automatic sampler 2 ml vials with 11 mm crimp caps (Hewlett-Packard Co., Avondale, PA). The samples were analyzed with an HP-1090 high performance liquid chromatograph (Hewlett-Packard Co., Avondale, PA) coupled to a diode-array UV detector and monitored at 375 and 277 nm (lambda max for 2- hydroxymuconic semialdehyde and catechol respectively). Samples were separated by an isocratic mobile phase (35% water with 0.1% acetic acid, 65% methanol) on an HP C18 reverse phase micro-bore column with C8 guard column at a flow rate of 0.40 ml min^{-1}. External catechol reference standards (NBS certified) were incorporated to bracket samples' concentrations and analyzed in triplicate every tenth injection.

RESULTS AND DISCUSSION

Marker instability, especially with markers carried on a plasmid, is a major problem in ecological studies since under nonselective environments where the trait borne on the plasmid need not be expressed, the antibiotic-resistant populations have been observed to disappear as they are out competed by the native populations. Because of the importance of genetic markers as selectable traits for tracking/detecting genetically engineered microorganisms (GEMs), measuring their stable expression or maintenance in the host is a prerequisite for studies prior to field releases.

We present here results of our study in which a comparison of the expression and stability of genetic markers, and assay systems is made for bacteria proposed for release into a microcosm. Results of our study suggest that the *xyl*E marker gene is stably expressed in some strains but not in others. Expression of *xyl*E gene was or approached 100% in all colonies of the seven strains when grown on selective media. Except for *E. coli* W311DHRalpha (PSEL2-4) and *P. aeruginosa* PA4032 (pRO1940::*xyl*E) all strains which carried the *xyl*E gene on a plasmid proved highly unstable with respect to the simultaneous expression of the *xyl*E and antibiotic resistant phenotypes. Such instability could be a function of the frequency of segregation of plasmid-free cells and the difference in growth rate between plasmid-free and plasmid-containing cells. In contrast, chromosomally inserted marker genes, such as the *xyl*E gene in the haploid (*P. syringae* Cit7#69) and merodiploid (*P. syringae* Cit7#17) colonies, consistently maintained both the *xyl*E and associated antibiotic marker gene phenotype, on various selective and nonselective growth media.

The activity of the 2,3-catechol dioxygenase depends on the cell density and also on the growth phase. The activity of the enzyme was optimal when the cell concentration was greater than 10^8 CFU/ml and the cultures were in the stationary phase. It was also observed that 2,3-catechol dioxygenase activity was strain specific and its activity decreased in the late stationary phase of the culture. This loss of activity may be a function of the depletion of iron which is known to affect the specific activity of the enzyme.

The spectrophotometric limit of detection observed in this study indicates that a sample needs to contain a population density greater than 10^7 CFU/ml of the marked bacteria for direct enumeration without cultivation on plates. This condition is unlikely to occur in soil or in lake water. Even under laboratory conditions and with the use of a strain with vigorous catabolic activity of catechol such as in the merodiploid, the limit of detection of the assay system could not be improved. Additional limitation noted was the instability of the enzyme product, 2-hydroxymuconic semi-aldehyde. Where large numbers of samples are involved, the short half life characteristic of the product limits its application.

Although catechol dioxygenase activity could not be detected spectrophotometrically at whole cell

concentrations below 10^7 CFU/ml, HPLC analysis revealed that activity was detectable with cell density as low as 10^3 CFU/ml. The sensitivity attained via HPLC analysis is comparable to the spectrophotometric detection limit (10^3 cells ml^{-1}) observed with cell extracts (supernatant of sonicated cells) of filter concentrated lake water samples reported by others.

Of the seven strains studied, five carried the *xy*/E gene on a plasmid. Of the five assayed for expression and stability of the *xy*/E gene, only one consistently expressed both *xy*/E and antibiotic resistance phenotype. All constructs bearing chromosomally-inserted *xy*/E gene and antibiotic markers expressed catechol 2,3-dioxygenase activity and resistance to antibiotics. The merodiploid (*P. syringae* Cit7#17) showed superior catabolic activity of the substrate and produced the most 2-hydroxymuconic semi-aldehyde. The apparent hyper-expression of *xy*/E gene in this strain facilitated HPLC mediated detection of very low concentrations of marked bacteria. Although our study suggested that the *xy*/E-gene tagger system has limitations for spectral assay of whole cells (10^7 cells/ml), it also demonstrated that the *xy*/E gene assay is very useful for plate assays and is highly sensitive (10^3 cells/ml) for detection of merodiploid *xy*/E activity by HPLC analysis. Our study also confirmed the viability of the assay system as a stable and sensitive analytical tool for the study of anthropogenic strains. The high degree of sensitivity afforded by HPLC analysis presents an opportunity useful in the autecological investigation of released microbes.

FUTURE WORK

Future work will include a) increasing the sensitivity of the assay system by concentrating and stabilizing the product, b) determining the factors responsible for the differential expression of *xy*/E gene in the haploid versus the merodiploid, and c) testing the assay system in a microcosm setting using plant, lake water or soil.

PUBLICATIONS

This study was initiated this year and there are no publications at this time.

POTPOURRI OF BIOAEROSOL RESEARCH AT ERL-CORVALLIS IN 1990

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Environmental Research Laboratory
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INTRODUCTION

Viable microbial bioaerosols generated by many human (and natural) activities are dispersed by the atmosphere to unwanted downwind locations. In order to estimate the near source downwind risk associated with the deposition of those viable bioaerosols, the following areas of research are being pursued: increasing bioaerosol sampling efficiency, developing better tools to evaluate bioaerosol generation potential, and developing predictive simulation models of bioaerosol dispersal and deposition. The models act as integrators of new information generated with better tools and sampler efficiencies.

The remainder of this report will describe the progress in the past year in: (1) increased bioaerosol sampling efficiency, (2) tool development of an automated dynamic aerosol torroid (auto-DAT), (3) development of an Environmental Bioaerosol Research Chamber (EBARC), and (4) bioaerosol simulation models. In the following, bioaerosol specifically means bacterial bioaerosols.

PROGRESS DESCRIPTIONS

Bioaerosol Sampling

The atmospheric environment is more or less inhospitable for maintenance of viability in bioaerosols. However, at least some organisms in bioaerosols are only partly damaged while airborne and will not grow when cultured, although they are viable. In an effort to resuscitate some bioaerosols, betaine has been added to culture media increasing sampling efficiency in terms of viable counts by up to 30% (Marthi and Lighthart, 1990)

Catalase is another compound that has been used to resuscitate stressed bacteria. When we add catalase to our bioaerosol recovery media for both pure cultures and wastewater treatment plant bioaerosols, sampling efficiency increased up to 113% (Table 1).

Table 1. Resuscitation of aerosolized Pseudomonas syringae or of wastewater treatment plant (WWTRP) bacteria in 1000 units of catalase containing collection fluid (as percent of 0 min. non-catalase control).

Catalase Contact Time (minutes)	Catalase	
	+	-
A. WWTP		
0	0.0	0.0
30	98.4	10.1
60	112.9	8.2
B. *Ps. syringae*		
0	0.0	0.0
30	110.9	-8.5
60	112.1	-28.0

A speculative explanation of the catalase resuscitation mechanism is as follows:

1. Many bacteria produce toxic hydrogen peroxide.

2. Some bacteria produce membrane associated catalase to dissipate hydrogen peroxide.

3. On aerosolization, the bacterial membrane is disrupted affecting catalase function, peroxide accumulates, and nonculturability occurs.

4. Exogenous catalase penetrates damaged cells, catalyzes

   dissipation of toxic hydrogen peroxide, and subsequent culturability occurs.

5. Upon addition of betaine (an osmoprotectant) to aerosol suspension fluid, the catalase effect is reduced, indicating that the betaine may negate membrane disruption and consequent need for exogenous catalase.

Tool Development: Auto-DAT

Large, slowly rotating airtight drums have been used to evaluate the survival of airborne microbes (Figure 1). In order to increase the precision of the drum system, we have automated it using a microcomputer. The auto-DAT has just been used for the first time with some of the resuscitation experiments above. A protocol describing the construction of the auto-DAT audits use to evaluate the survival of bioaerosol is forthcoming.

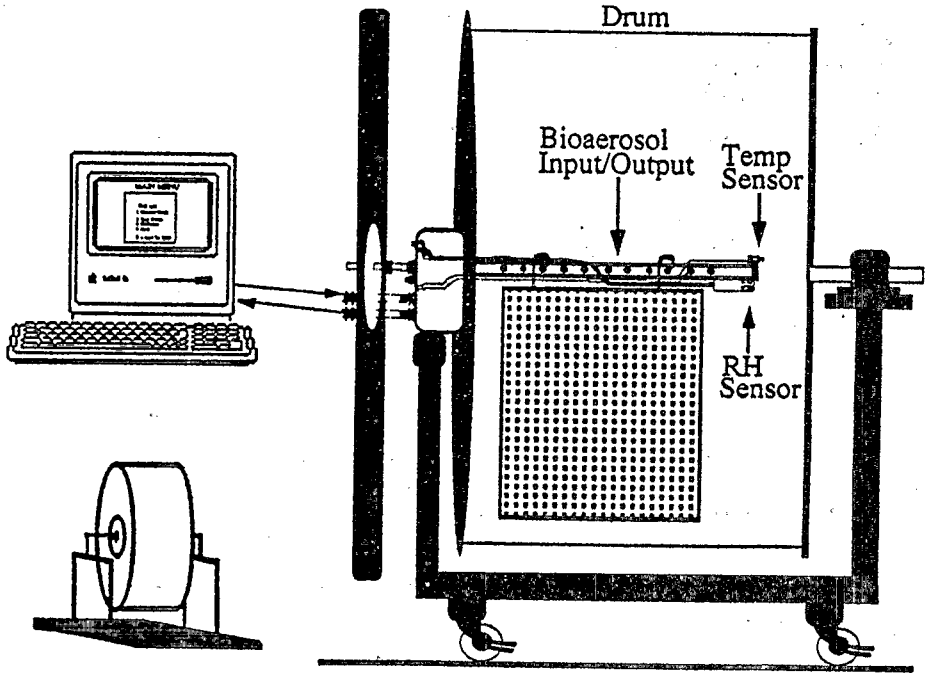

Figure 1. An Automated Dynamic Aerosol Torroid (Auto-DAT).

Tool Development: EBARC

In an effort to determine some of the conditions that would lead to bioaerosol entrainment from plant surfaces sprayed with microbial suspensions, the EBARC was designed to give a set of plants from one to many puffs of air. It is thought that the accelerated force (drag and thrust) occurring at the leading edge of a puff wind field would be the most effective mechanism to force microbial release from plant surfaces. Figure 2 (next page) is a diagram of the EBARC showing the rotating "lazy susan" with plants passing through influent air streams emitted from the influent air manifold. Samples downwind from the ports collect the entrained bioaerosol.

To date, we have used the EBARC to evaluate the variation between Andersen samplers (Table 2) and the particle size distribution of bacteria size fluorescent spheres sprayed onto plants and subsequently blown off (Figure 3).

Preliminary data in Table 2 shows that there are significant differences between Andersen sampler stages. Figure 3 (page 65) shows that many bacteria-sized particles are released from plants on larger rafting particles. This is also found in nature, where approximately 60% of the bacteria are particles of $\geq$ 7 μm aerodynamic diameter.

Table 2. Comparison of 8 randomly selected Andersen samplers used in the EBARC to evaluate *BACILLUS SUBTILIS* var. *NIGER* spores blown off oat leaves.

Andersen Sampler	Stage Number-(Particle Size), Percent Total						Total Colonies (Log 10)
	6- (0.65 μm)	5- (1.0 μm)	4- (2.1 μm)	3- (3.3 μm)	2- (4.7 μm)	1- (7.0 μm)	
1*	1.0	16.2	0.4	20.7	6.8	54.8	2.97+0.04
2	2.6	14.3	11.8	10.8	8.5	51.9	2.78+0.15
3	9.9	5.0	10.3	12.3	6.7	55.7	2.84+0.14
4	5.1	12.6	7.4	8.8	9.4	56.7	3.04+0.05
5	3.2	13.1	10.7	10.1	5.6	57.3	2.96+0.04
6	1.1	13.8	11.0	9.7	7.1	57.3	3.01+0.04
7	9.3	10.8	10.4	13.7	11.8	44.0	2.96+0.04
8	1.0	15.3	9.7	9.5	8.3	56.2	2.97+0.05
Average (CV)	4.2+1.0 (23.8)	12.6+0.9 (7.1)	9.0+0.5 (5.5)	12.0+0.6 (5.0)	8.0+0.4 (5.0)	54.3+1.5 (2.8)	2.95+0.03 (1.0)

* 10 Replicates per sampler

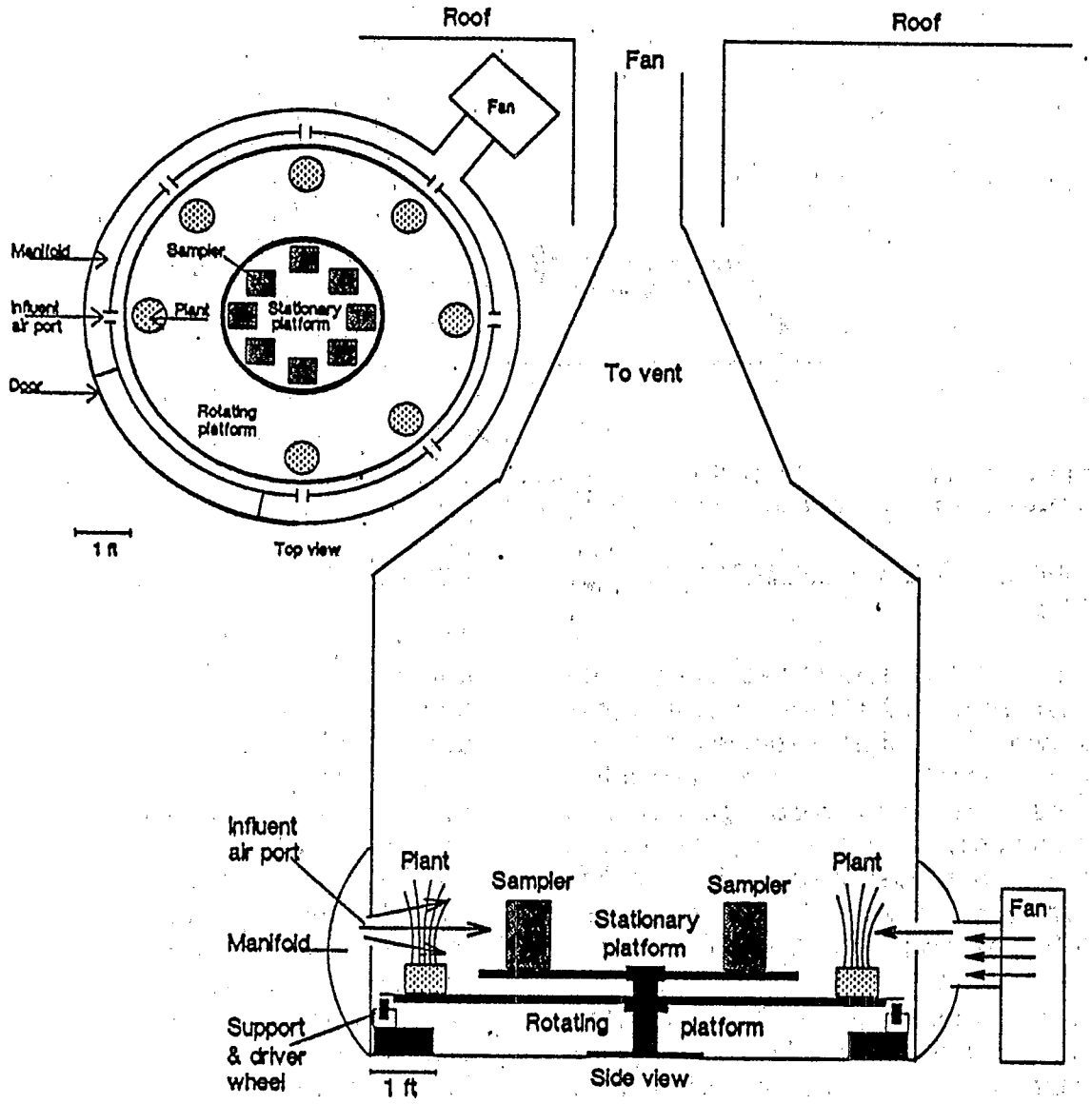

Figure 2. Environmental Bio-Aerosol Research Chamber (EBARC).

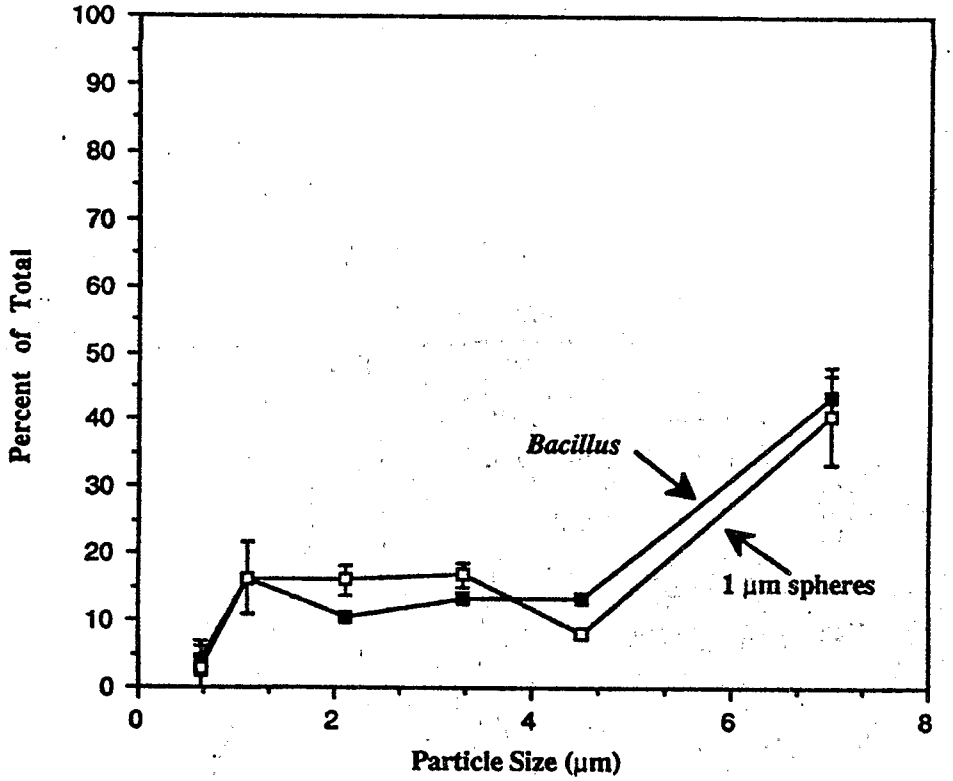

Figure 3. Comparison of the particle size distribution of *Bacillus subtilis* var. *niger* spores and 1 μm fluorescent microspheres blown off oat plants after multiple wind puff treatments using the EBARC.

Simulation Models: General Survival Model

Using DAT determined death rate values from ca. 70 literature cited cases involving 17 bacterial species in which gram reaction, relative humidity, temperature, and aerosol age were the experimental variables (Figure 4b), a regression model was fit having R^2-value 0.94 (Figure 4a)(Lighthart, B., 1989, Aerobiol. 5:138-144). Death Rate Constant ($\log_{10}$) = Constant - Log^{10} (Aerosol Age) + Temperature - Gram Reaction * RH. A less demanding model with an R^2 if 0.90 was fit to: Death Rate Constant ($\log_{10}$) = Constant - Log^{10} (Aerosol Age)

These models may be inserted into the dispersion model to give realistic survival properties of the bioaerosol as a function of temperature, relative humidity, gram reaction over time in the atmosphere.

Simulation Models: Trajectory Model

In an effort to obtain a more realistic characterization how a polydispersed bioaerosol might wander in the atmosphere, the trajectory of various size droplets containing various numbers of microorganisms in atmospheres of different relative humidity, temperatures, and wind speeds was prepared (Lighthart et al., in press). The results of this effort are shown in Figure 5a (simulated) where it is seen that droplets of $\leq$ 80 μ diameter in a 50% relative humidity and 20 °C atmosphere, evaporate to the bacterial residue of about 1 μm before they can fall to the ground and therefore remain airborne becoming part of the aeroplankton. Those greater then 80 μm fall to the ground. Observations confirm the model (Figures 5b, c observed).

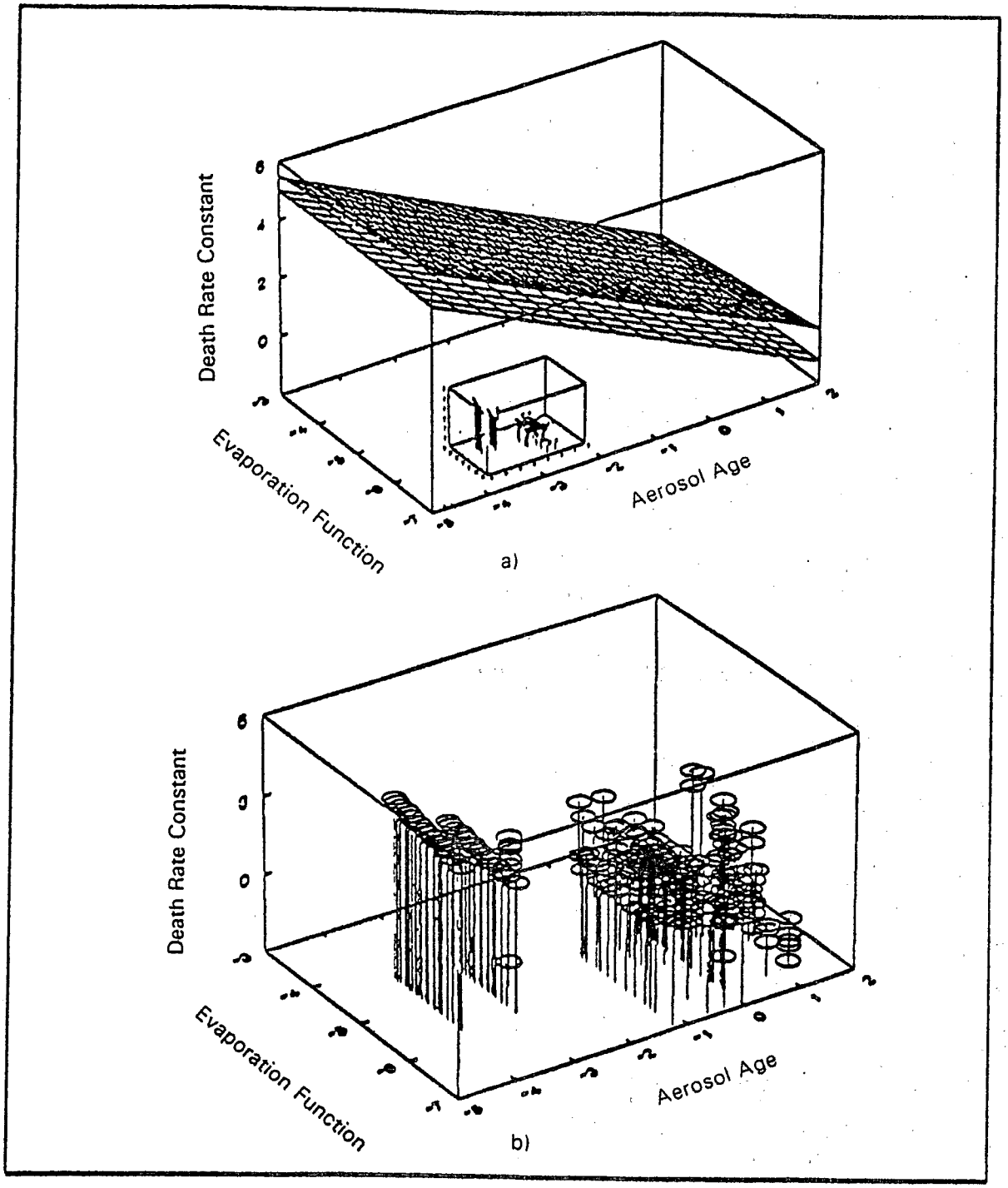

Figure 4. a) Response surfaces for aerosol age, evaporation function and bacterial death rate constants for Gram positive (lower surface) and Gram negative (upper surface) bacteria using the general death rate equation model. The two surfaces result to be not parallel; b) enlarged picture of insert shown in fig. 4A.

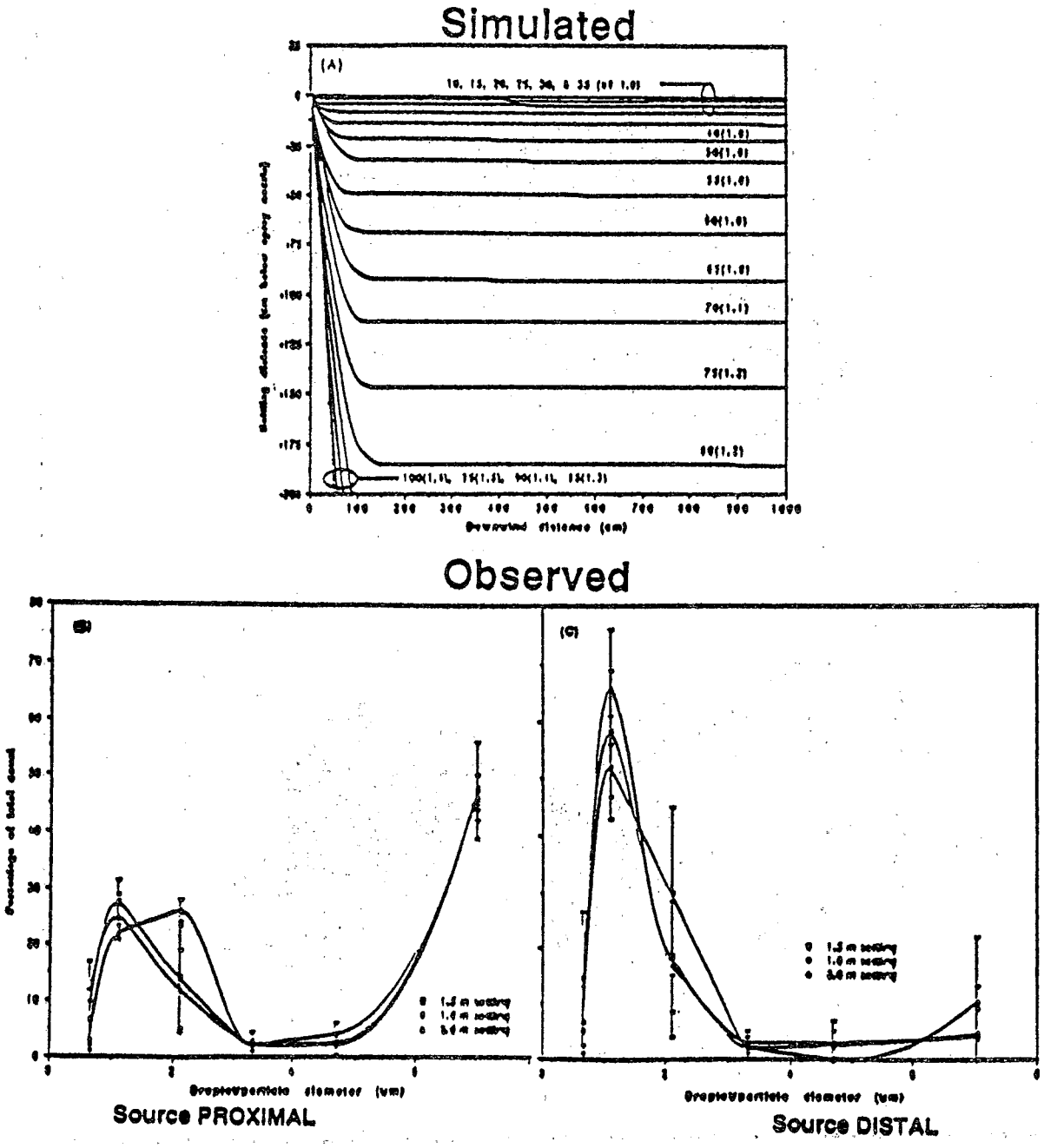

Figure 5. Trajectory model

Simulation Models: Deposition Model

A bioaerosol dispersion model has been developed and crudely compared with observations made at the Tulelake, CA, ice-minus bacterium spray-out (Lighthart and Kim, 1989). recently, a controlled spray of bacterial spores has been carried out by the U.S. Army at Dugway Proving Ground to provide data to quantitatively evaluate reliability of the bioaerosol dispersion model with a good set of observations. Presently, the model has been run using the observed meteorological data (Figure 6); the statistical comparison between simulated and observed will be completed in the near future.

FUTURE RESEARCH

* Evaluate the effects of genetic construct on airborne microbial survival.

* Evaluate the airborne survival of insect pathogens including MPCAs.

* Prepare a protocol to evaluate the survival of airborne microorganisms.

* Modify the bioaerosol dispersion model for use in a microcomputer.

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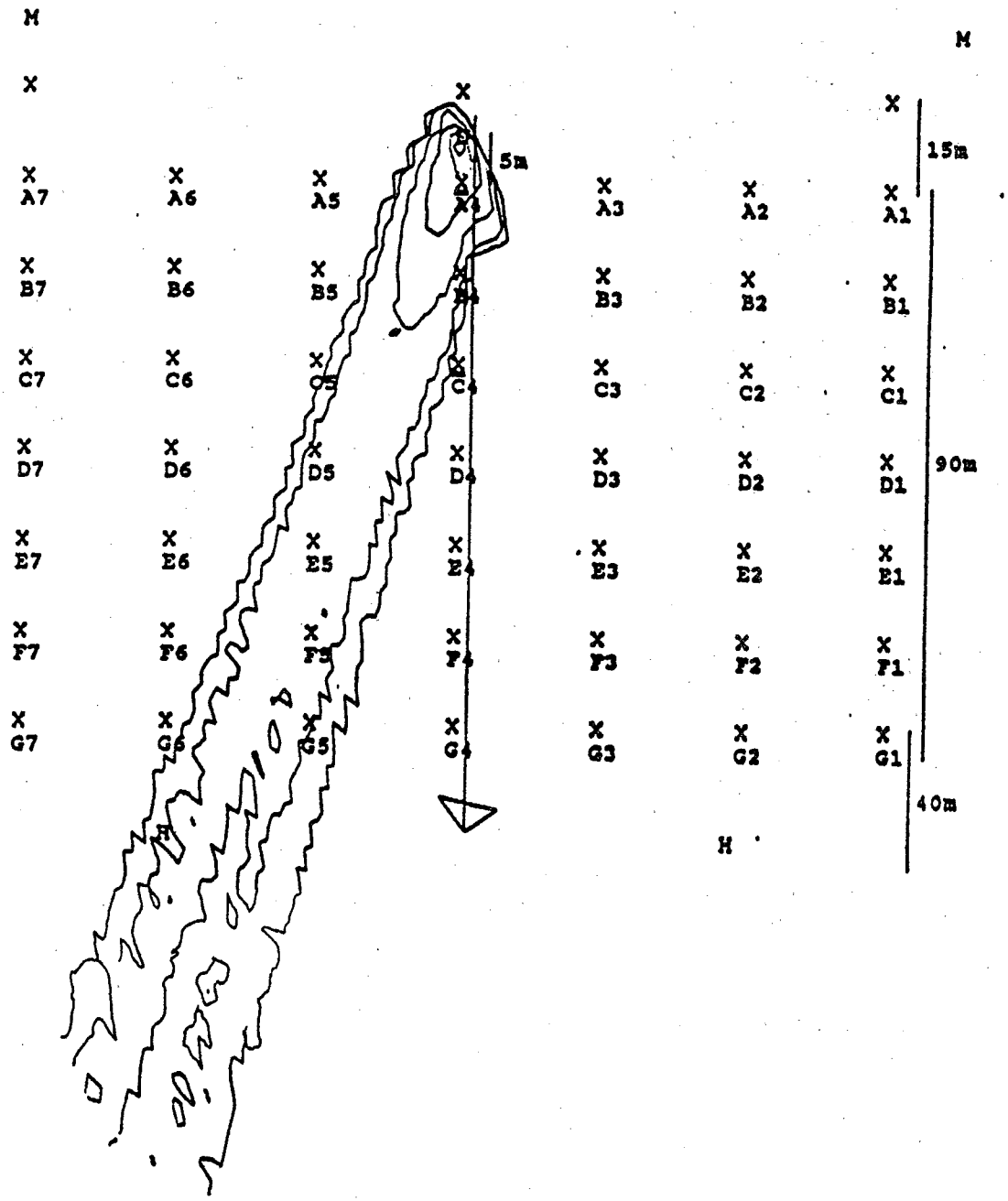

X - Andersen Sampler, X - Aerodynamic Particle Sizer and Andersen Sampler, H - High Volume Sampler, M - Meteorological Tower, D - Aerosol Disseminator

Figure 6. Sampler Arrangement

MEASURING ENTRAINMENT OF BACTERIA FROM THE PHYLLOSPHERE

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INTRODUCTION

Monitoring the dispersal patterns of genetically engineered microorganisms (GEMs) from a release site is an important aspect of understanding what impact GEMs may have on the environment. The possibility exists that after application, GEMs could become resuspended (entrained) from soil or leaf surfaces and transported downwind to colonize other locations.

Plant canopies as well as soil have been shown to be sources of entrained bacteria. The number of bacteria entrained is influenced by the season of the year, moisture content of the soil, and type of plant canopy. In addition, activities such as harvesting have been known to influence the number of entrained bacteria detected.

Measurements taken on days 1,2 and 4 following the application of recombinant strains of *Pseudomonas* indicated that low numbers of the GEM were being entrained into the atmosphere. The number of entrained bacteria decreased with time after application. In addition, entrained bacteria were detected primarily on gravity settling plates (GSPs), which are 150 mm petri dishes containing selective agar. Mechanical air samplers that proved very sensitive during the actual applications of the GEM either failed to detect entrained GEMs or detected very few of them compared to the GSPs.

It has been demonstrated that under greenhouse conditions, bacteria can be injured during aerosolization and that this injury can adversely effect their subsequent survival. Entrainment of bacteria results in their resuspension into the atmosphere, therefore it is possible that this process could cause injury or death to GEMs after release, or render them incapable of surviving in competition with indigenous microorganisms.

The objectives of this study were to: A) develop a method of artificially inducing bacterial entrainment from the phyllosphere, B) compare sampling efficiencies of GSPs, all glass impingers (AGIs) and Andersen samplers to determine which method of sampling would be most sensitive in detecting entrained bacteria, C) compare the magnitude of entrainment from different species of host plants, and, D) determine if the viability of bacteria is affected during entrainment.

METHODS

Media and Cultures

All experiments were conducted using a nonrecombinant strain of *Pseudomonas syringae* TLP2, resistant to 100 μg/ml of rifampicin (RIF).

Several studies were conducted using spores of *Bacillus subtilis var. niger*. *P. syringae* were cultured and enumerated using 100 μg/ml of RIF in Luria-Bertani broth and agar (LB-RIF) respectively. *B. subtilis* spores were obtained from spread plates on casitone agar (CAS) incubated at 30°C for seven days. Spores were recovered by scraping 10 plates with 10 ml of sterile distilled water and suspending them in 100 ml of distilled water.

Plant Application and Sampling

P. syringae were applied to three-week-old beans (*Phaseolus vulgaris humilus* cv. Bush Blue Lake) and three-week-old oats (*Avena sativa* L. cv. Cayuse) planted in 61 X 30 X 13 cm. pulp trays grown in a greenhouse. Plants were sprayed until run-off with twice washed *P. syringae* following incubation for 18 hours at 30°C. A population of 1 x 10^8/ml was applied using a CO_2 backpack sprayer pressurized to 36 psi and equipped with a Tee-Jet nozzle #8004-SS. Plants were allowed to dry a minimum of two hours before sampling was initiated. Bacterial populations were determined by randomly sampling three bean leaves or five oat stalks. Leaves were diluted with 20 ml of phosphate buffer and blended for one min. in a stomacher blender. Samples were serially diluted and plated on selective media and incubated for 48 hours at 30°C. Counts were standardized on a per gram basis. Spore populations were determined by heating at 80°C for 20 min. to heat-shock spores into germination and kill undesired bacteria. Samples were filtered using 0.45 μm filters and filters placed on CAS agar plates.

Aerosol Samplers

Six-stage Andersen samplers containing six agar plates filled with 20 ml of LB RIF were operated at 28.3 L/min. The AGIs were loaded with 20 ml of sterile 10 mM phosphate buffer and run at 12.5 L/min. Following sampling, the buffer was filtered through 37 mm diameter, 0.45 μm cellulosic filters, then transferred to the appropriate agar. GSPs used in these experiments consisted of 150 mm petri dishes filled with 75 ml of LB-RIF agar. Plates were uncovered immediately prior to sampling and covered immediately after sampling events. All plates were incubated for 48 hours at 30°C and colonies counted.

Bacterial Entrainment

Bacterial entrainment was induced by placing a 20 inch electric fan 0.5 m from sprayed plants. Air flow through the plants was measured at 4 m/sec and entrainment was measured for 10 min. In all experiments except sampler efficiency, plants were mounted on two 9 cm^2 wood blocks. Experiments were conducted in a 10 X 30 m greenhouse.

Sampler Efficiency

A two-level 122 X 183 cm. platform was constructed to adjust the intakes of the three samplers to the same height. The upper level of the platform consisted of three parallel rows of three circular holes located 0.25, 0.6, and 1.0 m from sprayed plants. The AGI

and Andersen samplers were placed on the lower level of the platform with intakes protruding through the holes level with the height of the gravity settling plates. Entrainment was measured at 2, 24, and 120 hours after application of the *P. syringae* using a separate set of plants for each time point. Results were compared using $\log_{10}$ transformations of the number of CFU observed on a per sampler basis.

Factors Affecting Bacterial Entrainment

Eight AGIs or two Andersen samplers were placed at 0.25, 1.0, 2.0, and 3.0 m from inoculated plants. AGIs were mounted in two groups of 4, at 26 cm. and 53 cm. above the ground, each group of four separated by 35 cm. Andersen samplers were placed 35 cm. apart on the soil surface at each of the four distances.

Effect of Host Plant

The effect of host plant was measured by comparing the magnitude of bacterial entrainment from beans with that of oats. Experiments were conducted to measure total bacterial populations being entrained from each plant using AGIs, and difference in particle size distributions were measured with Andersen samplers.

Dilution vs Die-off

To determine if decreases in entrained bacterial counts were due to dilution or loss of viability, the number of entrained vegetative bacteria were compared with the number of entrained spores of *B. subtilis*. Decreases in the spore populations were due to dilution, while decreases in the vegetative bacterial populations were due to loss of viability.

Time After Application

The effect of time after application was measured by sampling bean plants sprayed with *P. syringae* at 2, 24, and 120 hours after application using only AGIs. In these experiments, all four distances were sampled simultaneously, since the aim of the experiment was to detect the total number being entrained. The number of bacteria entrained were compared over time and related to the population of bacteria found on the leaf surface.

RESULTS AND DISCUSSION

Sampler Efficiency

In general Andersen samplers, AGIs and GSPs had the same pattern of response throughout the experiments. In all cases, there were significant effects due to both time after application and distance from the sprayed plants. On a per volume basis (CFU/m^3), there was no difference between the number of entrained bacteria detected in AGI and Andersen samplers. AGIs exhibited the most variable data over both distance and time. However, variability tended to increase for all three samplers as the distance from the sprayed plants and time after inoculation increased. No entrained bacteria were detected on GSPs beyond 1 m from plants.

Factors Affecting Bacterial Entrainment

Over a distance of 3 m from sprayed plants, both AGI and Andersen samplers exhibited similar dispersal patterns of entrained bacteria. Both samplers detected substantial numbers of bacteria 3 m from plants. Significantly higher numbers of entrained bacteria were detected 0.25 m from plants than at the other three sampling distances. There was no significant difference in the number of entrained bacteria detected 1 to 3 m from plants.

Graphical examination of the data collected from AGI and Andersen samplers demonstrated that the distributions of data were not the same. This difference indicates that the two populations being sampled are not equivalent, and rendered it impossible to make a direct comparison between sampler types. It appears that the AGI, which is a liquid based sampler, is sampling actual numbers of bacteria entrained, while the Andersen sampler, which impacts bacteria directly onto an agar plate, is sampling the number of infective particles being entrained.

Effect of Host Plant

An average of 79% more *P. syringae* were entrained from beans than from oats. For each distance sampled, the number of entrained bacteria from beans was significantly higher than the number of bacteria detected from oats. A significantly higher population of *P. syringae* were detected on bean leaves as compared with oat leaves. If the number of bacteria entrained from each plant is compared as a frequency of the relative population on the plant, then the amount of entrainment was actually higher from oat plants than bean plants. Bacteria entrained from oats followed the same dispersal pattern as from beans but with significantly higher numbers of entrained bacteria detected at 0.25 m from plants than from 1 to 3 m. Again, no difference was found in the number of bacteria detected 1-3 m from plants. There was a significantly different particle size distribution from each plant. The size of particles entrained from bean plants tended to decrease in size over distance while those from oats tended to have a particle size of 7 μm or greater.

Dilutions vs. Loss of Viable Count

The decrease in entrained spores was significantly greater than the decrease in vegetative *P. syringae*. At 0.25 m, virtually the same number of spores and vegetative cells were detected. However, as the distance increased, the number of vegetative *P. syringae* decreased below the number of *B. subtilis*. This indicates that differential loss of viable count was occurring. By dividing the number of vegetative cells by the number of spores detected at each distance, it was possible to generate a survival ratio over distance. If the number of vegetative cells decreased relative to the number of spores at each distance, then the ratio would become smaller over distance, which is what was observed. Therefore, loss of viable count is occurring beyond a distance of 0.25 m from plants.

Time After Application

Significantly more bacteria were entrained 2 hours after application than 24 and 120 hours after application. However, the number of bacteria entrained 24 hours after application was not significantly different than the number entrained 120 hours after application. Bacterial populations on plants reflected the decline in the number of bacteria entrained to a limited extent. The number of bacteria detected 2 hours after application was significantly higher than the number detected at 24 hours, and these populations were significantly higher than those detected after 120 hours. However, while plant populations continued to decline from 24 to 120 hours, the numbers of bacteria being entrained did not.

Results from these experiments suggest that: A) GEMs are entrained following leaf applications in response to moderate amounts of wind, B) the type of sampler used to detect bacterial entrainment must be based on the type of data desired, C) the magnitude of bacterial entrainment may be dependent on the host plant, D) the process of entrainment may cause death or injury to vegetative bacteria, and E) the magnitude of bacterial entrainment may not decrease over time.

Future research should be directed toward using this method to measure the colonization of plant surfaces by entrained bacteria, identifying environmental conditions most influential on bacterial entrainment, and conducting field experiments to validate the greenhouse data.

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UPDATE ON THE ASSESSMENT OF BACTERIAL DISPERSAL FROM FOLIAGE BY RAIN

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INTRODUCTION

Recent advances in genetic engineering techniques have made possible the modification of bacteria for use as novel pest and disease control agents in crop plants, or as potential replacements for chemicals currently used in agriculture. However, before such agents are released into the environment, possibly on a large scale, it is important to know if they are likely to have deleterious effects on non-target organisms or plants.

The release of genetically engineered microorganisms requires their containment within the area of release, and thus an assessment of their potential for spread is of great importance. Rain splash as a factor in the spread of disease in crops has long been recognized and it has been shown that it can play a major part in the spread of many fungal and bacterial plant pathogens. However, little is known of the efficiency of rain in removing and dispersing bacteria from leaf surfaces. A project to study the potential of rain splash for removing and distributing bacteria from the foliage of crop plants is underway at the Institute of Arable Crops Research, Rothamsted Experimental Station.

METHODS

All experiments were done in the Rain tower/wind tunnel facility at Rothamsted. Mono-sized water drops (simulating rain drops) were allowed to fall 11m onto target leaves carrying populations of bacteria. Rain drops of between 2 and 5mm diameter could be produced. Droplets, dispersed by splash, were collected at different distances from the targets and their bacterial content assessed. The bacterial content of water which collected on, and subsequently ran off, the leaves was also assessed.

Isolates of *Pseudomonas syringae*, *Klebsiella planticola* and *Bacillus subtilis var. niger*, all resistant to 100μg/ml of the antibiotic rifampicin were used in these experiments.

Two species of glasshouse grown crop plants were used: *Phaseolus vulgaris* cv. Prince, and *Brassica napus* cv. Cobra. The plants were chosen principally because of their different leaf surface characteristics: *P. vulgaris* has a hairy surface and no pronounced wax cuticle, while *B. napus* has a waxy cuticle, and is not hairy.

Bacteria were grown overnight in nutrient broth (Oxoid) at 30°C to a concentration of between 10^7 and 10^8 CFU/ml. The target plants were sprayed, inside a closed perspex

chamber, with the nutrient broth and left for about 22 hours to allow the bacterial population to stabilise. The plants were removed from the chamber about 6 hours before each experiment to allow surface moisture to dry from the leaves. Leaves were removed from the sprayed plants and placed in an overlapping layer on a horizontal nylon mesh covered frame at the base of the rain tower. The population of antibiotic resistant bacteria on the target leaves was assessed by taking a sample of four leaves before exposing the rest to splash. Each sample leaf was weighed and placed in a sterile glass conical flask containing 50ml of sterile quarter strength Ringer's solution (Oxoid) and washed on a flask shaker for 1 hour. Samples of the wash water were plated out on selective medium (N+R) consisting of nutrient agar (Oxoid) with 100μg/ml actidione to suppress fungal growth, and 100μg/ml rifampicin as a selective agent for the inoculated bacteria. The plates were incubated at 30°C overnight and the bacterial colonies counted.

The target leaves were exposed to splash from artificial rain drops for 30 minutes. Immediately after exposure, a further sample of four leaves was removed and washed to assess the numbers of inoculated bacteria remaining on the leaves. A sample of water from the rain generator was also plated out onto N+R as a check for contamination by antibiotic resistant organisms.

Splash droplets were collected in either sterile 9cm plastic Petri dishes containing 20ml of N+R or in sterile 9cm polypropylene funnels supported in sterile glass universals. The Petri dishes were used to estimate the numbers of bacteria-carrying droplets splashed and the funnels to estimate the total number of cells dispersed. The collecting vessels were supported at the same height as the leaves, at 8, 24, 40, 56, 72 and 88cm from the edge of the target area. During an experiment the two Petri dishes closest to the target were replaced with fresh plates at three minute intervals and the third closest plate was replaced after 15 minutes. Four pre-weighed funnel/universal assemblies were arranged underneath the leaf support frame to collect samples of the water running off the leaves. Run off water was also collected in plastic trays placed under the target. The sum of the volume of water collected in the trays and funnel/universal assemblies was taken as the total amount of run off water.

The Petri dish droplet samplers were incubated at 30°C overnight and the colonies growing on the plates counted. The droplet collecting funnels were rinsed with a known volume of sterile distilled water which was added to the collected water and samples of the resultant bacterial suspension plated out onto N+R medium. Samples of run-off water were diluted as appropriate and also plated out onto N+R. The plates were incubated overnight at 30°C and colonies counted.

RESULTS

Twenty-five experiments were done using the six different combinations of two plant species, *P. vulgaris* and *B. napus* and three bacteria species *P. syringae*, *K. planticola* and *B. subtilis*.

For all experiments the rain drop size was 3mm and air temperature was about 18°C with about 85% relative humidity. The effect of rain drop size was studied in a further 24 tests. Three different rain drop sizes were used: 2.4, 2.9 and 4.7mm diameter. Eight experiments were done with each drop size using *B. subtilis* on *B. napus* as the target. Experiments were done at about 21°C and 75% relative humidity.

Proportion of Bacterial Populations Removed by Splash

There was a large variation in the estimate of the proportion of bacteria removed by splash for replicate experiments and no statistical differences were found either between bacteria species or between plants. The variation in bacterial numbers between individual leaves, used to assess pre- and post-splash bacterial populations, was often large and frequently the differences between pre- and post-splash bacterial numbers were not statistically significant. However, the results suggest that up to 90% of bacteria may be washed off the leaves. Surprisingly, there was little effect of drop size in removing *B. subtilis* cells from *B. napus* leaves. It was estimated that, for all drop sizes, about 75% of the bacteria were removed during each experiment.

Bacteria Removed by Run-off

In all experiments substantial numbers of bacteria were found in the run-off water. In the first set of experiments the numbers of bacteria recovered per unit area of target was frequently (70% of experiments) greater than the estimated population density at the start of the experiment, suggesting that the method of leaf washing may have underestimated the initial populations. There was no significant difference between the proportion of bacteria in the run-off water (P_r) for the two plant species and the same organism. However, P_r for *P. syringae* and *K. planticola*, was nearly always greater than for *B. subtilis*. In the second series of experiments the value of P_r increased with drop size (mean values of 0.27, 0.44 and 0.84 for 2.4, 2.9 and 4.7mm drops respectively).

Dispersal by Splash

The numbers of inoculated bacteria recovered on exposed agar plates or from the funnel assemblies decreased rapidly with distance from the target leaves. The number of bacteria deposited per square centimetre, D, decreased exponentially with distance from the target: $D = A \exp(-\alpha x)$ where A, α are constants. The equation has the property that D decreases by half at regular distances, $d_{1/2}$ ($= 0.693/\alpha$), from the source. The half distance, $d_{1/2}$, is an easily visualised measure of the deposition gradient.

Deposition gradients for both plates and funnels for all tests were steep: $d_{1/2}$ ranged between about 5 and 17 cm. For a given organism the values of $d_{1/2}$ for funnels were similar for each plant species: for *P. syringae* and *K. planticola* the average value of $d_{1/2}$ was about 7cm for *B. napus* but slightly larger (≈9cm) for *P. vulgaris*. Because

the numbers of *B. subtilis* cells caught in the funnels were small at distances greater than about 20cm from the target $d_{1/2}$ values were unreliable. The values of $d_{1/2}$ for plates were similar ($d_{1/2} \approx 7$cm) for *K. planticola* and *B. subtilis* inoculated leaves, but, for *P. syringae* on both plant species deposition gradients were significantly shallower ($d_{1/2} \approx 12$cm). For both funnels and plates mean (of 8 replicate experiments) $d_{1/2}$ values were largest for 4.7mm rain drops and smallest for the 2.4mm drops: 8.2, 7.9 and 5.1cm for the funnels and 10.3, 7.5 and 7.2cm for the plates for 4.7, 2.9 and 2.4mm rain drops respectively.

Proportion Dispersed by Splash

The total numbers of bacteria dispersed in splash droplets per centimetre width of the target was estimated by numerically integrating the deposit with distance from the edge of the target. The numbers of bacteria dispersed depended on the initial population present on the leaves. In nearly all cases, (80%), it was estimated that less than 5% of the initial inoculated bacterial population was splashed. The average fraction of bacteria splashed was about 1.5% (range 0.02 - 8%). There appeared to be little systematic difference in proportions splashed between either bacteria species or plant species, except for *B. subtilis* where the mean values for *B. napus* were significantly larger than for *P. vulgaris* (2% compared to 0.3%). In the second set of experiments a greater proportion of *B. subtilis* cells were splashed from target leaves by large rain drops than the smaller ones: about 1.7% for 4.9mm drops compared to 0.5 and 0.2% for 2.9 and 2.4mm drops.

The number of cell carrying droplets splashed was much smaller than the number of cells for both *P. syringae* and *K. planticola*: between 5 and 200 times as many *P. syringae* cells and between 2 and 50 *K. planticola* cells were splashed than droplets. For *B. subtilis* the numbers of droplets and cells splashed were similar. This difference between organisms was probably due to differences in initial populations. The initial populations of both *P. syringae* and *K. planticola* were usually larger than those of *B. subtilis*. The number of cells carried in each splash droplet will depend on the leaf population density: larger densities will lead to more cells per drop. Thus at high densities the number of cells splashed will be larger than the number of cell carrying droplets splashed. As with the cells larger rain drops splashed more bacteria carrying drops than smaller ones. About twice as many cells as bacteria carrying droplets were splashed for the three drop sizes.

Dispersal Rates with Time

The pattern of dispersal with time, as indicated by the change in deposition rate on the plates nearest the target, was substantially different for the experiments with *P. syringae* than for the other two organisms. With *P. syringae* the deposition rates decreased almost linearly with time and for both plant species were reduced by about 20% over the 30 min. exposure period. Deposition rates of the other two organisms decreased roughly linearly

with time when splashed from *P. vulgaris* plants: rates were reduced by about 70% during the experiments. For *B. napus* the decrease in deposition rates followed a concave pattern with rates at the end of the test only about 10% of those at the beginning, suggesting that the available bacteria were washed off *B. napus* leaves more readily. There were also differences in the changes in deposition rate with time for the rain drop experiments. For the 4.7mm drops deposition rates decreased in a similar manner to the first set of experiments, except that the differences between beginning and end rates were smaller; about a 60% reduction. With the other drop sizes deposition rate initially increased reaching a maximum about 5 minutes and 10 minutes from the start of the experiment for the 2.9 and 2.4mm droplets respectively. Deposition rates then decreased and at the end of the tests were about 45% of the maximum value for the 2.9mm drops and about 60% for the 2.4mm drops.

DISCUSSION

Our experiments show that rain splash can be an efficient mechanism for removing bacteria from leaf surfaces. Patterns of dispersal and the distances travelled by bacteria in splash droplets were similar to those which have been reported for both fungal and bacterial pathogens. Although we only examined the effect of splash on the removal of three species of bacteria from two plant species, the results suggest that splash dispersal patterns and splash processes may be similar for a wide range of organisms and plants. The proportions of the leaf populations dispersed in splash droplets were small (usually <10%) as were the distances travelled by most of the bacteria. However, large numbers of cells were found in the run-off water collected from underneath the target area. Thus, rain water running off leaves to the soil around crop plants could be an important source of bacteria for further spread through travel in ground water, or by secondary splashing.

Large rain drops appear to be more effective in removing and splashing bacteria from leaves than small ones. However, bacteria were more quickly removed by the large drops, suggesting that for longer periods of rainfall drop size may not be critical for the removal (but not necessarily the dispersal) of bacteria from leaves. Indeed, when the proportions of bacteria removed during the experiments were adjusted for the volumes of rain falling on the target, the small drops were just as efficient at removing bacteria as the large ones.

These experiments show that rainfall has the potential to remove and disperse bacteria from leaf surfaces. However, as studies of the dispersal of fungal pathogens show, the splash process is complex and its effectiveness depends upon the nature of the rainfall or irrigation and on the nature of the crop or vegetation canopy. More work is needed to answer questions such as how does crop structure effect bacteria spread? Which rainfall events favour the dispersal of bacteria? Are natural bacteria dispersed in a similar manner to inoculated bacteria? How efficiently do bacterial cells survive dispersal? Wind may also have a role in the spread of bacteria from leaf

surfaces. Thus, an understanding of the effects of the combination of wind and rain to the removal and spread of bacteria is needed before the potential for dispersal of genetically manipulated organisms can be quantified.

FUTURE WORK

As the co-operative agreement terminates in July 1991 there is little scope for expanding the studies much further. However, experiments to examine the influence of environmental factors such as relative humidity on bacterial spread are planned. We also intend to examine the potential for longer distance transport by very small airborne droplets. Funding to expand these studies is being sought from other sources.

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GENETIC AND MOLECULAR ANALYSIS OF SURVIVAL MECHANISMS OF PSEUDOMONADS

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INTRODUCTION

Colonization of plant roots by certain isolates of fluorescent pseudomonads promotes plant growth and suppresses pathogens. These beneficial effects have raised interest in the agricultural value of crop inoculation using native or genetically engineered strains to improve quality and yield. In order to assess some of the potential hazards associated with release of genetically engineered pseudomonads we have researched basic processes involved in colonization of plant roots by pseudomonads. Colonization is important in the attainment of effective populations of the bacteria and in the persistence and spread of organism in the environment.

We have concentrated upon two aspects of pseudomonad colonization in the plant root environment: 1) mechanisms of pseudomonad attachment to the plant root involving plant agglutinin, and 2) the role of catalase and superoxide dismutase (SOD) in pseudomonads in protection of the bacteria against root produced activated oxygen species.

METHODS, RESULTS and DISCUSSION

Mechanism of Attachment to the Plant Root Involving Agglutinin

Certain isolates of *Pseudomonas putida* are agglutinated by plant surface glycoproteins termed agglutinins. Purification of the agglutinin has been initiated to determine structure and as a primary step in isolation of the plant genes encoding agglutinin-active components. We have examined wild type agglutinable isolates, mutants which lack the agglutination phenotype (Agg^-) and agglutination complemented mutants to probe the molecular and genetic basis of the agglutination phenotype in the bacterium.

A) The agglutinin is a ß-lectin arabinogalactan protein

Agglutinin activity is detected in water washes of intact plant roots. Components in bean root washes have been purified by passage through a sizing filter to remove molecules of less than 10,000 Mr, followed by Concanavalin A and DEAE-Sephadex chromatography. These approaches demonstrate that agglutinin activity is associated with multiple fractions. The majority of the agglutinin activity elutes without adsorption to both ConA and DEAE-Sephadex. This nonadsorbed material retains ability to react with Yariv's reagent: a reaction indicative of ß-lectin activity associated with arabinogalactan proteins. IEF of the nonadsorbed material fractionates the

preparation into four bands of agglutinin -active components with pI 7-8 and a larger agglutinin-active heterogenous fraction with a more basic pI.

A second line of evidence supports the agglutinin to be an arabinogalactan protein. Both crude and more purified preparations react with a monoclonal antibody MAC 207, which is derived to an arabinose epitope of a membrane-associated arabinogalactan protein from carrot. This antibody was generated in the laboratory of Dr. Keith Roberts, John Innes Institute of Norwich England, with whom collaborative studies are being continued. Agglutinin-active fractions which elute without adsorption to ConA, but which are retained or adsorbed to DEAE-Sephadex react differently to two other mononclonals derived by Robert's et al. to pectin : thus these studies indicate that differences in carbohydrate structure between agglutinin-active components exists. Data from studies of Robert's et al. indicate that MAC 207 antibody-reactive materials are widespread in the plant kingdom: these data agree with our previous observations that extracts from a wide array of plant species possess agglutinin activity.

In collaboration with Dr. Schippers at the University of Utrecht we have surveyed several pseudomonad isolates from wheat, tomato and potato for agglutinability. Certain of these European strains display agglutinability with our crude and more purified preparations of agglutinin from bean, as well as with crude root wash preparations from wheat, tomato and potato prepared at Utrecht. These data support that the agglutination phenotype in pseudomonads has relative little plant species specificity. Additional collaborative studies with our Agg^+ wild type and Agg^- mutants of *P. putida* have revealed that the agglutination phenotype has little influence on whether the bacteria become endorhizosphere colonists or remain on the rhizoplane.

B) Genetic and molecular studies of the bacterial agglutination phenotype.

Genetic and molecular techniques have been used to explore the nature of the bacterial phenotype which is responsible for agglutination. Genetic analysis has revealed two distinct loci associated with the agglutinable phenotype. One locus, termed *aggA* has been analyzed in mutant Tn5 5123. A second locus *aggB* is detected in Tn5 mutants 1104 and 6000.

The *aggA* locus is present in a 2.7 kbp EcoRI-Hind III subclone of parental DNA. Deletion analysis using exonucleases revealed only 1.45 kbp was needed for complementation of the Agg^- mutant 5123 to the Agg^+ phenotype. The presence of the subclone in transconjugants of 5123 restored the agglutinability of the cells. Adherence to intact bean roots surfaces in a 15 minute exposure was also restored to wild type level in the transconjugants. Thus, a role of the locus in binding, which was suggested by previous studies with Agg^- mutants, was confirmed.

Sequence analysis of the 2.7 kbp fragment reveals an open reading frame on one strand of 1356 nucleotides encoding a predicted 50,509 Da protein and pI of 5.24. A consensus sequence

for a ribosome binding site (AGGGAG) was located 8bp upstream of the projected ATG translational start site. Portions of the canonical sequences recognized by the δ^{54} and δ^{70} RNA polymerase holoenzymes were present 5′ of the ATG. The predicted protein contains one membrane associated helix, from residues 2-17, and there is consensus for a prokaryotic cleavage site located between residues 22 and 23.

Another reading frame was present on the opposing strand of the 2.7 kbp subclone but it lacked a ribosome binding site, promoter sequences and would use GTG as a potential translational initiation site. No translational stop codons were observed in 86 bases 5 to the GTG site. These data suggest that the second ORF may be incomplete and not be involved in the agglutination phenotypes. A search of the NBRF and GenEMBL data bases has revealed no homologous sequences with either of the predicted ORFs.

Hybridization analysis with the *aggA* locus to the genomes of plant-associated bacteria indicated that with thirty isolates examined from bean, wheat, tomato and potato, only the agglutinable isolates demonstrated strong to moderate hybridization. Thus this locus has good specificity for detection of the agglutinable phenotype.

The second locus *aggB* from *P.putida* also is involved in the agglutination phenotype. Complementation of the phenotype has not been achieved *in trans* using cosmid clones from a wild type *P. putida* library. Marker exchange studies with the locus *aggB*: Tn5 disrupted the Agg^+ wild type chromosome and the recombinants were nonagglutinable. Preliminary data suggest the presence of repetitive sequences in the region of the insertion.

Molecular studies have demonstrated that cell envelope proteins are associated with agglutination phenotype. Agg^-mutants Tn5 5123, EMS 1202 and EMS 1236 possessed a 16,000 Mr protein band which was less apparent in the Agg^+ parental strain. Fractionation of the cells indicates this 16,000 Mr protein is located in the periplasm. Agg^- mutants Tn5 4312, Tn5 5123 and EMS 1236 displayed an outermembrane protein band of Mr 42,000 which was not observed in the parent. Transconjugants of Agg^- 5123 which were restored to agglutinabilty (Agg^+) by the presence of clone pRKAGG201 bearing a wild type 2.7 kbp EcoRI -Hind III fragment did not produce the 42,000 Mr protein but still expressed the 16,000 Mr protein. These data suggests that the 42,000 Mr protein is associated with the agglutination phenotype.

Catalase and SOD Activities from Root Colonizing Pseudomonads

The plant root is potentially a toxic environment for microbes because of the production of activated oxygen species from the root surface. Consequently, we have examined the role of catalase and SOD in root colonizing pseudomonads as a protective mechanism to permit survival in the rhizosphere. Each of the pseudomonad isolates examined have catalase and SOD activities. We have

surveyed five beneficial isolates from wheat, six isolates from sugar beet, the bean colonizing isolate of *P. putida* and isolates from plants grown at a mine spoil site with acidic pH and high copper content. The mine spoil site was examined because of the potential of heavy metals to catalyze interconversions of activated oxygen species which would perhaps put oxidative stress on the bacteria. The pseudomonads from the agricultural soils had similar levels of catalase whereas three of four mine spoil isolates displayed three to ten fold more activity. Multiple isozymes of catalase of differing mobilities between isolates were observed for cells grown to stationary phase in rich medium, whereas one or two isozymes of SOD were obtained.

Studies with *P. putida* and *P. fluorescens* isolates with beneficial properties demonstrate that SOD is rapidly increased in activity upon exposure to bean roots and remains at high level during root colonization. Catalase activity is low in cells that have established colonization. However, upon short term exposure in the bacteria the activity transiently increases, in part because of production of a second isozyme and enhanced levels of the normally expressed isozyme. We are exploring the differential functions of these catalase isozymes. Catalase isozyme A is the first isozyme produced during logarithmic growth and has a cytoplasmic location. Additional isozymes, which are less anodic on nondenaturing gels and are termed B,C etc., are produced later in growth phase. Catalase B is located in the membrane envelope of *P. putida*. Increased activities of catalase A and production of catalase B and C occur in logarithmic phase cells of *P. putida* after exposure to H_2O_2. Thus these changes in isozyme expression may be important for the survival of the bacterium to exogenous H_2O_2, a situation occurring upon exposure to plant roots.

It seems likely that catalase A and B are products of different genes, rather than being derived from one gene product. The isozymes are differentially inhibited by aminotriazole and organic solvents. Also we can separately mutate the activities in *P. putida*. Mutant J1, obtained by EMS mutagenesis of the parental strain, lacks catalase A activity under liquid growth conditions. Nondenaturing electrophoresis indicated that catalase A was absent, although catalase B and C were produced during late growth periods. However, mutant J1 was able to colonize plant roots at wild type rates. This ability may relate to production of catalase A in J1 after exposure to external sources of H_2O_2. A similar induction of catalase A in J1 occurs on contact of J1 with plant roots. Cells of J1 examined from the root surface after twelve hours of exposure displayed both catalase B and catalase A isozymes.

A further mutant J6 obtained by Tn5 insertion into J1 lacked all detectable catalase activities when grown on liquid growth media even to stationary phase. Thus J6 appears to have a mutation which prevents the production of catalases B and C. This mutant only colonized roots at levels much reduced from that of the wild type. Also J6

was reduced in its ability to survive H_2O_2 treatments although it was able to produce catalase A after H_2O_2 exposure. These data suggest that regulation of catalases B and C as well as catalase A is important in the survival of bacteria to H_2O_2 and on plant roots.

We are exploring the altered gene expression of SOD upon root colonization by the generation of SOD deficient mutants, through marker exchange of a mutated gene into the parental cell. Currently we have isolated a fragment from a cosmid library of *P. putida* which will complement the SOD deficiency in a SOD-mutant of *Escherichia coli*. The complemented cells produce a SOD activity which migrates on nondenaturing gel electrophoresis to the same location as the Fe SOD from the pseudomonad cell. This migration pattern is quite distinct from that of the *E. coli* SODs. We are currently subcloning the fragment and will perform transposon mutagenesis to delete the SOD activity.

CONCLUSION

We have demonstrated that root colonizing pseudomonad cells have several levels of interaction with the plant. Interaction between a root surface arabinogalactan protein and surface features of root colonizing bacteria is involved in strong attachment and improved colonization potential. The agglutination phenotype in the bacteria is related to discrete genetic loci and we have identified one locus as being a specific marker for the agglutinability trait. Other genes in the bacteria are also regulated upon root colonization. These include genes for catalase and SOD which encode proteins involved in the survival of the bacteria on the root surface.

FUTURE STUDIES

Our goals are to:

1) continue the purification of the agglutinin so that amino acid sequences and glycosylation structure can be determined. We will initiate progress towards isolation of plant genes involved in agglutinin synthesis.

2) isolate genes encoding catalase A and FeSOD from *P. putida*. Mutated genes will be inserted into the chromosome of pseudomonads by marker exchange to probe their function. We also will examine the regulation of the promoters of the genes through gene fusion constructs.

3) examine the function of the promoter regions for the two loci that are involved in the agglutination phenotype in the bacteria. We will use promoter fusions to determine if expression of the agglutination genes is regulated as the bacteria colonize root surfaces.

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STABILITY OF FOREIGN DNA IN THE FUNGUS *Colletotrichum gloeosporioides*

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INTRODUCTION

Progress in the genetic engineering of fungi has been rapid in recent years. With these advances, researchers are observing that the foreign DNA is often unstable in the transformed fungi. It is typical for the introduced DNA to be lost or rearranged within the chromosome. Also, transformants are often relatively sickly as demonstrated by their poor survival, abnormal pigmentation, and strange colony morphology. We selected the fungus *Colletotrichum gloeosporioides var. aeschynemone* (CGA) as a model organism to study the factors that determine the stability of transformed marker genes in CGA sprayed on plants and in microcosm chambers.

METHODS

Transformation of Protoplasts and Detection of Foreign DNA

The fungus was cultured for 4 days at 30°C on Torula yeast agar (15 g Torula yeast, 15 g starch, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot H_2O$, 15 g agar/L) or in YpSS broth. To prepare protoplasts, spores were harvested from plates with sterile water, centrifuged for 5 min. at 1000xg, suspended in a solution consisting of 2 parts 1.2 M $MgSO_4$-Tris-Cl (10 mM, pH 5.8) and 1 part 0.6 $MgSO_4$-Tris-Cl (10 mM, pH 5.8.) containing Novozym 234 (50 mg/ml), and incubated for 5 hours at 32°C. Protoplasts were centrifuged for 1 min. at 2000xg and washed five times by centrifugation in 1.2 M sorbitol-Tris-Cl (10 mM, pH 7.0) to remove nucleases present in the Novozym. Cells were resuspended in the sorbitol solution for DNA transformation.

Cells were transformed with pBT. This plasmid was made by inserting a 2.6 kb Sal I fragment that encodes the β-tubulin gene from a *Neurospora crassa* mutant (gene confers resistance to benomyl) into the Sal I site of pBS(+/-) (Stratagene). Twenty μg pBT DNA were added to about 10^6 protoplasts. After adding an equal volume of 30% PEG in 10 mM $CaCl_2$, the suspension was incubated 30 min. at 25°C. Protoplasts were centrifuged for 3 min. at 3000xg, resuspended in 1.2 M sorbitol-Tris-HCl (10 mM; pH 7), and spread on agar containing 0.8 M sucrose. After 24 h, four ml volumes of 1% YpSS overlay agar containing 10 μg benomyl/ml were layered on the agar surfaces. These were incubated at 30°C for 5 days. Putative transformants were restreaked three times on the selective medium.

To extract DNA, fungi were cultured in YpSS broth containing 10 μg

benomyl/ml and collected by filtration, dried, and ground with a mortar-pestle. The powder was suspended in a hexadecyltri-methylammoniumbromide (CTAB) solution (10% CTAB, 0.7 M NaCl), heated 1 hour at 55°C, mixed with an equal volume of chloroform:isoamyl alcohol (24:1), centrifuged 10 min., and the supernatant liquid collected. An equal volume of 1% CTAB-Tris-Cl (50 mM, pH 8.0) was added. After 30 min. at 25°C, the suspension was centrifuged at 5000xg for 5 min. The pellet was dissolved in cesium chloride solution and the DNA purified by ultra-centrifugation.

Presence of the β-tubulin gene in transformants was confirmed by DNA hybridization. DNA was restricted, electrophoresed, transferred to a Nytran membrane, and probed with the Sal I fragment carrying the β-tubulin gene. The probe was labelled with ^{32}P by random primer extension. The final wash in the hybridization step was performed for 15 min. at 65°C in 2 X SSC and 0.1% SDS. Hybridization was detected by autoradiography.

Construction of Plasmid pBC

Plasmid pBC was prepared by first restricting the Sph I site in the multiple cloning site (MCS) of pBS(+/-) and inserting a 2.2 kb Sph I fragment which encoded the cutinase gene from *C. gloeosporioides*. The direction of the cutinase reading frame in this construct was away from the single Eco RI site in the MCS. This DNA was then doubly-digested with Eco RI and Eco RV (site about 1 kb from the Eco RI site in the MCS) and blunt end-ligated to eliminate the 1 kb RI-RV fragment. This step removed the Sal I site in the MCS and shifted the unique Nar I site in the cutinase gene to a position near the middle of the fungus DNA insert. Next, we restricted the Nar I site, removed the tails, and inserted Sal I linkers. Finally, the Sal I fragment carrying the *N. crassa* β-tubulin gene was inserted into the new Sal I site of the cutinase sequence.

Microcosm Studies

Microcosm studies were performed in a glass chamber (1.0 x 0.75 x 0.75 m) that was irradiated with a 1000-watt Sylvania metal halide lamp (18 h/6 hours light/dark cycle). Crimson clover plants were raised in wooden trays filled with 6-7 cm. of soil. To spray plants, CGA spores were washed off Torula yeast agar plates with sterile water, centrifuged twice in water, and suspended at $2\text{-}5 \times 10^7$ spores/ml. Plants and soil were sprayed with about 100 ml suspension/tray using a plastic misting bottle. Three plant samples (2-3 g/sample) were aseptically taken at random from each chamber on each sampling day, put in sterile plastic bags with 20 ml water, and blended for 1 min. in a Stomacher blender (Model 80, Tekmar Co.). Using a spatula, three soil samples (1-2 g/sample) were aseptically collected at random from the top centimeter of soil and vortex-mixed for 1 min. in 18 x 150 mm screw-capped tubes containing 5 ml water and glass beads. To enumerate colonies, samples were diluted in water, spread in duplicate on Martin's agar amended with 100 μg benomyl/ml, and incubated for 36 hours at 30°C. Colony counts

from a pair of plates were averaged for calculations of colony forming units (CFU).

RESULTS AND DISCUSSION

Transformation of Protoplasts and Detection of Foreign DNA

We obtained benomyl® strains of CGA following transformation with plasmid pBT (*N. crassa* β-tubulin gene on Sal I insert in pBS(+/-)). To date, three putative transformants have been analyzed. They tolerate up to 100 μg benomyl/ml and hybridize with the β-tubulin gene probe. However, in the presence of the antibiotic, only transformant II-4 has the orange pigmentation of the parent strain and does not have retarded growth. This isolate also grows without significant inhibition in the presence of 300 μg benomyl/ml. Based on DNA hybridization of Southern transfers, the transforming DNAs in the three isolates inserted into sites other than the genomic β-tubulin sequences. We are currently determining if the foreign DNA changes the pathogenesis of the transformants by inoculating northern joint vetch plants (susceptible to invasion by CGA) with spores from transformants.

We are at the early stages of the transformation studies and have not yet transformed CGA with pBC (pBS(+/-) carrying *N. crassa* β-tubulin gene with flanking *C. gloeosporioides* cutinase sequences). As we acquire these transformants, they will be compared with isolates containing DNA from plasmid pBT (pBS(+/-) carrying *Neurospora* β-tubulin without the flanking *C. gloeosporioides* cutinase sequences). This will allow us to determine the influence of the associated, homologous cutinase DNA on a) frequency of β-tubulin gene transformation, b) site of integration, c) stability of the transforming DNA in the chromosome, and d) persistence of the benomyl phenotype.

Microcosm Studies

In the second aspect of our research, soil/plant microcosms are being used to compare survival of CGA transformants with the nonrecombinant CGA parent. We have already developed methods for spraying spores on plants and processing soil and plant samples. This preliminary work was done by inoculating crimson clover plants with 10^6 CFU/g of leaf. Populations dropped about 100-fold by day 22. During the same period, populations in the soil decreased from about 10^4 to 10^3 CFU/g of soil.

Currently, microcosm experiments are being used to compare populations of the parent and transformant II-4 strains. When the CGA populations in the microcosms drop to near-threshold levels, we will culture isolates from leaf and soil samples and extract the DNAs. RFLPs of these DNAs will then be analyzed by DNA hybridization with the β-tubulin gene as a probe. This will allow us to determine if the location of the transformed gene changes during exposure of the fungus to leaf surfaces and soil.

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Dr. George Rohrmann's expert advice on cloning strategy was crucial for the successful construction of pBC.

FUTURE WORK

Plans for this project include: a) determine genetic factors (e.g., homologous versus heterologous integration) related to stability of recombinant DNA in transformed fungi; b) assess survival recombinant fungi in the field; c) study persistence of recombinant fungi in microcosms that simulate on-going field conditions (RH, air temperature, light intensity, and soil moisture); d) use recombinant fungi to enhance bioremediation and restoration of polluted environments.

PERSPECTIVES ON PLASMID STABILITY: A STUDY OF THE EPA BENCHMARK PLASMIDS

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INTRODUCTION

Microbial degradation of toxic pollutants released into the environment by various human activities proceeds through co-oxidation and / or mineralization (Alexander, 1981). Often resulting from non-specific degradation by enzymes of common pathways, co-oxidation produces intermediates which can no longer be broken down by enzymes with high substrate specificity further down the pathway and which accumulate in the medium. In some cases, the intermediates are even more toxic than their precursors, which makes co-oxidation quite undesirable. It follows that co-oxidation is rather slow, fails to completely degrade toxic pollutants and cannot provide microflora with energy or metabolites. In contrast, through mineralization, toxic pollutants are completely broken down by microflora which can derive energy and metabolites from them. Thus microbial mineralization appears to be a more attractive process to detoxify the environment.

Although, in several documented instances, microbial mineralization could be shown to result from the combined action of chromosomal and plasmid-borne genes in the same strain (Ghosal et al., 1985), it is seldom observed that a naturally occurring pure culture can completely break down a target compound. Rather, mineralization usually results from complementary action of members of a mixed culture (Chatterjee et al., 1981). Through intensive selection in the laboratory, one can isolate a pure strain that can use a target compound as sole energy and carbon source. Often, such a strain could subsequently be shown to harbor a plasmid carrying all the genes necessary for the mineralization process.

By using selection to engineer a strain that can metabolize a target compound, one is faced with the problem that the resulting strain might require a much higher concentration of substrate for growth than could be found in the worst polluted site. Compounding that problem, alternative carbon sources readily available in the environment might be used in preference to the target compound, thus defeating the purpose of the engineered strain. Also, a strain developed in a laboratory environment might lose its competitiveness over indigent flora of a polluted site, thus would be rapidly outgrown before it has time to complete its intended detoxification task.

While making them more amenable to various studies the plasmid location of those genes of interest also increases their likelihood to be lost. Our current

research focuses on the problem of plasmid loss from a population of genetically engineered microorganisms. With plasmid copy number restored to its pre-division state shortly after cell division, plasmid loss could result from random unequal partitioning of copies to daughter cells, or segregation. It could also be accelerated by a higher growth rate of the plasmid-free cells, as compared to that of plasmid-bearing ones, or selection.

Most often, plasmid loss results from a combined effect of segregation and selection. Knowing which phenomenon plays a major role in plasmid loss helps one decide on the most appropriate course of action to enhance plasmid stability. If segregation is the major cause of plasmid loss, one could consider ways to increase copy number to lessen the chance of loss by unequal partitioning or to incorporate a function that ensures equal partitioning. If a plasmid reduces the growth rate of its host, increasing its copy number or incorporating a partitioning function might have the opposite effect of making plasmid-bearing cells even less competitive, thus exacerbating the problem.

As model plasmids, we used a series of EPA benchmark plasmids (pRO2313, pRO2317, pRO2318, pRO2320 and pRO2321; Zylstra et al., in press), maintained in two bacterial model backgrounds, *Pseudomonas aeruginosa* strain PAO1c and *P. putida* strain PRS2015. We determined that all the above benchmark plasmids are more or less unstable in either host background. Whether segregation or selection is the major cause of plasmid loss depends very much on the plasmid, the host background and the growth medium.

METHODS

Bacterial Strains and Plasmids

Dr. R. Olsen (Medical School, Univ. of Michigan) kindly provided plasmid-free P. aeruginosa PAO1c and PAO1c strains bearing benchmark plasmids pRO2313, pRO2317, pRO2318, pRO2320 and pRO2321. Dr. S. Cuskey (Environmental Research Laboratory, Gulf Breeze, FL) kindly provided plasmid-free P. putida PRS2015 and PRS2015 strains bearing the same set of the above benchmark plasmids. To ensure true isogenicity, plasmid DNA was isolated from plasmid bearing strains by the rapid isolation technique (Maniatis et al., 1982) and used to re-transform plasmid-free strains by the $CaCl_2$ technique (Mandel and Higa, 1970). Transformants were isolated, streak-purified on antibiotic plates, grown to saturation in L broth with antibiotic, mixed with an equal volume of glycerol and frozen at -80 °C. Glycerol stocks of plasmid-free strains were made in a similar way except no antibiotic was used. All experiments were initiated with single colonies streaked on antibiotic plates from such glycerol stocks.

Media and Antibiotics

LB broth contains per liter 10 g Tryptone, 5 g Yeast Extract and 10 g NaCl. LB agar is LB broth with 14 g Bacto agar per liter. PG contains per liter 30.7 mmoles K_2HPO_4, 14.7 mmoles KH_2PO_4, 18.7 mmoles NH_4Cl, 1 mmole $MgSO_4$, 2 micromoles $FeSO_4$,

2 mg thiamine hydrochloride and 1 g glucose. Anhydrous ampicillin (Ap) is dissolved in dimethyl sulfoxide at 100 g/l, stored at -80 °C, and used at a final concentration of 500 mg/l. Carbenicillin (Cb) and piperacillin (Pp) are dissolved in water at 600 g/l and 100 g/l, stored at -80 °C and used a final concentrations of 600 mg/l and 20 mg/ l, respectively. Tetracycline (Tc) is dissolved in methanol at 10 g/l, stored at -80 °C, and used at a final concentration of 80 mg/l for PAO1c or 10 mg/l for PRS2015 strains bearing Tc- resistant plasmids.

Stability- and Competition-type Experiments

Stability- and competition-type experiments were conducted in a pairwise fashion. Essentially, plasmid-bearing cells were grown to saturation in LB supplemented with antibiotic, harvested by centrifugation, washed three times with an equal volume of 8.5 g/l NaCl. Plasmid-free cells were grown up similarly but without antibiotic and harvested in the same manner. For a stability-type experiment, plasmid bearing cells were inoculated on day 0 as pure culture at OD550 = 0.05. A competition-type experiment started with a culture of the same OD550 but consisting of a 1:1 mixture of plasmid-bearing and plasmid-free cells. Cultures were incubated at 32 °C, aerated with a 240 RPM gyratory motion. Every subsequent 24 hours, cells were subcultured at 1:100, as described above, into fresh medium. This regimen resulted in an equivalent of log2(100) = 6.64 generations per 24 h. Each day, saturated cultures were serially diluted in 8.5 g/l NaCl and plated on LB agar to obtain single colonies; the daily frequencies of antibiotic resistant cells were determined by toothpick streaking 100 or 200 colonies on plates of LB agar supplemented with antibiotic.

Data analysis. According to Lenski and Bouma (1987), the rate of plasmid loss could be described by the following differential equation:

$$[1] \qquad \frac{dP}{dt} = -uP - sP(1 - P)$$

where P is the frequency of plasmid-bearing cells, u the segregation rate and s the selection coefficient. Equation [1] can be integrated to yield:

$$[2] \qquad P = \frac{(u + s)P_o}{[u + s(1 - P_o)]e^{(u+s)t} + sP_o}$$

where P_o is the initial frequency of plasmid-bearing cells. We computed estimates of the segregation rate, u, and selection coefficient, s, by least squares non-linear regression (Dixon, 1985) fit to equation [2]. Through the use of a dummy variable (Kleinbaum and Kupper, 1978), the intercept, P_o, could be varied according to whether a particular set of points came from a stability- or competition-type experiment. This allowed paired stability- and competition-type experiments to be used simultaneously in the estimation of either u alone (model I, in which s is constrained to be zero) or both u and s (model II). Partial

F-tests were conducted to determine if model II significantly improves the fit over model I with the null hypothesis being rejected only at $p<0.05$.

RESULTS AND DISCUSSION

For each pair of stability- and competition-type experiments, we used partial F-test to determine whether the data points fit model II (segregation and selection) significantly better than model I (segregation alone). For each combination of plasmid, host and growth medium, we tabulated how many pairs of experiments fall into the above category. If over 5% of the total pairs of experiments does, we conclude that for that combination, selection plays a significant role in plasmid loss. The results are shown in Table 1. The process leads to assembling the host-plasmid-medium combinations into two groups: Group A comprising those with a pattern of instability fitting model I (segregation alone); group B comprising those with a pattern of instability fitting model II (segregation and selection).

The above arrangement facilitates comparison among member combinations of a same group for their instability. This could readily be done with group A since it involves direct comparison of the segregation rates. However, the instabilities of member combinations in group B result from an interaction between segregation and selection. To arrive a meaningful comparison, we define a new quantity, l, a loss factor in equation [3]:

TABLE 1. PROPORTIONS OF EXPERIMENTS SHOWING SIGNIFICANT CONTRIBUTION OF SELECTION IN PLASMID LOSS

Plasmid	Total	Experiments with selection as major cause	Percentage
	host: PRS2015 medium: PG		
pRO2313	6	0	0.0%
pRO2317	6	0	0.0%
pRO2318	6	0	0.0%
pRO2320	6	1	16.7%
pRO2321	6	0	0.0%
	host: PRS2015 medium: LB		
pRO2313	5	1	20.0%
pRO2317	6	2	33.3%
pRO2318	18	7	38.9%
pRO2320	20	0	0.0%
pRO2321	6	5	83.3%
	host: PAO1c medium: PG		
pRO2313	4	1	25.0%
pRO2317	10	3	33.3%
pRO2318	4	0	0.0%
pRO2320	15	9	60.0%
pRO2321	4	0	0.0%

$$[3]\ I = -\lim_{P \to 0} \frac{dP}{P\,dt} = -\lim_{P \to 0} -u - s(1-P) = u + s$$

where u, s and P are defined previously.

Since the segregation rates and consequently the loss factors as well, are not normally distributed, we chose non- parametric methods to conduct our statistically analyses. We used Kruskal-Wallis method to determine if there was a significant difference among the segregation rates of group A and the loss factors of group B. In both cases, the null hypothesis was rejected at $p<0.0005$. We then proceeded to carry out pairwise two-tailed Mann-Whitney test within each group to determine the relative ranking in stability. In this case, the null hypothesis was rejected at $p<0.05$. The results are summarized in Table 2 (page 96).

Our analyses of the EPA benchmark plasmids indicate that stability of a plasmid-bearing strain is the result of a complex interaction among plasmid, host and growth medium. It is thus very difficult to generalize and to predict if a particular background would support two plasmids equally well even if they were derived from a common precursor. For each particular combination, it is therefore necessary to determine empirically the pattern by which a plasmid-bearing strain behaved.

FUTURE WORK

From our studies, we identified several host-plasmid-medium combinations with minimal instabilities. We plan to use them in experiments to test for the destabilizing effects of gene(s) of environmental utility. We also identified several combinations with high instabilities. These would be ideal backgrounds to test for the stabilizing effects of various genetic functions.

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TABLE 2. HOST-PLASMID-MEDIUM COMBINATIONS[a] WITH DIFFERENT PATTERNS OF PLASMID LOSS

Host	Plasmid	Medium	(Generation-1)[b]
	Group A (segregation alone)		
PRS2015	pRO2313	PG	0.002
PRS2015	pRO2317	PG	0.002
PRS2015	pRO2321	PG	0.002
PRS2015	pRO2318	PG	0.003
PAO1c	pRO2321	PG	0.044
PRS2015	pRO2320	LB	0.053
PAO1c	pRO2318	PG	0.15
	Group B (segregation and selection)		
PRS2015	pRO2321	LB	-0.011
PRS2015	pRO2317	LB	-0.002
PRS2015	pRO2318	LB	0.006
PRS2015	pRO2320	PG	0.017
PAO1c	pRO2317	PG	0.037
PRS2015	pRO2313	LB	0.055
PAO1c	pRO2313	PG	0.069
PAO1c	pRO2320	PG	0.15

[a] Combinations showing no statistical difference in segregation rates or loss factors are clustered together.

[b] Median segregation rate u for Group A and median loss factor l for Group B.

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MODELING THE FATE OF BACTERIA IN SURFACE WATERS

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INTRODUCTION

Potential ecological and public health impacts resulting from the release of a genetically engineered microorganism (GEM) to the environment are dependent on the fate of the organism and the relationship between organism density or activity and a particular effect. A fundamental consideration in this regard is the ability of the organism to survive and compete in a natural setting and possibly transfer the engineered genetic trait to other organisms. Prediction of the fate of the organism and its engineered trait in natural systems is a major component of a quantitative risk assessment.

Surface water systems are an important environmental compartment with regard to organism fate because they typically provide a suitable habitat for a variety of organisms and they provide a means of rapid transport. Predicting the fate of a GEM in a surface water system requires an analysis of substrate and nutrient inputs, the response of the GEM and the indigenous community to these inputs and the impact of predation as a population control factor. A necessary component of this analysis is the competition for resources that defines the potential of the GEM to invade the community. The analysis must also describe the transfer of engineered genetic material between the GEM and indigenous organisms.

The overall objective of the research being conducted in this project is the step-wise development of the components of a framework to model the movement and growth of genetically-engineered bacteria that, either purposely or inadvertently, have been introduced to a surface water system. A primary goal has been to develop a modeling framework capable of predicting the long-term behavior of bacteria in surface water systems. We have made significant progress towards this goal. We have completed development of a framework which has been successfully applied to a microcosm study of bacterial dynamics.

The second goal is to extend the framework to include competition between bacterial populations. This involves the division of bacteria into a minimum of two groups (indigenous community and introduced population). Recombinant bacteria will be included as a third group if gene transfer is considered to be a potentially significant process. Achieving this objective requires the definition and quantification of the mechanisms of resource competition. Existing theory

and past laboratory studies will be used to establish working hypotheses that will be evaluated through laboratory experimentation to be conducted at the Gulf Breeze EPA.

Ultimately the framework must be expanded to incorporate mechanistic descriptions of gene transfer. In ongoing and recently completed research, models have been proposed to describe this process in simple laboratory systems. These formulations provide the basis for development of a complete biotechnology risk assessment modeling framework. Such a framework must then be evaluated based on its ability to predict the fate of engineered genetic material. This would be accomplished by application to laboratory systems in which the organisms have been introduced. Such laboratory data are being generated through the biotechnology program at the Gulf Breeze EPA and evaluation of the full modeling framework could be con-ducted in a future cooperative agreement.

RESULTS AND DISCUSSION

Bacterial Dynamics Modeling Framework. The primary goal of developing a mathematical model of the microbial food web capable of predicting bacterial dynamics in surface water systems has involved the following tasks:

1) a review of bacterial growth kinetics in laboratory systems,
2) development of a methodology for defining bacterial substrate,
3) formulation of the equation set describing the dynamics of the microbial food web, and
4) application of the modeling framework to a natural system.

We have completed the first three of these tasks and are nearing completion of the fourth. The major conclusions of this work follows.

The utilization of dissolved organic carbon (DOC) by pelagic bacteria is dependent on the both the compounds comprising the DOC and environment specific growth characteristics of the bacteria. Laboratory studies have shown that growth yield coefficients are related both to the energy content of the compound and the availability of other required nutrients such as nitrogen. Half-saturation constants describing substrate or nutrient limitation of growth rate appear to be a function of environment, higher values being associated with higher nominal substrate levels.

To make a modeling analysis of bacterial growth tractable it is necessary to define a scheme for classifying DOC according to its ability to be used as substrate. We have proposed three categories of DOC: L1, L2 and R carbon, in order of decreasing lability. The fractionation of carbon into these categories may be accomplished through a BOD assay in which breaks in the oxygen utilization curve are used to signify the exhaustion of first the L1 and then the L2 component.

Development of the BOD assay procedure is continuing at the Gulf Breeze EPA. A methodology has been established which appears to have the needed sensitivity and replicability. Samples (unfiltered and innoculated sterile filtered) are incubated at *in situ*

temperature in the dark and oxygen utilization is monitored at close (1-2 hour) intervals. A change in slope of the oxygen utilization curve is used to define exhaustion of L1 carbon. Total oxygen utilization is used to establish the degradable (L1 +L2) carbon. DOC measurements establish the oxygen:carbon stoichiometry and bacterial counts (AODC) confirm the exhaustion of substrate and cessation of growth. This methodology is being applied to natural water samples, samples spiked with various carbon sources (e.g., algal exudate, sea grass detritus) and will be applied to terrestrial carbon in non-point runoff and to treatment plant effluent.

Preliminary laboratory assay data suggest that the L1 substrate pool is small. The bacteria are able to readily use this substrate at maximum growth rates calculated to be about 4/d. Because of the small size of this substrate pool and its rapid use it is exhausted in the assays within a time scale of hours or even minutes.

A review of experimental studies of the fate of carbon produced through primary production indicates that a significant fraction of the DOC released from phytoplankton through exudation, cell lysis and sloppy feeding by grazers would be classified as L1 substrate. Further, a portion of the detrital POC generated through grazing appears to be rapidly solubilized to L1 DOC.

A comprehensive review of zooplankton bioenergetics has been used to establish equations defining trophic transfer and loss of biomass carbon. Allometric relationships between consumption rate and body weight and between respiration rate and body weight are being used to define parameter values for the three grazer levels (nano-, micro- and mesozooplankton) included in the framework.

Use of the L1-L2 substrate categorization, routing phytoplankton carbon to these classes in accordance with published experimental results and specification of bacteria and grazer dynamics according to a Monod formulation successfully modelled the microbial dynamics of a microcosm study.

Competition

The expansion of the modeling framework to include GEMs entails quantification of competition between the GEM and the indigenous population and gene transfer between them. We have focused on modeling competition between bacterial groupings. Growth of each group is defined using the Monod equations. Competition results through interaction with the substrate pools as defined by growth rates, yield coefficients and half-saturation constants. In addition, differences in predation, as defined by group specific grazing rates, are also considered. Coexistence of an introduced organism and the indigenous community or the isogenic counterpart of the GEM depends on each of the coefficient values and substrate specificity. It may be necessary to model multiple substrates to adequately define the potential of the GEM to survive and to invade the community.

FUTURE WORK

We are continuing to review the literature on zooplankton growth and grazing. We have found that the allometric relationship we used for maximum ingestion rate underestimated rates reported for microzooplankton. We are now using an alternate relationship with published growth rate data to estimate the assimilation efficiency of ingested carbon. Once this has been completed we will attempt to recalibrate the Bagsvaerd microcosm model with the new parameters.

A model of bacteria in the Delaware River estuary has been formulated. It consists of the bacterial kinetic equations which we previously developed and empirical specification of phytoplankton dynamics. Observed primary production rates define substrate input to the system. Observed phytoplankton biomass concentrations define algal prey concentrations for the zooplankton grazers. Segmentation of the estuary and bay has been completed and we are currently calibrating transport over the four month period being modeled (2/18/85 to 5/25/85). Salinity data from the five cruises conducted during this period are being used as the transport tracer. When this is completed we will begin to model the bacterial dynamics that have been observed in this system.

The laboratory experiments to examine bacterial uptake of various carbon sources (i.e., the substrate utilization assay) has begun to yield data. We will analyze these data to determine the distribution of carbon between operationally defined substrate categories and bacterial growth on these.

We will continue to examine competition between bacteria to better define the processes controlling survival and growth of an introduced organism.

We will continue to prepare manuscripts covering research conducted during this project. The first manuscript has just been completed and a manuscript describing the Bagsvard microcosm modeling study will be started shortly.

An abstract of a platform presentation of our modeling work has been submitted for consideration for inclusion in the Fifth International Workshop on the Measurement of Microbial Activities in the Carbon Cycle in Aquatic Environments "Microbial Ecology of Pelagic Environments" to be held from August 18-23, 1991 in Helsingor, Denmark.

PUBLICATION

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MODELING TRANSDUCTION IN AQUATIC ENVIRONMENTS

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INTRODUCTION

With the recent advances of genetic engineering have come new questions about genetic stability and exchange among microbes in natural ecosystems. We are using *Pseudomonas aeruginosa* as a model organism to study viral-mediated gene transfer (transduction) in freshwater microbial populations. Our studies have revealed a significant potential for transduction of both plasmid and chromosomal DNA in these environments. Initially, we explored three models for the source of transducing particles in freshwater environments: (a) cell-free lysates of bacteriophages grown on an appropriate DNA donor, (b) environmental induction of bacteriophages from a lysogenic DNA donor bacterium, and (c) environmental induction of bacteriophages from a lysogenic recipient bacterium. The transfer of plasmid and chromosomal DNA was documented in each of these systems *in situ*. The highest number of transductants were routinely recovered from systems where the recipient bacterium was a lysogen, probably due to the immunity imparted by the resident prophage. Transduction was observed in both the absence and presence of the natural microbial community. Reciprocal chromosomal transduction was observed in chambers inoculated with two lysogens. Apparently, both primary infection of a non-lysogen and prophage induction from a lysogen can generate sufficient numbers of transducing particles to allow gene exchange to be observed.

For transduction to take place in this system a unique sequence of events must take place. (a) Phage virions must be produced through spontaneous or stress-stimulated induction of the prophage from the lysogen. (b) These viral particles must infect, propagate, and lyse the plasmid-containing donor. (c) Transducing particles produced during this lytic infection must absorb and transfer DNA to the remaining lysogens. Hence, environmental lysogens serve as both efficient sources of transducing phages and as viable recipients for transduced DNA.

The finding that transduction can occur in natural environments is significant as this mechanism of gene transfer has been virtually ignored in both the design and preliminary testing of genetically engineered micro-organisms for environmental release. However, the ultimate question which must be addressed in determining the effects of environmental transduction is

whether phage-mediated horizontal gene transfer alters the probability or equilibrium frequency of an introduced genetic sequence in a natural bacterial community. We are now turning our attention to developing a model transduction system whose components are derived directly from the environment and to determining whether transduction is effective in altering the makeup of the gene pool available to natural populations of bacteria. These studies will allow a more accurate assessment of the real potential for genetic exchange to affect natural microbial populations.

METHODS

Our investigations of horizontal gene transfer are conducted in both laboratory and field formats and can be divided into three stages. First, the potential for transmission is evaluated by standard genetic protocols. Second, microcosm studies are conducted in the laboratory to evaluate protocols to be tested *in situ*. Third, field trials are conducted in biological containment chambers incubated at the freshwater field site. This stage is used to validate predictions made from the laboratory simulation experiments. The potential for transduction in freshwater *P. aeruginosa* populations was evaluated using a variant of the generalized transducing phage F116 originally designated DS1. For studies of plasmid transfer, the Tra- Mob$^-$ plasmid Rms149 is used. Plasmid transduction was confirmed by molecular analysis. Detailed methods have been published (see below). Natural bacteriophages were isolated from lake water collected at several of our test sites. Samples were used either directly or concentrated. Phages were identified by their ability to plaque on one or more indicator strains. Phages selected for further studies were purified by glycerol gradient ultracentrifugation. *P. aeruginosa* strains were isolated from lake water by means of selective media and *Pseudomonas* Isolation Agar (Difco, Detroit, MI) and further characterized as appropriate. In some experiments, mixed populations of either bacteriophages or bacteria isolated from the field sites were used. Lysogens were identified by their ability to release phage either spontaneously or following exposure to UV radiation and by their superinfection-immunity phenotype.

Continuous culture experiments are conducted in a New Brunswick Scientific (Edison, NJ) BioFlo Chemostat. Culture medium consists of *Pseudomonas* Minimal Medium Salts without citrate supplemented with yeast extract (10^{-5} to 10^{-7} g/ml).

RESULTS AND DISCUSSION

Developing a Natural Transduction Model

While our research has demonstrated the potential for transduction in natural freshwater habitats, it is still artificial as its components are well characterized laboratory strains. We are currently attempting to develop a model system based on components isolated directly from the environment.

To begin this study, we demonstrated the occurrence of bacteriophages, potential hosts, and lysogens in the aquatic environment.

We monitored these parameters in our field sties for an eight month period. During this period, we found total bacterial counts rangee from 10^5 to 10^7 CFU/ml and *Pseudomonas* counts from 5×10^2 to 5×10^4 CFU/ml. Titers of bacteriophages capable of forming plaques on laboratory strains of *P. aeruginosa* ranged from 10^1 to 10^4 PFU/ml. When lysogeny was evaluated using a laboratory strain of *P. aeruginosa* as a indicator, between 1 and 7% of the *Pseudomonas* isolates tested positive. However, we found that approximately 45% of *Pseudomonas* isolates from our field site tested positive in colony hybridization when probed with DNA from a naturally occurring phage isolate.

Second, we isolated transducing phages from our environmental samples. One of these, UT1, has been studied in some detail. This phage was isolated from Fort Loudoun Lake near Knoxville, Tennessee where we have carried out the majority of our *in situ* transduction experiments. Although this phage is virulent under laboratory conditions, it establishes pseudo-lysogeny in the environment. It is a generalized transducing phage capable of mediating the transfer of both plasmid (Rms149) and chromosomal DNA among *P. aeruginosa*. As might be expected with a virulent phage, the highest levels of transduction are observed at very low MOIs (10^{-3} PFU/CFU). Using standard laboratory protocols, both chromosomal and plasmid transduction was detected at frequencies as high as 10^{-4} transductants/PFU depending on the MOI used.

Third, we have used UT1 to initiate studies to determine the potential of the natural microbial community present at our field sites to act as recipients of transduced plasmid DNA. Cells from 10 liters of lake water were concentrated and used as a recipient pool for transduction using cell-free lysates of UT1 grown on a *P. aeruginosa* strain containing Rms149. Significant levels of transduction were observed and confirmed molecularly. These observations have been made using samples from several different field sites collected at different times of year. They suggest that the natural microbial community at our field sites contains organisms capable of acting as hosts and recipients for plasmid transduction.

For transduction to take place, interaction between the transducing phage and its host must take place. We examined, the ability of bacteriophages to interact with their hosts at low cell densities and under starvation conditions. The attachment and replication of three *P. aeruginosa* bacteriophages were investigated under conditions similar to those found in nature. Attachment and replication of bacteriophages were not impaired at host-cell densities equal to or lower ($<10^5$ CFU ml^{-1}) than those frequently found in aquatic environments when the host cells were physiologically competent to allow phage growth. Attachment to either actively growing or starved cells was not impaired in river water, indicating that attachment is efficient in natural freshwater habitats. However, the replication of bacteriophages was significantly altered in starved cells in

river water; the latency period was extended (broth, 70-110 min.; river water, 110-240 min.), and the burst size was reduced (broth, 27-65; river water 5-7). The findings of this study indicate that phages are likely to affect microbial ecology significantly in freshwater ecosystems.

We isolated strains of *P. aeruginosa* which were sensitive and resistant to UT1 from Fort Loudoun Lake and used them to monitor host-phage interactions over 45 days in lakewater microcosms. Temporal changes in host density, phage-to-bacterium ratio (PBR), and the appearance of apparent prophage carriers within the host population were analyzed. About 45% of sensitive bacteria incubated with phage UT1 were pseudolysogenic within 12 hours of incubation in natural lake water. Phage UT1 appeared to stabilize the density of host bacteria in lake water at a level of 10^4 CFU/ml. Bacterial coexistence with a mixed phage population isolated from Fort Loudoun Lake resulted in an oscillating equilibrium with the PBR stabilizing at about 3. The presence of extraneous homoimmune phages appeared to be detrimental to the stability of the pseudolysogens, which were maintained at a lower population density than prophage-free cells in lake water containing the mixed phage population.

A Mathematical Model for Transduction

We are currently developing a mathematical model for transduction. We have convincingly shown that transduction can act to stabilize (and even increase) the frequency of a genotype in a population from which that genotype would otherwise be lost. Our research to date suggests that, in populations of bacteria in which transduction is occurring, the change in the frequency of a transducable genotype (Ph_t) as a function of generation of growth "g" can be described by the formula:

$$dPh_t/dg = t - s$$

Where "t" is the fraction of new cells added to the "Ph_t" genotype due to transduction and "s" is the fraction due to selection.

FUTURE WORK

In the future, we plan to evaluate our natural model system *in situ* by introducing a plasmid donor (either a lysogen or pseudolysogen) and allowing the natural community to act as recipients. We are currently developing an appropriate contraselection for the introduced donor which will allow us to identify transductants in the natural population. In addition, we are determining the physiological, environmental, and genetic factors which regulate "t." Preliminary analysis of our data suggest that "t" cannot be described simply as a mass-action phenomenon but contains parameters which are highly dependent on the physiological state of the hosts. We will continue to explore these factors in the immediate future.

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ENVIRONMENTALLY INDUCED GENETIC INSTABILITY IN MICROORGANISMS

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INTRODUCTION

Organisms must maintain the integrity of their genetic material if species survival is to be assured. It is now apparent that microorganisms possess DNA repair systems which, although differing in capabilities and potentials, act to repair damage resulting from cell metabolism or environmental insult.

Most of the data available concerning DNA repair has utilized the enteric bacterium *Escherichia coli* under conditions which probably will rarely, if ever, occur in natural environments. However, some important principles have been revealed by these studies. It is clear that bacterial cells suffering DNA damage will undergo repair processes. In some cases, these repair systems are mutagenic. Further, the efficiency and activity of DNA repair systems will be different under differing growth conditions.

From work already performed, there can be no doubt that genetic systems exist in the eubacteria which are induced by environmental stress resulting in enormously increased rates of mutagenesis of the genetic material. The existence of such potentials for genetic instability complicates the task of risk assessment for environmental releases of genetically engineered microorganisms (GEMs) and raises questions concerning the stability of introduced genotypes in natural ecosystems. We have been utilizing *Pseudomonas aeruginosa* as a model system to study stress-induced genetic alterations in bacterial cells. The use of this model will allow a more accurate assessment of risk associated with GEM releases in the future.

METHODS

The frequency of mutation-containing cells in a population of *P. aeruginosa* was determined for cells incubated in growth medium in the laboratory and for cells placed in
in biological containment chambers and incubated either in the laboratory or *in situ* at our established field sites. The chambers utilized in these studies are permeable to gases and allow the transmittance of solar UV radiation. They were filled with sterilized lake water from our field sites and inoculated with genetically well characterized *P. aeruginosa* strains. Chambers incubated *in situ* were placed at locations receiving solar UV radiation for significant periods of the day or in locations substantially shielded from sunlight.

Cells were recovered from chambers by plating on one-tenth strength YEPG agar. Viable counts were determined for each sampling period. In order to determine the frequency of mutation-containing cells in the population, samples (0.1 ml) were plated on 0.1 X YEPG agar and incubated for 48 hours

to recover cells. The cells were then replica plated onto various selective media. Cells were screened for streptomycin resistance, rifampicin resistance and, in some cases, resistance to infection by the virulent phage UT1. The protocols for determination of drug resistance have been modified to include another round of replica plating on nonselective medium before recovered cells are plated on media containing antibiotics. Individual drug resistant clones used for further experimentation were carefully streaked for isolation. Extra replica plating steps are required to avoid artifactual contributions to the apparent mutation rate of cells that are physiologically adapted to starvation conditions but, contain no genetic mutations to drug resistance.

The ability of cells existing under starvation conditions to resist stress was determined by laboratory experimentation. Cells were grown in 0.1 X YEPG at room temperature to stationary phase. The cells were diluted 1:10 in sterile water from a field site and incubated at room temperature for various periods of time. After a period of such starvation, the cells were irradiated with UV, and the percentage survival was determined for several doses of UV radiation.

Induction of the *recA* gene product of *P. aeruginosa* and *E. coli* was examined under several growth conditions using the Western Blotting technique. Cells were grown in Luria Broth, low-strength medium (0.01 X YEPG) or starved as described above. They were then irradiated with UV light. Subsequent to UV exposure, the cells were incubated in the dark and samples were taken periodically to be used for Western Blotting. These samples were electrophoresed on denaturing polyacrylamide gels and the proteins transferred to nitrocellulose membranes. These membranes were subjected to Western analysis using a biotintylated-streptavidin-horseradish peroxidase-conjugated antibody detection system. Anti-*recA E. coli* antibody was used as the primary antibody.

Fusions of a promoterless β-galactosidase gene to stress-inducible genes of *P. aeruginosa* PAO were constructed using a modified Tn*3* transposon. This transposon will insert into target DNA yielding transcriptional fusions of the disrupted gene and β-galactosidase.

For prophage induction experiments, F116L or D3 lysogens of *P. aeruginosa* were placed in chambers and incubated in the laboratory or *in situ* at sites receiving solar UV irradiation. The ratio of infectious centers (IC) to total colony-forming units (CFU) was determined to detect induction of the prophages. Cell concentrations were determined as described above. ICs were enumerated by dilution in 0.85% NaCl and plating with phage-sensitive indicator strains. In some laboratory experiments, lysogens of *E. coli* were treated in a similar manner.

For starvation induction experiments, a D3 lysogen of *P. aeruginosa* was grown to early exponential phase in Luria broth. The cells were harvested by centrifugation and suspended in sterile river water to induce a putative stringent response. IC determinations were made at various times after medium shift.

RESULTS AND DISCUSSION

Mutation Frequency

Our original data suggesting increased rates of mutagenesis *in situ* were based on the reversion frequencies of amino acid auxotrophs. Although no apparent selection for the prototrophs could be detected, we were concerned that unknown factors were acting to increase the reversion frequency. Therefore, we have spent some time evaluating our procedures for determination of mutation frequency. The experimental protocols which we are now utilizing reflect experience gained from our field experiments. Specifically we have found that expression time after sampling the cells is required to detect mutation events. It has proven best to recover cells on low-strength medium (0.1 X YEPG) and apply selection later by replica plating onto appropriate media. The use of the replica plating technique allows a one-to-one mutation to resultant colony detection and allows testing of identical populations for the frequency of several different mutant phenotypes.

We have modified our screening procedures for detection of drug resistant mutants to include a longer recovery time before selection is applied. Cells undergoing starvation stress apparently adapt physiologically in such a way that their apparent drug resistance is increased. This change is not necessarily genetic in nature. We have clearly been able to isolate Rifampicin-resistant mutants of at least two distinct classes. By transductional analysis we have determined one class is resistant only to Rifampicin, the other class is resistant to several drugs simultaneously. The exact frequency of production of Rifampicin-resistant mutations subsequent to stress application is unknown at present. Our previous estimates were confounded by artifactual contributions from adapted cells not containing true genetic alterations.

Reversion of amino acid auxotrophies subsequent to starvation stress has proven to be reproducible. Under conditions of no apparent selection advantage for revertants, we find rapid production of prototrophic cells. It is unknown if these are true revertants, second site suppressor mutations, or expression of normally cryptic genes.

Several different mutant phenotypes have been examined. Screening for rifampicin resistance (Rif^r) has proven to be ideal since Rif^r bacteria are infrequent at our field sites. The use of streptomycin resistance is also convenient. Mutation to phage resistance is also an excellent marker. For these studies, we have used the extremely virulent environmental phage UT1. This phage is ideal since it is absorbed by and lyses even starved *P. aeruginosa* cells. Other virulent laboratory phages were unacceptable since changes in growth condition resulted in declined virulence or reproduction potential.

Enhanced Resistance to UV Stress

The effects of starvation on the response of bacterial cells to DNA damage are dramatic. The physiological adaptations required for viability maintenance result in a tremendously

enhanced ability to resist UV irradiation. Preliminary experiments suggest that the genes required for DNA repair (*recA*, *uvrABC*, etc. in *E. coli*) are expressed at enhanced, constitutive levels. Western Blotting experiments performed on both *E. coli* and *P. aeruginosa* support this conclusion.

Induction of Prophages

Laboratory and field experiments have also demonstrated that starvation of *E. coli* and *P. aeruginosa* lysogens greatly affects DNA-damage induction of resident prophages. Preliminary experiments using the temperate *P. aeruginosa* bacteriophage F116 have suggested that conditions encountered in natural aquatic environments may lead to enhanced activation of lytic phage production in these cells. One important question is whether this is a general characteristic of all phages. Our initial experiments have used three UV-inducible temperate phages: D3 and F116 of *P. aeruginosa* and of *E. coli*. Field experiments have demonstrated no increased induction of D3 prophages from lysogens of *P. aeruginosa* exposed to significant, long term solar irradiation as measured by the frequency of ICs in the population. Laboratory investigations have confirmed that starved cells lose the ability to support UV induction of prophage. This phenomenon has been observed for both *E. coli* and *P. aeruginosa* and has been termed "aptitude" by Andre Lwoff. Significantly, we find that starved lysogens of *P. aeruginosa* are capable of the spontaneous production of phage particles for long periods. In contrast, lysogens of *E. coli* rapidly become inviable under the same growth conditions.

Stress-Inducible Expression of *P. aeruginosa* Genes

To aid in the quantification of levels of expression of DNA-damage inducible (*din*) genes under environmental conditions such as starvation and exposure to solar UV, we have constructed and partially characterized transcriptional fusions of various *P. aeruginosa* PAO genes to β-galactosidase (*lacZ*) which are inducible by exposure to DNA damaging agents. These genes are clearly expressed at greatly increased levels subsequent to UV irradiation of Rec^+ *P. aeruginosa* and are inducible by other agents which damage DNA as well in this species. They are not inducible when transferred into *E. coli*. The molecular mechanism of this anomaly is currently under investigation.

Plasmid-Encoded UV Resistance

Laboratory studies of the UV-resistance plasmid R2 have revealed that it encodes a true *recA*-dependent, DNA damage-inducible repair system which is highly mutagenic. Laboratory and field studies of *P. aeruginosa* cells containing the UV-resistance plasmid R2 have demonstrated an enhanced mutagenesis activity under environmental conditions which result in exposure to solar-UV radiation. An understanding of the contribution of these plasmids to genetic instability of natural populations of bacteria and their effects on novel genetic sequences

introduced into these environments by the release of GEMs is absolutely vital in risk and fate assessment.

FUTURE WORK

We will continued to investigate the existence of a hypermutable state in cells undergoing starvation stress. Auxotrophic revertants will be mapped to better characterize their nature. We will continue to examine the expression of UV resistance plasmids exemplified by R2.

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GENOMIC PLASTICITY AND CATABOLIC POTENTIAL OF *Pseudomonas cepacia*

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INTRODUCTION

The genome of the bacterium *Pseudomonas cepacia* contains a large number of insertion sequences. The abilities of these elements to mediate genomic rearrangements and activate the expression of neighboring genes have been implicated in the evolution of novel catabolic functions and appear to underlie the extraordinary nutritional versatility and adaptability of this bacterium. We have been particularly interested in the roles of IS elements in the recruitment of foreign genes for new degradative pathways as well as their capacity to be transferred to and transpose and activate gene expression in other bacteria.

In order to gain information about the relationship of *P. cepacia* IS elements and insertion sequences from other bacteria we have determined the nucleotide sequences of IS*401*, IS*402*, IS*406*, and IS*407* and compared them to elements listed in the GenBank and EMBL data bases. The four elements examined had been shown to promote rearrangements of a cryptic plasmid present in *P. cepacia* 249 or to activate the expression of the *lac* genes of Tn*951* when this transposon was introduced into *P. cepacia* on the broad-host-range plasmid pGC91.14. The data obtained has also been used to construct IS*402* and IS*407* variants carrying the trimethoprim resistance (Tp^R) gene of pR388, which are being used to examine factors influencing transpositional activity.

To gain information about the organization of the *P. cepacia* genome we have undertaken the construction of a physical map of the chromosome of strain 249. Our aims are to examine the distribution of IS elements and genes encoding key catabolic enzymes. Such information should provide insights into the bases for the unusual catabolic potential of this bacterium.

METHODS

Analyses of the Nucleotide Sequences of Selected IS Elements

Insertion sequences were cloned from element-containing derivatives of pRP1 and pGC91.14 into the sequencing vector pBLUEKSP and inserts of suitable length for sequence analysis were generated by subcloning DNA fragments or by creating nested deletions. Nucleotide sequence data was obtained by the dideoxynucleotide procedure of Sanger using ccc-DNA as template. The data were compiled and analysed on a MicroVAX computer using programs provided by the Genetics Computer Group at the University of Wisconsin.

Construction of Marked IS Elements

The nucleotide sequences of IS*402* and IS*407* were examined to identify unique restriction enzyme sites located outside open reading frames and terminal inverted repeat sequences that might be important for transposition. Two such sites were identified: a *Dra*II site within IS*402* and a *Dra*I site within IS*407*. A 0.7-kb trimethoprim resistance (Tp^R) cassette from pR388 was ligated into these sites to form the derivatives IS*402.2* and IS*407.1*, which each carry the dihydrofolate reductase (*fol*) gene of pR388, and accordingly confer high level resistance to trimethoprim.

Physical Mapping of the Chromosome of *P. cepacia* 249

Bacteria were immobilized in agarose plugs and lysed under alkaline conditions by treatment with SDS and proteinase K. The plugs were washed in TE buffer and the chromosomal DNA was digested *in situ* with restriction enzymes such as *Pac*I, *Afl*II, *Xba*I, and *Dra*I, which cleaved the *P. cepacia* chromosome into a relatively small number of high molecular weight fragments. The agarose plugs were transferred into wells on agarose gels, and the DNA fragments were resolved by pulsed-field gel electrophoresis using a CHEF gel apparatus obtained from Owl Scientific Co., Cambridge, MA. Yeast chromosomes and concatamers of coliphage lambda DNA were used as size standards. Neighboring *Pac*I fragments were identified by Southern hybridization experiments using as DNA probes *Afl*II and *Xba*I fragments which overlapped the pertinent *Pac*I sites.

RESULTS AND DISCUSSION

Relationship between Elements from *P. cepacia* and other Bacteria

The results of our nucleotide sequence analyses indicate that IS*401* and IS*407* are members of the IS*3* family of insertion sequences, whereas IS*402* and IS*406* are distinct from previously described elements. The results indicate that at least some of the IS elements present in *P. cepacia* have been widely dispersed among bacteria. Table 1 compares some of the characteristics of IS*401* and IS*407* and other IS*3*-like elements. IS*407*, IS*476*, and IS*R1* comprise a subgroup of the IS*3* family. All three of these elements produced 4-bp duplications of target DNA upon insertion. IS*407* was most closely related to IS*476*. The deduced amino acid sequences of the major open reading frames of these two elements exhibited between 72 and 82% similarity. IS*401* was typical of the majority of IS*3*-like elements in that it generated 3-bp target duplications. IS*401* exhibited a high degree of homology with IS*51* and with a region of the Ti plasmids of Agrobacterium tumefaciens important for tumor induction in plants. In this context it is of interest that certain Ti plasmids confer ability to utilize octopine, a compound which supports rapid growth of *P. cepacia*. It is possible that IS*401* might have been involved in acquisition of octopine utilization genes.

Table 1. Characteristics and sources of IS*3*-like elements

Element	Size (bp)	IRs[a] (bp)	Target repeats[b]	Percent[c] Homology	Source
IS*407*	1236	12	4	100	*Pseudomonas cepacia*
IS*476*	1225	26	4	62	*Xanthomonas campestris*
IS*R1*	1260	13	4	55	*Rhizobium lupini*
IS*401*[d]	1316	26	3	44	*P. cepacia*
IS*51*	1311	26	3	40	*Pseudomonas syringae*
IS*3411*	1309	27	3	39	*E. coli*
IS*3*	1258	39	3	38	*E. coli*
IS*911*	1250	27	3	38	*Shigella disenteriae*

[a] Terminal inverted repeats
[b] Directly repeated duplications of target site DNA
[c] % Overall nucleotide sequence homology with IS*407*
[d] IS*401* was most closely related to IS*51* (65% homology)

Transposition of IS*402* and IS*407* Variants in *E. coli*

Donor strains containing pBluescript vectors carrying IS*402.2* and IS*407.1* (variants conferring resistance to trimethoprim) as well as IncP1 plasmids conferring resistance to tetracycline were mated with *E. coli* and *P. cepacia* recipients. Transconjugants containing cointegrate plasmids were obtained readily. IS*402.2*, a variant with the Tp^R gene of pR388 inserted into the unique *DraII* site of IS*402*, was ligated into pTGL133, an IncP1 plasmid temperature sensitive with respect to its replication to form pTGL154. *E. coli* derivatives bearing pTGL154, when propagated at 42°C, gave rise to Tp^R transposants lacking the Tc^R marker of the donor plasmid. The results suggest that IS*402* and IS*407* can transpose in *E. coli*. We are analysing the putative tranposants to confirm that this is the case.

Macrorestriction Analysis of the *P. cepacia* Genome

The restriction enzymes *Pac*I, *Dra*I, and *Ase*I cleaved the *P. cepacia* chromosome into 6, 41, and 35 fragments, respectively. The sums of the molecular weights of the fragments indicated that the size of the *P. cepacia* chromosome is 4.9 Mb. Thus the unusual degradative abilities of this bacterium are not a consequence of its having a large genome than that of less versatile bacteria. Prototrophic and auxotrophic derivatives were identified in which large deletions resulted in

fusion of neighboring *Pac*I fragments. *Afl*II and *Xba*I fragments containing *Pac*I sites have been used in Southern hybridization experiments to define linkages between *Pac*I fragments. All six *Pac*I fragments were shown to comprise a single linkage group. We have carried out Southern hybridization experiments to determine the distribution of IS*401*, IS*406*, IS*407*, IS*408*, and IS*415*. All copies of these elements were clustered within a limited region of the genome comprised by the two smallest *Pac*I fragments. Analyses of the distributions of additional elements and of essential genes should reveal whether such clustering reflects the location of essential genes in other regions of the chromosome or perhaps organization of elements into domains where their transposition can be regulated by localized changes in DNA topology -- e.g. alterations in extent of supercoiling.

FUTURE WORK

Our long term goal is to understand the factors underlying the unusual adaptability of *P. cepacia*. We are particularly interested in examining the roles of IS elements in the evolution of catabolic pathways. Towards this end we will extend the *Pac*I restriction map we have generated to include more data about the distribution of IS elements as well as the locations of genes encoding various biosynthetic and catabolic pathway enzymes. A major aim is understand how *P. cepacia* maintains relatively stable phenotypes despite its potential to undergo frequent IS element dependent rearrangements of its genome. Our working hypothesis is that transpositional activity is triggered in response to environmental stress -- e.g. prolonged starvation for nutrients and exposure to toxic agents. To examine whether transposition is regulated and gain information about functions required for transposition we are constructing strains whose chromosomes contain IS elements carrying a Tp^R resistance casette. These will be used to obtain data about the influence of various physiological conditions on transposition. (The transposition assay entails transfer of broad-host-range plasmids from the pertinent strains and screening of transconjugants for acquisition of the Tp^R determinant of the marked elements). It should also be possible to use such strains to gain information about the factors governing exchange of *P. cepacia* elements in complex bacterial populations.

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STUDIES ON CONJUGAL TRANSFER OF PLASMIDS FROM GEMs TO INDIGENOUS AQUATIC BACTERIA

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INTRODUCTION

The horizontal spread of recombinant genes from genetically engineered microbes may magnify ecological risks because their stability and expression are likely to increase in microorganisms indigenous to aquatic and terrestrial environments. Two experimental approaches have been used for evaluation of gene transfer in the environment: i. A retrospective approach comparing clonal identity with plasmid patterns of bacterial populations (Selander and Levin, Science 210:545-547 (1979)). ii. The addition of genetically marked donor and recipient strains to environmental samples and the scoring of recombinant strains. Due to problems posed by background indigenous microbes this last approach employs either environmental enclosures or sterilized environmental samples. Both approaches indicate that gene exchange does occur, albeit at low frequencies, in the environment (Levy and Miller (eds.), Gene Transfer in the Environment, McGraw-Hill Publishing Co. (1989)). However, the use of marked laboratory strains may underestimate frequencies of transfer because the strains are not adapted to life in the test environment. Thus, methods are needed to detect *in situ* horizontal gene transfer between indigenous microbes. Here, I describe an experimental approach for the detection of gene transfer from an engineered organism to microbes indigenous to aquatic environments.

The approach is based on the assembly of catabolic pathways when the donor and recipient genes are brought together through the formation of recombinant strains. If genes encoding part of the pathway are placed on a genetic element whose transfer is followed, and the indigenous community is acclimated to carry out the remaining reactions of the same pathway, then strains with new catabolic capabilities would emerge when indigenous microbes receive and replicate the transferred genes. I have used the assembly of *mer*B (encoding organomercurial lyase) on broad host range conjugal plasmids, and *mer*A (encoding mercuric reductase), to detect gene transfer from a donor pseudomonad strain to Hg(II) acclimated aquatic microbes by the ability of transconjugants to grow in presence of an organomercury compound, phenyl-mercuric acetate (PMA). This is based on a prior demonstration that Hg(II) acclimated microbial communities are enriched for microbes that reduce Hg(II) by the activity of the mercuric reductase enzyme and carry *mer*A genes (Barkay et al., Appl. Environ. Microbiol. 56:1695-1701 (1990)).

MATERIALS AND METHODS

Construction of conjugal broad host range plasmids containing a *mer*B-plant DNA cassette: Plasmids, pGTE15 and pGTE22, containing the *mer*B gene of the broad spectrum mercury resistance plasmid R831 and a 350 base pairs (bp) sequence of the plant napin gene, bracketed by multi cloning sites of the pUC plasmids were prepared. The *mer*B-plant DNA cassette was excised and cloned into appropriate cloning sites of the following broad host range plasmids, R388 (IncW), RP1 (IncP) and pKM101 (IncN) to create plasmids pGTE16, pGTE26 and pGTE25, respectively (Figure 1, page 120). Conjugal transfer of parental and recombinant plasmids between E. coli and pseudomonad strains was tested by the filter conjugation test as described by Simon et al., (Biotechnology 1:784-791 (1983)).

Conjugal transfer of *mer*B-plant DNA plasmids to aquatic microbes: The experimental procedure employed is outlined in Figure 2 (page 121). Freshwater bacteria were acclimated to Hg(II) using conditions described by Barkay (Appl. Environ. Microbiol. 53: 2725-2732 (1987)). *P. aeruginosa* containing the test plasmid (approximately 10^7 cells/ml) were added to acclimated water samples, and samples were filtered through 0.2 μ nitrocellulose filters using low vacuum pressure. This treatment did not injure either donor or acclimated microbes. Following a 4 hour incubation (a period demonstrated to allow for conjugal transfer), the cells were suspended in 1 ml broth, Hg(II) was added to 0.1 μM and suspensions were incubated for 30 minutes prior to plating on selective media. This incubation in presence of a sublethal concentration of Hg^{2+} was essential for expression of *mer* and subsequent selection of PMA resistant transconjugants. Conjugation with microbes from control communities (incubated in the laboratory in the absence of Hg^{2+}) were used as negative controls. Colonies grown on PMA plates (10 μg/ml) were confirmed as transconjugants by i. transfer to medium containing antibiotics (Tp - trimethoprim (1000 μg/ml), Cb - carbenicillin (600 μg/ml), and Kn - kanamycin (600 μg/ml), for pGTE16, pGTE25 and pGTE26, respectively), and ii. hybridization with the plant DNA probe (described by Barkay *ibid*).

RESULTS AND DISCUSSION

Evaluation of enrichment (Table 1) indicates that whereas Hg(II) resistant bacteria were selected during the 2 days acclimation period, selection for PMA resistance did not occur. Such selection was anticipated because *mer*B is a part of the *mer* operon that includes *mer*A, the gene enriched during acclimation (Barkay et al., 1989. Appl. Environ. Microbiol. 55: 1574-1577). Coselection would have resulted in a high background of PMA resistant bacteria and a possible masking of transconjugants.

Results of conjugation between PAO1(pGTE16) and indigenous Hg(II) are presented in (Table 2). Presumptive transconjugants were present at 10^2 to 10^3 CFU/ml whereas background PMA resistance was below detection. Thus, there is at least two orders of magnitude difference between the

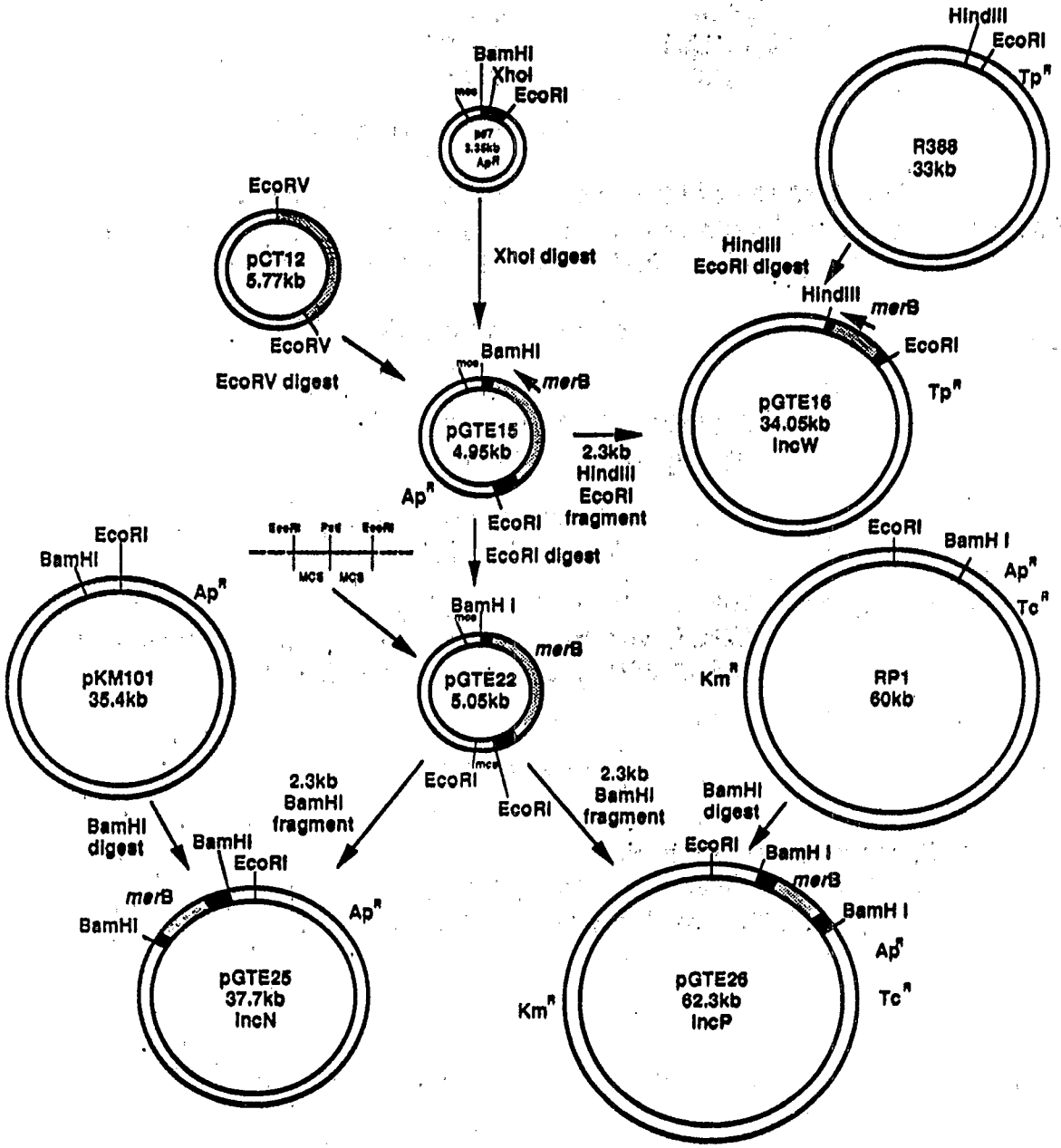

Figure 1. Construction of Broad Range Plasmids with a mer B-Plant DNA Cassette

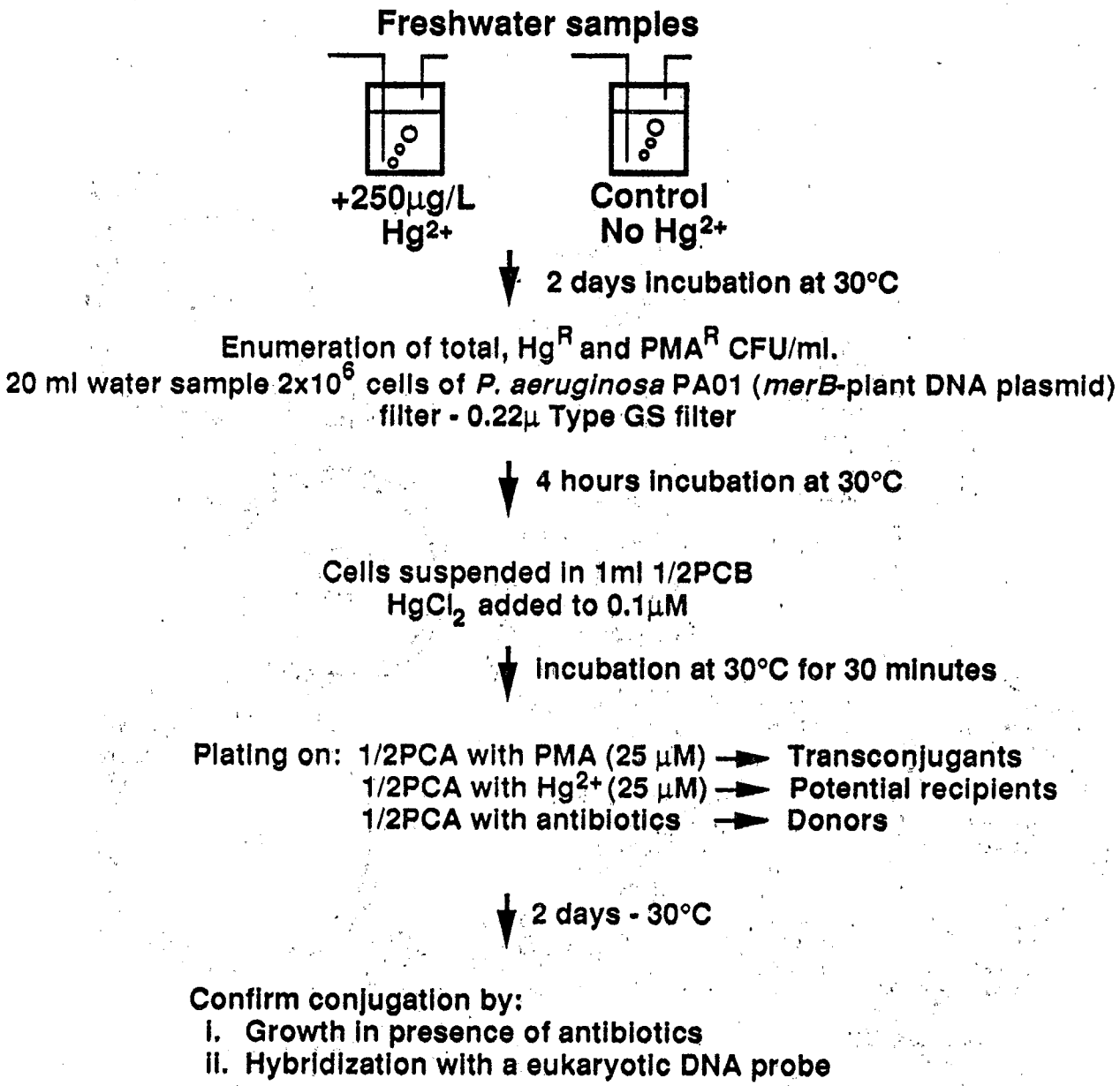

Figure 2. Conjugal Gene Transfer From GEMs to Aquatic Bacteria

Table 1. Enrichment of Hg(II) and PMA resistant heterotrophic bacteria after exposure to 250 μg/L Hg(II) for two days at 30°C.

		CFU/ml			
		Exp.1		Exp. 2	
		+Hg(II)	Control	+Hg(II)	Control
Day 0	Total	3.3×10^4		5.4×10^4	
	Hg2+-R	4.8×10^2		1.2×10^1	
	PMA-R	$2. \times 10^{-2}$		<0.01	
Day 2	Total	4.0×10^5	3.2×10^4	4.7×10^6	6.7×10^4
	Hg2+-R	8.6×10^4	1.0×10^2	8.0×10^2	<1.0
	PMA-R	<10.0	<0.01	0.9	<0.01

Table 2. Results of conjugation experiments between PA01(pGTE16) and indigenous Hg(II) resistant indigenous bacteria.

	+Hg(II)	Control	+Hg(II)	Control
Donor	3.1×10^6	4.0×10^7	7.8×10^6	1.2×10^7
"Recipient" (Hg^{2+}-R)	ND[2]	ND	9.5×10^3	ND
Transcon.	6.0×10^2	<1.25	3.5×10^2	ND
Background (PMA-R, no donor)	<1.25	<1.25	<1.42	ND
Efficiency (trans./donors)	1.9×10^{-4} ($<4.0 \times 10^{-7}$)[3]		3.6×10^{-2}	recipient

1 Hg(II) - indigenous flora from exposed microcosms; Control - indigenous flora from microcosms not exposed to Hg(II).
2 ND - not determined
3 appearance of background PMA-R/donor

number of transconjugants and background PMA resistant organisms, allowing for unmasked detection of transconjugants. Efficiency of transfer was at the range of 10^{-4} to 10^{-5} transconjugants/donor. In comparison, the efficiency of pGTE16 transfer to an E. coli HMS174 that contained pACYC184::Tn501 (encoding Hg(II) resistance and compatible with pGTE16) was $3x10^{-3}$ per donor. When the number of transconjugants is related to the number of potential recipients (in Exp. II, Table 2), it appears that 3% of the Hg(II) resistant strains received pGTE16. Thus, experiments using standard laboratory strains may overestimate efficiencies of transfer. All PMA resistant transconjugants were found to be resistant to Tp at 1000 μg/ml and they hybridized with the plant DNA probe, confirming that they were formed by conjugation between PAO1 and indigenous bacteria. When the number of transconjugants is related to the number of potential recipients (in Exp. II, Table 2), it appears that 3% of the Hg(II) resistant strains received pGTE16, indicating that there is a substantial potential for transfer of this IncW plasmid to indigenous flora.

In summary, the utility of catabolic gene assembly as a tool for detection of conjugal transfer of recombinant plasmids to indigenous micro-organisms was demonstrated under optimal conditions. This approach is currently applied to bacterial populations in environmental samples. This experimental system will allow determinations of the effect of environmental parameters on transfer of conjugal plasmids.

THE FATE, STABILITY & MOVEMENT OF FOREIGN DNA IN FILAMENTOUS FUNGI: AN ENVIRONMENTAL STUDY

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INTRODUCTION

Genetically engineered fungi (GEF) offer great potential as tools with which to study the fate of recombinant DNA (rDNA) in the environment. While techniques have been developed to detect and monitor genetically engineered bacteria, progress has been less rapid with fungi. Presently there is no eukaryotic model for studying the effects resulting from the introduction of rDNA into an ecosystem via a fungal vector. As simple, but true, eukaryotes, GEFs provide a unique opportunity for studies of strain development and use under field conditions. Prior to environmental release, however, detailed risk assessments are needed to design rational strategies for safe release.

We have been using both molecular and genetic techniques to study the impact of GEFs in the ecosystem. In order to predict the potential impact of a GEF following introduction into the environment, it is critical to evaluate the consequences of foreign DNA insertion into the organism. We have focused our studies on the stability of such foreign sequences following meiosis and mitosis, and are using sequences with and without significant homology to the recipient's genome to determine if the origin of the foreign sequences plays a significant role in determining stability.

For our studies we have been using fungal strains identified as *Gibberella fujikuroi* mating population "A" (Sawada) Ito in Ito & K. Kimura (asexual stage Fusarium moniliforme Sheldon). This organism can be readily manipulated under laboratory conditions, and sexual crosses completed with ease in a 4-6 week time frame. Numerous markers, including resistance to hygromycin B and benomyl®, as well as auxotrophs and spore color variants, are available in strains that we have developed. Also, this fungus can be divided into over 1000 different vegetative compatibility groups (VCGs). At least 10 genes underlie this VCG system and they determine whether stable heterokaryons can be formed and appear to limit the degree to which asexual transfer of genetic material can occur. We have also developed a reliable DNA-mediated gene transfer system for this fungus. These advantages make this organism a natural candidate for studies of the fate of foreign DNA in the environment.

Our overall objectives are: a.) to determine conditions for stable maintenance of rDNA in filamentous fungi and identify factors that influence genetic stability, and b.) to determine parameters affecting genetic transfer of rDNA within and between fungal populations.

Specifically we will: a). determine if copy number affects the stability of the foreign DNA through meiosis and mitosis, and b). Determine if rDNA can be passed within and between populations of *G. fujikuroi* through asexual or sexual genetic exchange.

METHODS

Strains and Plasmids

All *Gibberella fujikuroi* strains used in this study belong to mating population "A" (variety moniliformis) and were fertile as males. Most of the strains were also female fertile. Strains were stored in 15% glycerol at -80°C; vegetative cultures were maintained on complete medium. For hygromycin resistance studies, the vector pBHM-1 containing the hygromycin B phosphotransferase resistance gene from *E. coli* fused to promoter sequences from *Cochliobolus heterostrophus* was used. For studies of benomyl resistance the *Neurospora crassa* altered β-tubulin gene in the vector pBT6, was used. In addition, we have cloned as mutant β-tubulin gene from *G. fujikuroi* (see below).

Cultural Practices

Resistance to the amino glycoside antibiotic hygromycin B was scored three days following transfer from complete medium to minimal medium plus 100 mg/L of the drug. Plates containing hygromycin B were made fresh and not kept for more than two weeks because the drug's inhibitory properties in the media decrease with time. Resistance to benomyl® was done at a concentration of 1 mg/L. Strains carrying the nic1 mutation require nicotinic acid and were grown on MM supplemented with 10 mg/L nicotin-amide. Strains carrying the pdx1 mutation require pyridoxine and were grown on MM supplemented 10 mg/L pyridoxine HCl. Sexual crosses were made on carrot agar, and single random ascospores recovered using a micro-manipulator. Mutants and transformants were purified through the isolation and subculturing of uninucleate microconidia.

Mutant Induction

A benomyl resistant mutant of *G. fujikuroi* was obtained by UV irradiation of fungal spores.

DNA Manipulations

Fungal DNA was extracted using a "miniprep" procedure that we have modified for *G. fujikuroi*. Large and small scale plasmid preparations were made using the alkaline lysis procedure. For Southern analyses, DNA samples were digested to completion with appropriate restriction enzymes, separated on agarose gels, and transferred to nitrocellulose membranes. DNA probes were isolated and purified from agarose gels and radiolabeled by nick translation. Blots were

autoradiographed with Kodak X-(OMAT) film and Dupont Cronex intensifying screens at -70°C.

Transformation Procedures

Cells were transformed to either hygromycin or benomyl resistance via a whole cell transformation procedure adapted for *G. fujikuroi*. This procedure is relatively inefficient with respect to the number of transformants per μg of DNA, however it is rapid and technically simple.

RESULTS AND DISCUSSION

Recovery of Benomyl Resistant Mutants and Cloning of *G. fujikuroi* β-Tubulin Gene

To isolate the β-tubulin gene, a plasmid library in pUC119 was constructed from the benomyl resistant mutant. Using highly conserved sequences for β-tubulin, primers were designed and the polymerase chain reaction (PCR) was used to generate a probe from total genomic DNA of the mutant. Following the screening of the library a 2.1 kb clone was selected and mapped. Constitute expression of the gene was indicated by RNA blots and a transcript of 1.9 kb was observed. Validation of the functionality of this gene was demonstrated by transformation of wildtype *G. fujikuroi* to benomyl resistance.

Meiotic Stability of Transformed Strains of *G. fujikuroi*

To date, stability studies have focused on the hyg^r gene under meiotic conditions. Crosses with single copy transformants segregated hyg^r: hyg^s in a 1:1 manner consistent with that expected for a Mendelian locus in a haploid organism. Multiple-copy transformants, however, segregated hyg^r: hyg^s in a 1:2 manner that was not consistent with Mendelian expectations for a chromosomal marker, even though two unrelated auxotrophic nuclear genes were segregating normally. Segregation ratios in crosses in which hyg^r was introduced via the male parent did not differ significantly from crosses in which the transformed strain served as the female parent. Some of the sensitive progeny from the crosses with the multi-copy transformants carried hyg^r sequences. When these phenotypically sensitive progeny were crossed with a wild-type strain that carried no hyg^r sequences, some of the progeny were phenotypically hyg^r. Some progeny from some crosses were more resistant to hygromycin than were their sibs or the transformant strains that served as their parents. Transformants passaged through a maize plant only rarely segregated progeny with high levels of resistance. The mechanism underlying these genetic instabilities is not clear, but may involve unequal crossing-over and/or methylation.

FUTURE WORK

Foreign DNA can be inserted into fungal chromosomes in three basic patterns: a single copy at a single site, multiple copies at a single site, and multiple copies at multiple sites. We are presently constructing *G. fujikuroi*

multiple copies at multiple sites. We are presently constructing *G. fujikuroi* strains with each of these different patterns with respect to the hygr, *Neurospora* benr, and *G. fujikuroi* benr genes. We will analyze these different genomic configurations with respect to fitness characters, stability and transmission capability. We will cross these transformed strains with our auxotrophically marked strains to determine if the origin of the sequence and/or the amount of sequence homology between the genomic DNA and the foreign sequence affect the stability of the DNA. We will also further examine the structure of the second generation progeny with "reactivated" resistance to determine if the configuration of the foreign DNA has been altered in these strains as compared to the original transformant.

We will begin studies to determine if strains that belong to the same or different VCGs are capable of exchanging foreign DNA introduced via transformation. Strains marked with traditional genetic markers (auxotrophs and spore color mutants) will be paired with the foreign DNA included in the auxotrophic strain. Selection will be based on the concurrent recovery of resistant prototrophic colonies following growth under conditions that favor the formation of heterokaryons. Strains that differ at 0, 1, and 10 of the loci underlying the VCG phenotypes will be used to provide a range of possible inhibitions to the asexual genetic transfer process.

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SESSION II
EFFECTS RESEARCH

Ecological Processes

Researchers are evaluating the effects of introduced microorganisms on structural/functional aspects of freshwater, marine and terrestrial ecosystems. Improvements in the procedures that are developed in these ecological investigations may be incorporated into new testing methods. Further work is being conducted on smaller components of aquatic and terrestrial systems not directly related to ecosystem-level effects. This work includes pesticide degrading microorganisms and biological control agents such as insect growth regulators and microbial pest control agents.

Higher Organisms

A wide variety of microorganisms may be used in biotechnology and information on their toxicological, behavioral, pathogenic, and histopathological effects on higher organisms is unavailable. This research investigates the genetic and molecular basis of infectivity and pathogenicity, and is developing methods that determine the host range of microbial pest control agents. Experimental results will be used to develop protocols for testing effects of microorganisms on beneficial invertebrate and vertebrate species.

Human Health

Researchers are investigating the toxicological, pathological and cytogenic effects of exposing humans to selected recombinant and non-recombinant microorganisms using mammalian cells and surrogate organisms. Different exposure routes are evaluated in terms of measurable effects.

USE OF RIBOSOMAL rRNA SEQUENCES TO CHARACTERIZE DIVERSITY AND STABILITY OF MICROBIAL POPULATIONS

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INTRODUCTION

An explicit characterization of natural microbial community diversity has been elusive. In large part, this has been due to the constraints pure culture methodologies have imposed upon environmental microbiology - pure cultures needed to be representatively obtained from the natural population for subsequent laboratory identification and biochemical characterization. This approach was recognized to be limited in that only 10% (at best) of the bacteria observed in an environmental sample by direct counting could be obtained in laboratory pure cultures. Further, biochemical identifications of such pure cultures may have been imprecise and have lead to erroneous misclassifications in the past. Assessing the potential risk imposed upon natural microbial populations and the ecological processes they mediate requires a greater degree of resolution than offered by isolation of pure cultures.

Recent advances in the application of molecular techniques now permit long-standing issues in microbial ecology to be addressed. In particular, small subunit ribosomal RNA (16S-like rRNA) sequence comparisons facilitate the direct measure of natural microbial population diversity. Population diversity of sulfate-reducing bacteria (SRB) in anaerobic estuarine sediments is being explored through 16S rRNA sequences. SRB have direct roles in the cycling of sulfur and the terminal oxidation of organic matter. Currently, SRB population diversity is being measured with 16S rRNA-targeted hybridization probes. These studies are directed towards defining populations of SRB and assessing population stability in relation to ecological processes.

METHODS

Phylogenetic Probes

Oligonucleotide probes were redesigned such that occurrence of mixed, or multiple nucleotides at a common nucleotide position, were eliminated. This was found to provide more precise measurements of relative rRNA abundance for hybridizations to membrane bound nucleic acids. Probes for specific SRB groups were labeled at their 5′ ends using gamma ^{32}P-ATP and polynucleotide kinase. Additionally, fluorescent dye-conjugated bacterial domain- and delta subdivision-specific oligonucleotide probes were prepared for direct fluorescent microscopic identification of bacterial cells obtained from marine sediments.

Analysis of Natural SRB Populations

Nucleic acids were extracted directly from fractions of sediment cores by mechanical disruption (bead-beater lysis) in the presence of sodium acetate/EDTA buffer pH 5.2, sodium dodecyl sulfate, polyvinylpoly-pyrollidone, and beta mercaptoethanol. Lysates were extracted with phenol, phenol:chloroform, and chloroform. Nucleic acids were precipitated with ethanol. These were then applied to nylon membranes and hybridized with the SRB rRNA specific probes. Rates of sulfate reduction in the sediment core fractions were determined by isotopic assay. Measurements of sulfate and sulfide in the sediment porewater was by standard methods.

Preparation of a Sediment Bacterial Fraction

Sediment bacterial fractions were obtained for whole cell hybridizations with fluorescent dye-conjugated oligonucleotide probes. Sandy sediment samples (3.0 g wet weight) were placed into 50 ml flasks with 3 mm glass beads and 0.15 M NaCl. The contents of the flasks were vigorously shaken for 30 min. then allowed to settle for 15 min. A 3 ml aliquot was withdrawn from the upper portion of the settled suspensions. DAPI stained cells and cells hybridizing with the rRNA probes were determined. Efficiencies of cell recoveries were obtained by adding known amounts of sulfate-reducing bacteria to washed, sterile sand and to natural sediment samples. For enumeration of sulfate-reducing bacteria, preparation of cell fractions were prepared under anaerobic conditions. The bacterial fractions were passed through 8.0 μM filters to remove large particles. Filtrates were treated with combinations of SRB basal salts medium, nutrients, and nalidixic acid to stimulate production of ribosomes and enhance the detection of SRB.

RESULTS AND DISCUSSION

SRB Population and Function Profiles in Sediment Cores

Concentrations of sulfate were 15 mM in the surface water and in sediment porewater to a depth of 4 cm. Porewater concentrations of sulfate decreased from 10 mM at the 4-5 cm. depth interval to less than 5 mM in the 14-16 cm. depth interval. Porewater concentrations of sulfide were 0.1 to 0.2 mM in the 4-10 cm. depth intervals and increased markedly to 0.8 mM 12 to 16 cm. below the water-sediment interface. The peak in activity of sulfate reduction (3.5 mmol/ml/hr) occurred in the 2-3 cm. interval.

Desulfovibrio 16S rRNA was the most abundant SRB rRNA and peaked at the 3-4 cm. interval. Stratification of the SRB population with sediment depth was apparent. Amounts of 16S rRNA of fatty acid- and/or acetate-utilizing SRB (encompassing four SRB phylogenetic groups) decreased in the sediment fractions having the highest amounts of *Desulfovibrio* 16S rRNA.

The peak in activity of sulfate reduction did not occur within the same fraction as the predominant peak in *Desulfovibrio* rRNA. Acetate has previously been shown by other

investigators to be the primary substrate for sulfate reduction in estuarine sediments. Thus, the peak sulfate reduction rate was expected to coincide with a peak in the rRNA abundance of acetate-utilizing SRB. This relationship was not seen in the results and may be accounted for by several possibilities. Additional groups of SRB may exist in nature that are not represented in the culture collection that not are being accounted for by the probes. Also, since the data represent one sampling time, it is possible that a shift in the activity of sulfate reduction had occurred and that the population had yet to respond by an increase in rRNA abundance. Finally, accounting for energy released by the reduction of sulfate by different electron donors should be considered in evaluation of the community structure-function relationship. Hydrogen, for example, as an electron donor for sulfate reduction yields considerably more energy than acetate.

Whole Cell Hybridizations of Natural Populations

Laboratory grown SRB cells were recovered from washed, sterile sediment samples with efficiencies approaching 90%. For fixed sediment samples, cells hybridizing with the bacterial domain probe were 16% of the cells observed by DAPI staining. Cell counts hybridized with the delta subdivision-specific probe were 42% of the counts hybridized with the bacterial domain probe. Delta subdivision cell counts increased to 59% and 49% of the bacterial domain counts when samples were incubated in medium with or with nalidixic acid, respectively. When samples were incubated in medium containing molybdate, and inhibitor of sulfate reduction, delta subdivision cell counts were only 11% of the bacterial domain counts. The results demonstrate that fluorescent dye labeled rRNA probes could be used with natural samples and that specific bacterial populations can be stimulated to enhance their detection.

FUTURE WORK

Research planned will address the community structure-function relationship of SRB populations. PCR amplification and sequencing of 16S rRNA genes from natural populations will be used to determine natural diversity not represented in the culture collections. Natural populations will be characterized during temporal sampling to determine base line variations in populations with respect to sulfate reduction rates and turnover rates of substrates. Laboratory sediment systems will be used to control parameters, such as availability of specific electron donors, to alter SRB populations. Hybridization of fluorescent labeled probes sediment bacteria as a means to increase the number of samples which can be analyzed will be explored.

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HABITAT SPECIFIC DIFFERENCES IN PERSISTENCE AND EFFECTS OF INTRODUCED CELLULOLYTIC BACTERIA USED AS SURROGATES FOR GEMS

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INTRODUCTION

These studies have three primary objectives: a) to determine the persistence of introduced bacteria in natural benthic aquatic communities, b) to test several community and ecosystem level response variables in order to pinpoint those most useful for detecting effects of the introduced organism, and c) to evaluate the utility of mesocosms for assessing the fate and effects of genetically engineered microorganisms (GEMs) in natural systems. We have worked with surrogates for a GEM with enhanced cellulose degrading capability. Cellulose "superdegrader" GEMs and their products are of industrial interest and may find application in the conversion of biomass to fuel. The use of a surrogate expedites field studies, although we recognize that a surrogate will not mimic a GEM in all physiological and ecological respects. Our research focuses on stream ecosystems since they often receive waste discharges and non-point source inputs. White Clay Creek, the study stream, drains a protected rural watershed in southeastern (Chester Co.) Pennsylvania.

We screened over 20 isolates of aerobic cellulolytic bacteria from culture collections by assaying their lytic activity on pure cellulose, ability to degrade algal detritus and leaf litter, and their growth rates and ranked them according to their performance in these tests. Fluorescent antisera (FA) were produced against the most active isolates, *Cellulomonas flavigena* (NRC 2403), *C. fimi* (NRRL B402), *C. sp.* (NRC 2406), and *C. uda* (NRRL B404) to enable their detection in benthic communities. Preliminary studies of the population dynamics of *C. sp.* (NRC 2406), *C. uda* (NRRL B404), and *C. sp.* CS1-1 (an organism that cross reacted with *C. uda* antiserum) in streambed sediments were conducted in 2.95 L microcosms.

C. sp. (NRC 2406) was selected for initial studies in larger (35 L) mesocosm streams. The first experiment, conducted during the summer months indicated that the isolate could persist for several weeks in sediments and benthic algal growths, although densities declined fairly rapidly from high post-inoculation levels. Effects on benthic community metabolism (primary productivity and community respiration), associated changes in DOC, and bacterial productivity were not pronounced. High densities of FA stained cells were found in algal growths in both an inoculated and a control mesocosm 3 months after introduction. We have also found cells

that stained with *C. uda* (NRRL B404) and *C. sp.* (NRC 2406) antisera in leaf packs and streambank soil from White Clay Creek. While there may be a natural source of FA stained cells in White Clay Creek, our observations suggested habitat specific differences in persistence deserved study. The experiment described below focused on population dynamics of the introduced organism in three different micro-habitats; algae, sediments, and leaves, and on alteration of the rate of degradation of leaf litter.

METHODS

Six stream mesocosms (2.23 m long x 0.203 m wide x 0.127 m deep), designed to simulate a slow run habitat in White Clay Creek, were constructed of vinyl coated steel and Plexiglas end plates. They were filled with 2 cm. of gravel and coarse sand from White Clay Creek, over which 40 plastic trays (0.10 m square x 0.038 m deep) containing surface sediments from White Clay Creek were placed. The bottoms of the trays were removed and replaced with 400 µm mesh nylon screening to allow exchange of water, nutrients, dissolved gases and organisms within the sediments. Stream water (35 L) was recirculated in each system; 900 ml/min of new stream water was added continuously to replace the entire volume approximately every 40 min. Water depth approximated 1.5 cm. Water returned to White Clay Creek was first filtered through cartridge filters (which were autoclaved before discarding) and irradiated with ultraviolet light, a treatment found to effectively kill the study bacteria. The streams, housed in a greenhouse, were immersed in water jackets continuously replenished with White Clay Creek stream water to maintain ambient stream water temperatures. The systems were separated by clear plastic sheeting suspended from a wooden frame to hinder spread of organisms between microcosms.

Population and community structural and functional parameters (including densities of FA stained cells, total bacteria, primary productivity, community respiration, ATP, chlorophyll a, and cellulase activity) were measured by sampling sediments and filamentous algae (dominated by *Cladophora sp.*) prior to addition of the isolate. Just before inoculation of the isolate, packs (approximately 5 g each) of tulip poplar (*Liriodendron tulipifera*) leaves that had been previously leached and redried to obtain weights, were placed in the mesocosms. The surrogate was placed in the water in two mesocosms, maintaining two others as uninoculated controls. Cells for inoculation were grown at 20°C on Solka-Floc in basal salts medium for 5 days with agitation. After allowing the cellulose to settle, cells were harvested, resuspended in autoclaved stream water, and added to duplicate mesocosms. After recirculating the inoculum for approximately 72 h with no stream water addition, the systems were returned to flow-through mode and 4 h later, an intensive (3 - 5 day) study of densities of the surrogate and total bacteria in surface sediments, leaf packs, and algae was initiated. Then, at approximately 10 - 14 day intervals the response variables noted above

were measured, and samples for leaf pack chemical composition and weight loss were collected. Light, temperature, and selected water chemical characteristics were monitored. Samples of sediment and *Cladophora sp.* were obtained by filling plastic rings (1.15 cm. i.d. x 0.2 cm. deep) with sample. Leaf packs were sampled by coring replicate samples (1.10 cm. i.d. x approximately 2 cm.) with a cork borer.

Primary productivity and community respiration of sediments and *Cladophora sp.* were estimated from dissolved oxygen changes in flowing water microcosms from which ecosystem P/R status was determined. Leaf pack respiration rates were determined in the same systems. For bacterial enumeration, formalin fixed samples were sonicated for 45 sec. at 30 W with 0.1 M dibasic ammonium phosphate as a surfactant, centrifuged once (412 x g for 5 min at 4°C) in 30% glycerol, and twice more in 60% glycerol. Aliquots of supernatant fluids were filtered onto 0.2 µm pore size Nuclepore filters and stained with FA or DAPI ,for epifluorescence microscopic counts of the target cell or total bacteria on from 6-10 or 3 replicate samples, respectively. Algal biomass was assessed from chlorophyll a determination, and total viable biomass from ATP, each on 5 aliquots. Cellulase activity was determined using the Cellulose Azure (CA) assay; data were corrected for color contributed by samples using a two wavelength correction procedure for leaf packs or a regression of color against sample weight for sediments. Leaf pack decomposition was determined from weight loss and cellulose content. Cellulose was obtained by combustion at 450°C for 4 h following sequential extraction of non-cell-wall material, hemicellulose, and lignin using detergent solutions and permangenate oxidation.

RESULTS AND DISCUSSION

Epifluorescence microscopic counts indicated that the *C. sp.* (NRC 2406) inoculum contained 1.97 ± 0.15 x 10^9 cells/ml, and viable counts from spread plates indicated 4.77 ± 0.68 x 10^9 colony forming units/ml. Using the average of these estimates, we calculated that the initial density in the inoculated mesocosms was approximately 4.57 x 107 cells/ml of water.

Six water changes after exposure, the densities (cells/cm3) associated with sediments, *Cladophora sp.*, and leaf packs in the duplicate inoculated mesocosms averaged 1.06 ± 0.16 x 10^6, 4.32 ± 2.32 x 10^6, and 2.07 ± 0.97 x 10^6, respectively. Based on sample weights, the densities (cells/g dry weight) were 8.06 ± 4.16 x 10^5, 3.52 ± 2.83 x 10^7, and 1.30 ± 6.34 x 10^7, for those sample types, respectively. Given the densities of total bacteria in these samples, which ranged between 1 x 10^8 and 1 x 10^{10} in all three sample types, the isolate composed maximally 0.17% and 0.44% of the total bacterial community in *Cladophora* in each inoculated stream, 0.35 % and 0.49 % of in leaf packs, and only 0.04% and 0.04% of the sediment bacterial communities in each of the duplicate inoculated mesocosms. FA stained cell densities (cells/g dry weight) in the duplicate

control mesocosms averaged only 3.8 ± 17.1 x 10^4 and 4.58 ± 4.20 x 10^6 in sediments and *Cladophora sp.* respectively and the organism was never detected in leaf packs.

Densities decreased rapidly from post inoculation maxima in sediments, and slowly in leaf packs, with an intermediate rate of decline in *Cladophora*. Regression analyses of cell densities on both a dry weight and a volumetric basis indicated half lives (T1/2) of 13.5 (r2 = 0.829, 0.778), 1.5 (r2 = 0.599, 0.602), and 8.8 (r2 = 0.875, 0.989) days for the isolate in those habitats, respectively. While densities were near background (control) levels in sediments and *Cladophora* after 10 and 30 days respectively, FA stained cells were found in a leaf pack at 70 days.

Effects on community respiration and primary productivity of sediment were not statistically significant (Before-After, Control-Impact test, BACI, a = 0.05). These measures were made on *Cladophora* samples less frequently, and although rates were much higher the isolate had no pronounced effect on either respiration or primary productivity. Similarly, there were no strong effects on chlorophyll a concentrations and assimilation ratios and hourly P/R ratios were in a similar range in both inoculated and control mesocosms throughout the study. Leaf pack respiration per gram dry weight sample was higher on packs exposed to the isolate (0.450 ± 0.205 vs. 0.367 ± 0.181, n = 6), but the difference was only statistically significant at the a = 0.10 level (paired sample t-test). There was overlap in ATP values between streams and no striking difference between data collected before and after exposure.

Cellulase activity tended to be slightly higher overall in the inoculated streams for each sample type. However, the difference was statistically significant only for sediment samples (paired t test, a = 0.05) and since it occurred both before and after inoculation the effect cannot be attributed to introduction of the isolate. Correlation analyses including all sample types indicated that isolate densities whether expressed on a volumetric or dry weight basis, were weakly but significantly correlated with cellulase activity (r = 0.40 and 0.32, a = 0.01 and 0.05 respectively). The relationship was highly significant for sediment samples (r = 447; a = 0.01, n = 32) although not for leaf packs, perhaps because other cellulolytic bacteria and fungi were more numerous there.

Regression analyses indicated that leaf packs lost weight at a similar rate in both the experimental (T1/2 = 99 days) and control (T1/2 = 115 days) systems, and predicted slightly less cellulose (68.9%) in exposed leaf packs than in unexposed (75.6%). Sample sizes were small however, and these indications require confirmation. While other cellulolytic bacteria and fungi were present in the packs, correlation analyses showed that both weight loss and cellulose content of leaf packs were weakly correlated (r = 0.45 and 0.50, a = 0.08 and 0.10) with FA + cell densities and those densities expressed as a percentage of the total bacterial flora (r = 0.64, a = 0.05, n = 11).

FUTURE WORK

A more detailed study of FA population dynamics in leaf packs and effects on leaf pack decomposition has been performed, including a comparison of those parameters in mesocosms and the field. Samples from that experiment are still being processed. More work with *Cladophora* or other algae with a cellulose cell wall is needed.

Obviously, for an introduced organism to affect community or ecosystem properties the impact must be either immediate and acute or the organism must be able to persist and exert an effect over time. Work to date indicates the need to understand in greater detail the factors controlling bacterial population dynamics in natural systems, including the physiological and ecologically relevant characteristics of the organism as they interact with ecosystem properties such as nutrient supply and grazing rates. The application of rRNA probes in those studies is desirable, to increase the specificity of identification of the isolate in natural samples. Finally, we anticipate the extension of these studies to work with a GEM.

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FIELD CALIBRATION OF SOIL-CORE MICROCOSMS FOR EVALUATING FATE AND EFFECTS OF GENETICALLY ENGINEERED MICROORGANISMS IN TERRESTRIAL ECOSYSTEMS

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INTRODUCTION

Microcosms are an attractive option for obtaining preliminary data on the fate and ecological effects issues associated with the release of genetically engineered microorganisms (GEMs) into the environment, because tests and microorganisms can be contained within the laboratory. Historically, microcosms have been used to assess the fate, transport, and toxicity of chemicals and pollutants. Recently; intact soil-core microcosms incubated in the laboratory were used to evaluate the fate and ecological effects of transposon mutants of *Azospirillum lipoferum* and a wheat (*Triticum aestivum*) -root colonizing *Pseudomonas sp.* However, before microcosms can be used as a standard tool for biotechnology risk assessment, they must be compared with the field to ensure that they are effective field models.

The principal objective of this project was to calibrate soil-core microcosms with their field counter part for microbial fate and ecosystem structural and functional properties and the resultant effect of introduced microorganisms on these properties. In the first year of this project (October 1988 to September 1989), the fate of rhizobacterium *Pseudomonas sp.* RC1 in soil and on the wheat rhizoplane was determined as a function of time in intact soil-core microcosms at ambient temperature (22°C) and in a growth chamber with temperature fluctuations that simulated average conditions in the field, field lysimeters, and field plots (Bolton et al., 1991a). The effect of the introduced RC1 on ecosystem structural and functional properties as well as a comparison between the four systems was also conducted the first year (Bolton et al., 1991b).

In the second year of this project (October 1989 to September 1990) two experiments were conducted with soil-cores as microcosms located in a growth chamber and as lysimeters in the field. First, RC1 was used to determine how year-to-year field variability influenced the calibration of microcosms with the field. Second, the Gram-positive bacterium *Streptomyces lividans* TK24 was used to allow us to compare microcosms as field surrogates for both Gram-negative (RC1) and Gram-positive (TK24) bacterial survival and ecological effects. The complete results of this project are contained in the final report (Bolton et al. 1991c).

MATERIALS AND METHODS

The bacterial strains used in this project included a spontaneous rifampicin resistant mutant (100 mg/L) of *Pseudomonas sp.* strain RC1 (RC1)

grown on Sands rif[50] medium and *Streptomyces lividans* TK24 (TK24), which was selectively grown on starch-casein mineral salts medium with carbenicillin (25 mg/L), streptomycin (50 mg/L), nystatin (50 mg/L), Benomyl (50 mg/L), and cycloheximide (500 mg/L).

Intact soil cores for use as microcosms and field lysimeters (17.5 cm. diameter, 60 cm. length) were obtained from the field site in southeastern Washington state using a steel coring device. The surface soil (top 15 cm.) was removed from all cores and combined, mixed, and sieved to pass a 2-mm screen. The RC1 study had two soil treatments including inoculation with 2.3×10^7 colony forming units (CFU) of RC1/g dry soil and uninoculated. The surface soil for the separate TK24 study was amended with 1.0% cellulose and either inoculated with 2.5×10^6 CFU of TK24 spores/g dry soil or left uninoculated. All treatments were brought to a final soil moisture content of 16% with tap water.

The microcosms were incubated in a growth chamber under a cycling temperature regime that approximated the average daily minimum temperature during the dark cycle and the average daily high temperature during the photoperiod at the Hanford Site. The lower temperature limit in the growth chamber was 5°C. The field lysimeters consisted of intact soil cores within polyethylene pipe placed back into the soil at the field site. The bottoms of the microcosms and field lysimeters were covered with polyester monofilament cloth (160 mesh) to prevent root (for RC1) and soil (for TK24) loss from the core.

The mineralization of ^{14}C-labelled cellulose in microcosms and lysimeters was determined in the TK24 study in minitubes (4.3 cm. diameter, 24 cm. length PVC pipe) with the $^{14}CO_2$ evolved determined as a function of time.

All treatments were replicated six times. The RC1 study was seeded with five "Daws" winter wheat seeds in each soil core on October 25, 1989 that were later thinned to two wheat seedlings. The TK24 study was started on November 2, 1989. Microcosms and field lysimeters were routinely watered with tap water to a final soil moisture content of 16%.

The survival of RC1 and TK24 in surface soil was determined as a function of time by plating onto their selective media.

For the RC1 study, the populations of RC1, *Pseudomonads*, and total aerobic heterotrophs on the wheat rhizoplane were enumerated at the three-leaf stage of growth on seedlings and at the boot stage of growth on roots from three sections (top: 0 to 15 cm., middle: 15 to 35 cm., and bottom: 35 to 55 cm.). The biomass of wheat shoots and soil dehydrogenase activity in surface (0 to 15 cm. depth) soil were determined at the three-leaf and boot stage of wheat growth.

For the TK24 study, surface soil samplings occurred in late November, early March, early June, and early September. The surface soil was sampled at these four times for populations of total aerobic heterotrophs and *Actinomycetes*, the soil enzyme activities of dehydrogenase and b-glucosidase, and ^{14}C-labelled

microbial biomass.

RESULTS AND DISCUSSION

The decline in the population of RC1 in surface soil was similar in the microcosms and the field. However, the population of RC1 in surface soil was significantly higher in the microcosms than the field at the three-leaf stage of wheat growth (5.9 log CFU/g > 4.5 log CFU/g), while the population decreased to the same value at the boot stage (4.2 and 3.6 log CFU/g for microcosms and the field). The rate of decline was similar to the rate found in the first year study (Bolton et al. 1991a), suggesting that small differences in inoculum level (7.4 and 7.7 log CFU RC1/g in this and the previous study) and year-to-year climatic variability in the field did not greatly influence the rate of decline of RC1 in soil. There was no difference in the colonization of wheat roots by RC1 at the three-leaf stage in microcosms or the field, similar to first year results. The population of RC1 on the rhizoplane at the boot stage of wheat was different from that of the first year with significantly higher populations associated with the microcosms than the field. Also, the distribution of RC1 on the rhizoplane as a function of depth in the microcosms was different from the field with the population of RC1 generally higher in the microcosms than the field.

Pseudomonad and total aerobic heterotroph rhizoplane populations were very similar in the microcosms and field, and similar to the previous year's results. Shoot biomass and dehydrogenase activity of soils were no different in the microcosm and the field at the three-leaf stage of wheat growth, but were lower and higher, respectively, in microcosms versus the field at the boot stage. These results were different from those of the previous year where the growth chamber and the field had a similar final biomass. The previous year the soil dehydrogenase activity was significantly higher in the microcosms than the field at the three-leaf stage of wheat growth. Soil dehydrogenase activity at the boot stage sampling was not determined the first year. Year-to-year variability was found in comparing this study with previous years (Bolton et al., 1991a, 1991b) including differences in the colonization of the rhizoplane by RC1 at the boot stage and wheat biomass production at the boot stag. However, for the majority of parameters, results from microcosms and the field were similar in both years.

Soil populations of TK24 declined less than 2 log units over the ten-month study. This slow decline in the soil population of TK24 compared to RC1 is not surprizing because TK24 is a gram-positive spore-forming bacterium and was added to soil as spores. There were no differences between the microcosms and the field for the soil population of TK24 until after 45 weeks when the population was 1 log unit higher in the microcosms. At the initial sampling in the fall, soil dehydrogenase activity was greater in the microcosms than the field and inoculation with TK24 depressed activity. Dehydrogenase activities were higher in microcosms than in the field during the winter and summer, but there were no differences at the spring sampling. Soil

ß-glucosidase activity was higher in microcosms than the field during the winter and summer, but were the same during the fall and spring. Aerobic heterotroph and *Actinomycete* populations were higher in microcosms than in field soils during winter and summer, while the field had larger populations in spring. The rate of ^{14}C-cellulose mineralization was similar in the microcosms and the field until early May when there was a flush of $^{14}CO_2$ in the field. The ^{14}C-labelled soil microbial biomass was significantly larger in the microcosm than the field, while inoculation with TK24 had no effect.

In summary, the microcosms simulated the field quite well for microbial fate and effects. Exceptions include a flush of $^{14}CO_2$ from the field soils in early May and the higher populations of TK24 in microcosm soils at the summer sampling, both of which could be attributed to differences in temperature and water regimes. The differences in microbial populations and enzyme activities at the winter and summer sampling points were attributed to the inability of the growth chamber to mimic temperature extremes in the field during these periods.

The results from studies conducted the second year of this project suggest that microcosms can accurately simulate the field with respect to the fate and effects on ecosystem structural and functional properties of both gram-negative and gram-positive introduced micro-organisms. However, better controls of environmental variables including temperature and moisture will be necessary to more closely simulate the field for future use of microcosms for risk assessment.

FUTURE WORK

This project was completed and a final report submitted to EPA-Corvallis in March 1991.

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THE EFFECT OF A GENETICALLY ALTERED BACTERIUM ON NITROGEN TRANSFORMATION RATES, INDIGENOUS MICROBIOTA AND MICROBIAL BIOMASS IN A XERIC SOIL

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INTRODUCTION

Genetically engineered microorganisms (GEMs) released into the environment could influence microbial populations and processes in soil, by altering nutrient availability through competition, removal of substrate(s), and/or addition of metabolites. The presence of GEMs may also alter predation patterns and immobilization and mineralization processes, affecting soil tilth and fertility. Thus, an understanding of the interrelationship between GEM function, differences in environment (*e.g.*, soil type and microbial community), and soil processes is critical.

We chose to study *Pseudomonas putida* PPO301(pRO103), engineered to degrade the herbicide 2,4-dichlorophenoxyacetate (2,4-D); an ability not found in its plasmidless, parental strain (PPO301). Previous work with PPO301 (pRO103) demonstrated that total fungal propagule numbers were depressed (both in the absence and especially in the presence of 2,4-D) in a low carbon soil inoculated with this GEM. Our study sought to both repeat this effect, and to examine other ecological endpoints not previously investigated.

In this study, we determined the dynamics of the indigenous populations of bacteria (active and total; nitrifying and denitrifying populations); fungi (active and total); protozoa, and nematodes over an 89-day period in the following treatments: (a) unamended and uninoculated soil, (b) 2,4-D-amended, uninoculated soil, (c) PPO301 inoculated, unamended soil, (d) PPO301 inoculated, 2,4-D-amended soil, (e) PPO301(pRO103) inoculated, unamended soil, and (f) PPO301 (pRO103) inoculated, 2,4-D-amended soil. Additionally, biomass estimates, concentrations of inorganic nitrogen species, respired carbon (CO_2), and pH were determined.

MATERIALS AND METHODS

Soil

The Xeric/Aridic Frigid soil used in this study was collected in March 1990 from the same site (Millican Limited Use Area, near Bend, OR), and characterized and stored in the same manner as that (obtained in July 1987) used in previous work.

Bacterial Cultures

Pseudomonas putida PP0301 (pRO103) and its parental strain PP0301 were derived previously from *P. putida* PP0300 (ATCC 17514). These strains are resistant to nalidixic acid (500 μg/mL). Plasmid pRO103 encodes resistance to both tetracycline (25 μg/mL) and mercury (25 μg/mL), and the constitutive degradation of 2,4-D to chloromaleylacetate. Both strains were maintained on either brain heart infusion or tryptone nutrient agar supplemented with the appropriate selective agents. The presence of the plasmid in the GEM was confirmed by DNA extraction and agarose gel electrophoresis prior to the start of the experiment. Inocula of both bacterial cultures were prepared as in previous work.

Soil Preparation and Microcosms

Sieved (2mm-mesh) soil was adjusted to the -33 kPa water tension, and left to equilibrate at 4°C, with daily mixings for 72 hours. On Day 0, the soil was inoculated with ca. 10^8 CFU/g oven-dry equivalent (ODE) soil and amended with 2,4-D (500 μg/g ODE soil) where appropriate. Following ample mixing, 60 g ODE soil was placed into each of 144 pint-size Mason jars and incubated at 25°C in a light-free chamber. Each Mason jar was closed with an air-tight lid fitted with a rubber septum to permit headspace sampling for CO_2 analysis. Carbon dioxide measurements were conducted by gas chromatography (Hewlett-Packard model 5840; thermal conductivity detector; Porapak Q (80/100 mesh) column; helium gas mobile phase).

Sampling Description

During the 89-day study period, soils were sampled eight times and CO_2 analysis was performed 23 times. To prevent the accumulation of metabolically inhibitory amounts of CO_2 (i.e., $\geq$ 3-4%), all jars were periodically allowed to off-gas for 1-2 minutes in a moist chamber. At each soil-sampling period, three replicate jars per treatment were processed for: % moisture; pH; 2,4-D and dichlorophenol (DCP) concentrations (by HPLC); inorganic nitrogen analysis; enumeration of the indigenous bacteria (nitrifying, denitrifying, active, and total), the introduced bacteria, the active and total fungi (including hyphal length measurements), the protozoa, and the nematodes; and microbial biomass estimations. Only one jar per treatment was subsampled for CO_2 analysis, and enough jars were used such that, except for the last three sampling periods, none were sampled for CO_2 on more than one occassion.

Enumeration of Indigenous Bacterial and Fungal Populations

Bacteria and fungi were enumerated both by viable plate counts (soil extract agar + cycloheximide (100 μg/mL), and Martin's medium, respectively) and by direct counts. Nitrifying and denitrifying bacterial populations were estimated by standard MPN techniques. Ten-fold (v/v) serial dilutions of each replicate were prepared in either 1 mM potassium phosphate buffer, pH 7.2 (for nitrifier and denitrifier enumerations

only) or sterile tap water (for all other estimates, including protozoa). Aliquots of 1-5 mL were removed from the first soil dilution and prepared as suspension-agar films for estimates of active (fluorescein diacetate stained) and total (phase contrast microscopy) fungi and for hyphal length determinations by standard methods. Active bacterial numbers and diameters were determined by the same method, under oil immersion. Total numbers of bacteria were determined using fluorescein isothiocyanate direct counts. Diameters of fungal hyphae and bacteria were measured to convert estimates of bacterial numbers or fungal volumes into biomass, using the average values of 0.41 g biomass/cm^3 hyphae and 0.33 g/cm^3 bacteria.

Enumeration of Protozoa and Nematodes

Protozoa were enumerated by an MPN technique and characterized as flagellates, amoebae, or ciliates. Nematode numbers were determined by extracting 5 gram subsamples of soil in Baermann extraction funnels.

Inorganic Nitrogen Determinations

Soil aliquots of 15 grams/sample were extracted in 30 mL of 0.5 M K_2SO_4 and filtered by gravity-flow. Filtrates were brought to final volumes of 30 mL, preserved in 0.2% (v/v) H_2SO_4, and stored at 4°C prior to colorimetric analysis for ammonium and nitrate species by an autoanalyzer (Alpkem Rapid Flow Analyzer: RFA-300; methods A303-S021 and A303-S170, respectively). Nitrite, unstable under these conditions, was oxidized to nitrate, and expressed as part of the sum of the two species.

Statistical Comparisons

Statistical comparisons (ANOVA and correlation coefficients) were performed using the Statistical Package for the Social Sciences (SPSS; Nie *et. al.* 1975. 2nd ed., McGraw-Hill, NY). Two-way analysis of variance was performed using time and treatment as the main effects. Only those main or interactive effects found significant by the F-test at $P < 0.05$ were considered. The mean separation test used was Least Significant Differences calculated for a significance level of $P < 0.01$.

RESULTS

Concentrations of 2,4-D and DCP

During the 89-day study, no reduction of 2,4-D concentration was observed in any of the amended soils. Extraction efficiencies of 2,4-D were ca. 36% of the added material. Also, no DCP was detectable in 2,4-D-amended soil inoculated with the GEM.

Soil Moisture, pH, and Respiration

In spite of the periodic opening of jars to relieve accumulating CO_2 levels, soil moisture did not vary significantly during the study. Soil pH values did not vary widely over time within the treatments, although all of the 2,4-D-amended soils were more alkaline (perhaps reflecting concurrent reductions in nitrifying bacterial populations and increases in NH_4^+

levels). Furthermore, within the 2,4-D-amended soils, those inoculated with either the GEM or the parent were significantly higher in pH than the uninoculated soil. All 2,4-D-amended treatments showed lower rates of CO_2 evolution.

Fungal Activity, Hyphal Length and Number of Propagules

During the first week of the study, some contrasting changes in bacterial and fungal numbers occurred in treatments with introduced bacteria, *i.e.*, as fungal numbers increased, bacterial numbers decreased, and vice versa. However, these population changes were transient, and in no case was a prolonged GEM-specific effect observed. Contrary to previous findings with PPO301(pRO103), no decline in fungal numbers was seen in soil inoculated with the GEM. In fact, the numbers of fungal propagules were significantly higher in 2,4-D-amended soils inoculated with either the GEM or the parent than in any other treatments, when enumerated by the viable plate count procedure. Counts of the active fungi suggested, however, that these increases were only transient (possibly reflecting sporulation). Both direct counts of total fungi and total hyphal length measurements revealed no strong differences between any of the treatments (2,4-D-amended or not). In general, the hyphal lengths of active fungi were decreased in all soils amended with 2,4-D.

Introduced Bacteria

The numbers of both the GEM and parent strains ranged between approximately 10^6 to 10^7 CFU/g ODE soil for most of the study. Occassion-ally there was a transient decrease in the number of both strains to 10^5. On Day 89, the numbers of both of the introduced bacteria in the unamended treatments were undetectable. The numbers of introduced bacteria in soil amended with 2,4-D remained constant, suggesting that the presence of the herbicide gave these organisms a selective advantage. At each soil sampling period, the GEM was phenotypically distinguishable from the parental strain.

Indigenous Bacteria, Protozoa, and Nematodes

No GEM effects, including remediation of 2,4-D effects, were observed. Bacterial biomass estimates for total bacteria and (especially) active bacteria, as well as direct counts, were reduced in the presence of 2,4-D. The numbers of total, nitrifying, and denitrifying bacteria were also reduced in the presence of 2,4-D.

Nematode numbers were only affected by 2,4-D on Day 89. Neither amelioration of this latent 2,4-D effect nor significant selection for nematode species were observed in GEM (or parent) inoculated treatments.

The community structure of protozoa was modified in soils inoculated with the GEM. Two species of amoebae appeared in greater numbers in GEM-inoculated soils relative to soils inoculated with the parental strain, regardless of 2,4-D amendment. However, protozoal numbers were reduced in all 2,4-D-

amended soils.

Nitrogen Pools

Nitrogen transformations in both parent- and GEM-inoculated soils were affected by the presence of these organisms; however, no GEM-specific effects were seen. Relative to the uninoculated, unamended control, NH_4^+ levels were higher in both parent- and GEM-inoculated soils, regardless of 2,4-D amendment. After Day 10, this was also true for (NO_2^- + NO_3^-) levels in unamended soils. The former might be attributed to nitrogen supplied by the introduced populations of bacteria, but more probably reflects reduced nitrification in the 2,4-D treatments as nitrifying bacteria were suppressed. The levels of (NO_2^- + NO_3^-) were consistently reduced in all 2,4-D-amended soils. However, the levels of NH_4^+ were consistently increased by 2,4-D in these soils.

DISCUSSION

Contrary to earlier work with PPO301(pRO103), we saw no GEM-mediated influences on 2,4-D degradation or numbers of fungi. Yet, the GEM maintained its phenotypic traits throughout the experiment, and the pRO103 plasmid has been shown to be stable in PPO301. Given the similarities (*e.g.*, the same soil source and handling; manner of both addition and extraction/analysis from soil, as well as concentration of 2,4-D; size and preparation of inocula, etc.) between these experiments, these findings are surprising. In earlier studies, addition of 500 ppm 2,4-D resulted in the recovery of 66% of the added chemical, while we recovered only 36% of the added 2,4-D on Day 0 and in subsequent samplings. Short *et. al.* (1991) found a steady decline of 2,4-D from 500 ppm to <100 ppm in 53 days. They attributed this to the degradation of 2,4-D (and subsequent appearance of the metabolite DCP) by the added GEM.

We believe our findings may reflect changes in Millican soil, which caused the extraction of 2,4-D to be compromised in our study. Of the differences that we are aware by chemical analysis between Millican soil collected in 1987 and in 1990, perhaps two are most significant. Grazing pressure, or at least cattle usage patterns, on this land may have changed. Inorganic nitrogen (*i.e.*, NO_3^- and NH_4^+) levels were 2-3 times lower in soil collected in 1990. Also, this soil had a higher organic content (1.8 $\pm$ 0.2% in 1990 vs. 1.3 $\pm$ 0.1% in 1987).

An increased (albeit low) level of organic matter may have reduced the extraction efficiency (and availability to the GEM) of 2,4-D by promoting increased soil binding and incorporation of the compound (*e.g.*, to humic acid). We never recovered more than 200 ppm 2,4-D. Short *et al.* (1991) found that as 2,4-D decreased below 150 ppm, the concentration of DCP decreased as well. Although this may reflect the instability of DCP, another explanation could be that a minimal titer is required to activate the catabolism of 2,4-D by PPO301(pRO103). In our study, no DCP was produced, no 2,4-D was removed, and no DCP effects were observed. We speculate that not

enough 2,4-D was available for the GEM to be activated.

FUTURE WORK

We hypothesize that if 2,4-D were available (i.e., extractable) in Millican soil to PPO301(pRO103) in sufficient concentrations, the GEM-mediated effects of 2,4-D degradation, DCP accumulation, and decreased fungal populations would occur. We plan to test this in the series of short experiments (using Millican soil collected in 1990), detailed below. Unfortunately, our reserves of Millican soil collected in 1987 are depleted. However, prior to the initiation of this work, we will verify that our present strains of PPO301(pRO103) have the ability to degrade 2,4-D in culture.

(a) Titration study of 2,4-D in Millican soil.
Objective: To determine that amount of 2,4-D recoverable as 500 ppm.

(b) Comparative extraction of 2,4-D (amount recoverable Millican soil as 500 ppm) from three different "soils": low carbon (Millican); high carbon (Thatuna -Naff); no carbon (silica sand).
Objective: To verify that soil composition affects the extraction efficiency of 2,4-D.

(c) Small scale repeat of present study, but using the 2,4-D amount which can be recovered as 500 ppm.
Objective: To determine if higher levels of 2,4-D amendment will result in the GEM-mediated effect of depressed fungi.

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EFFECTS OF A LIGNIN-DEGRADING RECOMBINANT *Streptomyces* ON MICROBIAL ACTIVITY AND NUTRIENT CYCLING IN SOIL

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INTRODUCTION

This research was a collaborative effort between the University of Idaho, the Battelle Pacific Northwest Laboratory, and EPA's Environmental Research Laboratory-Corvallis. The research was designed to provide information on the magnitude, extent, and mechanisms by which a recombinant lignin-degradation enhanced *Streptomyces* strain effected the integrity of a soil ecosystem. the need for the study stemmed from previous findings in D.L. Crawford's laboratory at the University of Idaho (EPA Cooperative Agreement CR-815300-01-05) showing that recombinant *S. lividans* strains expressing a plasmid-encoded gene coding for bacterial lignin peroxidase (1), when released into soil, transiently, but significantly affected the rate of carbon mineralization in the soil as measured by an increased rate of CO_2 evolution from the soils (2). The effect was particularly significant in nonsterile soils containing recently deposited lignocelulosic residues. It was found that this carbon mineralization effect resulted from increased production of lignin peroxidase by the recombinant in soil. It was hypothesized that this resulted in a transient increase in the rate of lignin depolymerization, which in turn more quickly opened the lignin barrier and temporarily stimulated lignocellulose decomposition by the native cellulolytic soil microflora. The most affected lignin was likely the relatively undegraded lignin recently deposited in the soil. In effect, the recombinant transiently relieved a rate-limiting step in carbon cycling in soil, that is lignin depolymerization. This in turn temporarily made more cellulosic polysaccharides available to the non-ligninolytic, cellulolytic soil microbial flora. This resulted in a short term spike in microbial metabolic activity.

In addition to carbon mineralization effects, this genetically engineered microorganism (GEM) might have other effects on the soil ecosystem. The objective of the just completed multi-investigator project was to determine what those effects, if any, were. Ecological parameters examined included 1) degradation of low molecular weight aromatic and/or

chloroaromatic compounds in the soil, 2) incorporation of organic carbon into soil humus, 3) effects on other biogeochemical cycles including the nitrogen, sulfur, and phosphorus cycles, and, 4) effects on total microbial biomass, specific microbial populations, and the activities of soil microbial enzymes within the soil environment. Results showed that while no long term environmental effects on these parameters apparently resulted from the release of the GEM, certain statistically significant, but transient effects were measured. These findings confirmed the previous observations of Crawford's group, but extended the findings of measurable transient effects to several other components of the soil ecosystem.

METHODS

The soil used was from a single batch of a Palouse silty loam rich in organic matter (3). This soil was air-dried, sieved, and split into equal lots, which were distributed to investigators and then stored at 4°C until used. ^{14}C-lignin and ^{14}C-cellulose labeled lignocelluloses were prepared from poplar and chemically characterized as previously described (4). The wild-type microbial strain used was *Streptomyces lividans* TK23, a prototroph expressing chromosomally encoded resistance to spectinomycin. The recombinant strain was *Streptomyces lividans* TK23.1 which expresses pIJ702.LP encoding a chromosomal lignin peroxidase gene of *Streptomyces viridosporus* T7A, this plasmid also encodes thiostrepton resistance as a selectable marker. Maintenance and characterization of the strains was as described previously (1,2). Enumeration of the *Streptomyces* in soil was monitored via plate counts onto selective media, using procedures similar to those described previously (2,3). Maintenance and expression of pIJ701.LP by the recombinant cells in soil was also monitored as previously described (2,5). CO_2 evolution from sterile and nonsterile control soils and from soils inoculated with the recombinant or wildtype strain was monitored as described previously (2,3). $^{14}CO_2$ evolution and incorporation of ^{14}C into soil humic and fulvic acids was measured as described previously (6,7). In humification experiments, incorporation of ^{14}C-lignocellulose derived ^{14}C into the different fractions of soil organic matter was examined by extracting and analyzing the soil for ^{14}C labeling of the soil fulvic acids, humic acids, and humans (7). Soil microbial biomass (C and ^{14}C-labeled) was quantified by the soil chloroform fumigation technique (8), while total soil carbon (C and ^{14}C) was determined by soil oxidation. Soil inorganic (NH_4-N and NO_3-N) determinations were by the method of Keeney and Nelson (9), soil biomass nitrogen by the soil chloroform fumigation technique (8), and total soil nitrogen by Dumas combustion. Soil enzyme assays included those for acid and alkaline phosphatase, dehydrogenase, β-glucosidase, peroxidase, arylsulfatase, and cellulase. These assays were carried out as described previously (1,10-13). In addition, in some experiments soluble phenolics, pH, and water content of the soils were monitored. In some experiments involving unamended soils or soils

amended with lignocellulose (uninoculated or inoculated with the wildtype or recombinant *Streptomyces* strains), specific culturable, aerobic microbial populations were monitored using plate counts, and included total bacteria, total fungi, cellulose decomposers, chitin utilizing bacteria, total spore-forming bacteria, nitrifying bacteria, and denitrifying bacteria. The media used for these enumerations have been described (3,9,10,13-16). The experiments were carried out in "Master jar" microcosms (17). All experiments were done in 3 or more replicates with values reported as averages ± standard deviations. Additional statistical analyses were also performed on all data, and each experiment was repeated at least two times.

RESULTS

The objective of this research was to evaluate the effects of release of *S. lividans* TK23.1 (expressing pIJ702.LP) on microbiological activities and biogeochemical cycling or carbon and nitrogen in soil. While some reproducible, statistically significant transient effects were indeed observed, among the environmental parameters examined, this GEM appears to have had no detrimental long term (>60-90 days) effects on the soil ecosystem.

The GEM specific enhancement of short term CO_2 evolution rates from soils inoculated with strain TK23.1, as compared to control soils and those inoculated with strain TK23 (2), was confirmed.

For nonsterile soils inoculated with the wildtype or recombinant strain, and supplemented with ^{14}C-lignin labeled lignocellulose, the rate of $^{14}CO_2$ evolution from the soils was not significantly different from the rate observed for nonsterile uninoculated soils over a 30 day periods. Thus, neither the parent or GEM measurably altered the net rate of lignin mineralization. $^{14}CO_2$ evolution rates from soils supplemented with ^{14}C-cellulose labeled lignocellulose were significantly greater for soils inoculated with TK23.1 as compared to TK23; however, the rate for TK23.1 inoculated soils was not significantly different from the uninoculated control soil.

After 30 days, incorporation of ^{14}C into the humus fractions of these same soils was determined. In soils supplemented with ^{14}C-lignin lignocellulose, the amount of ^{14}C-humic acid recovered was greater for soils inoculated with the GEM (TK23.1) than with the parental strain (TK23), and the value was higher for TK23-inoculated soil than for nonsterile, uninoculated soil. However, by 60 days this difference had disappeared, such that the control soil value was greater then the TK23.1 value, which was still greater than the TK23 value. After 30 days, the amount of ^{14}C recovered in fulvic acid fractions was somewhat lower for soils inoculated with TK23 and TK23.1 as compared to the control soil; however, after 60 days the soils inoculated with TK23.1 contained somewhat more ^{14}C-fulvic acids that either the TK23-inoculated or uninoculated soils. For soils supplemented with ^{14}C-cellulose labeled lignocellulose, after 30 days the amount of ^{14}C incorporated into soil humic acids was significantly greater

for TK23.1-inoculated soil as compared to TK23-inoculated soil, and the value for TK23-inoculated soil was greater than for the uninoculated soil. However, after 60 days there were no significant differences between the three soils. After 30 days the level of ^{14}C incorporation into fulvic acids was higher for TK23.1-inoculated soils than for the TK23-inoculated or control soils. Again, however, the values were not significantly different after 60 days.

In the water content, soil pH, soluble soil phenolics, and TK23/Tk23.1 population studies, it was observed that the water content of the soil remained stable throughout the experiments. The presence of lignocellulose in the soil affected soil pH, which rose about 0.5 units over the first 35 days of the experiment before stabilizing. In unamended soil there appeared to be a transient increase in pH due to presence of the GEM. In lignocellulose amended soil, the GEM appeared to significantly increase soil pH relative to controls. Thus, the effects of lignocellulose on the other measured variables could be related to this pH effect. The survival of both TK23 and TK23.1 was similar throughout the study. The numbers of the GEM in unamended soil remained greater than those in lignocellulose amended soil. After 14 days, the numbers of the GEM in lignocellulose amended soil approximated those of the parental strain in both unamended and lignocellulose amended soil. Thus, the presence of lignocellulose did not give the GEM a selective advantage over the parental strain. There was no significant effect of the GEM or parental strain on the total phenolics content of the soil in either amended or unamended soils over the time of the experiment (90 days).

The enzyme studies showed some transitory GEM specific effects. Greater arylsulfatase activity was present in soil amended with lignocellulose as compared to unamended soil, regardless of whether the soils were inoculated or not inoculated. There was a transient (3 day) enhancing effect of the GEM on arylsulfatase activity in both unamended and amended soils. There was an initial increase in acid phosphatase activity in all of the soils, but the activity than leveled off for the remainder of the study. There was no measurable effect of the GEM on acid phosphatase activity in either amended or unamended soils. In general a greater amount alkaline phosphatase activity was detected in soils amended with lignocellulose than in unamended soils, regardless of whether the soil was inoculated or not inoculated. There was typically a small increase in activity which then leveled off for the remainder of the study. Alkaline phosphatase activity was, however, transiently enhanced during the first week of incubation in GEM-inoculated soils amended with lignocellulose. Similarly, soil dehydrogenase was transiently increased in GEM inoculated soils. Soil peroxidase activity was not significantly affected by the GEM, although it is likely that peroxidase was tightly bound to lignin-derived organic matter in the soil and therefore not easily measured by the techniques employed.

In all soils, over time there was typically a slight increase in total

bacterial numbers followed by a decline. By day 91, however, the numbers of culturable bacteria had generally stabilized. There were measurable increases in bacterial numbers, as compared to the other soils, in soils amended with lignocellulose and inoculated with either the GEM (TK23.1) or the parental strain (TK23). However, bacterial numbers in the soil amended with lignocellulose and inoculated with TK23.1, were transiently lower than for those in lignocellulose amended soil inoculated with TK23. When the numbers of added bacteria (TK23 and TK23.1) were subtracted from the numbers of total bacteria, the numbers of total bacteria in soils inoculated with TK23.1 were transiently lower than those in uninoculated, unamended soils or unamended soils inoculated with TK23. As expected, bacterial numbers in soils amended with lignocellulose were generally higher than in unamended soils.

In all soils from time zero there was typically an initial 10-fold increase in culturable fungal propagules, followed by a decline and stabilization over time. Relative to controls, the numbers of fungal propagules were transiently depressed in the presence of the GEM in soils amended with lignocellulose. This may be seen in the result of increased competition form the more active bacterial microflora.

In general, from time zero there was an initial increase in the numbers of spore-forming bacteria, followed by a decline, subsequent recovery, and then stabilization of numbers. In unamended soil inoculated with the GEM, the numbers of spore-formers were significantly greater then those in soil inoculated with the parental strain, but only between days 14 and 35. In soil amended with lignocellulose and inoculated with the GEM, the number of spore-formers were fewer than in amended soil inoculated with the parental TK23 strain, but only on sampling days 14. 35, and 63. By day 63, the numbers of spore-formers were lower in soil amended with lignocellulose than in the unamended soil. The numbers of added bacteria (TK23 or TK23.1) had essentially no measurable effect on the numbers of spore-formers.

As would be expected, there were some general differences in number of cellulose decomposing bacteria in unamended soils versus those amended with lignocellulose. However, in general no differences were seen in the numbers of cellulose-degraders in soil amended with lignocellulose and inoculated with the GEM as compared with those that were uninoculated or inoculated with the parental strain. There was, however, a long term difference in the numbers of cellulose degraders in unamended soils inoculated with the GEM as compared to unamended uninoculated soil or unamended soil inoculated wi the parent. When the total numbers of added bacteria were subtracted from the numbers of cellulose-degraders, the effect due to the GEM in uninoculated soil disappeared, suggesting that the addition of TK23.1 to unamended soil may affect the numbers of cellulose-degrading bacteria in that one treatment.

Again, there were some differences between populations of chitin utilizing

bacterial populations in soil amended with lignocellulose as compared to unamended soil. There was a transient effect of the GEM in unamended soil, but only on one sampling day (day 14). This effect remained statistically valid when the number of added bacteria were subtracted from the numbers of chitin utilizing bacteria.

In general, the numbers of nitrifying and denitrifying bacteria were not significantly affected by introduction of the GEM or parental strain into any of the soils, and no appreciable effects on nitrogen cycling were observed.

Overall, the results show that a few statistically significant effects on the measured environmental parameters were observed during the course of this research. In all cases, the effects tended to be small and transient. These results generally support our hypothesis that the GEM transiently alters the rate of lignin depolymerization in the soil and, thereby, temporarily stimulates the saprophytic soil microflora. The data, particularly from the microbial populations and soil enzyme activity studies, support this conclusion and extend our original observation concerning the CO_2 mineralization effect (2). The transitory nature of the measurable effects, and also the humification study, support the conclusion that these effect are of little, if any, significance to the long term stability and health of this soil ecosystem.

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ANTICIPATED FUTURE RESEARCH

It would be of interest to examine the long term (e.g., a year or more) fate of the GEM (TK23.1) and its plasmid in several different soil types. More importantly, since humification is a slow process, it would be of interest to examine the effects of the GEM on the humification process over much longer periods of time than could be done in the present project. Effects not seen over 30 days might show up over longer period of time. Finally, this project has shown that the procedures employed are sufficiently sensitive to detect effects if they occur. Thus, it would be of value to utilize these procedures to evaluate environmental effects of other GEMs in soil ecosystems.

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MECHANISMS OF EFFECTS OF RECOMBINANT *Streptomyces* ON THE CARBON CYCLE IN SOIL

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INTRODUCTION

In our recently completed EPA cooperative agreement CR-815300-01-05, we carried out research to elucidate the mechanism by which a recombinant *Streptomyces* strain, genetically engineered to overproduce an extracellular lignin peroxidase, transiently enhanced the rate of carbon turnover in soil. The gene encoding the lignin peroxidase was obtained from the chromosome of the lignin-solubilizing actinomycete *Streptomyces viridosporus* T7A and was cloned into *Streptomyces lividans* TK64 in plasmid pIJ702 (1). This enzyme catalyzes the oxidative depolymerization of lignin during lignocellulose degradation and is involved in the ligning solubilization process of *S. viridosporus* (1,2). The need for the present study stemmed from our previous observation that certain genetic variants of *S. viridosporus* temporarily enhanced the rate of carbon turnover in soil, as measured by CO_2 evolution rates from soils inoculated with the variants (3). The enhancement was particularly significant when the variants were released into nonsterile soils amended with lignocellulose. Evidence implicated overproduction of lignin peroxidase by the variants as a possible mechanism for the effect, since they characteristically, but transiently overproduced lignin peroxidase as the result of spontaneous chromosomal gene amplifications. However, because the variations were unstable, the mechanisms of their effect on carbon turnover rates could not be easily studied. The availability of a recombinant lignin peroxidase-overproducing *S. lividans* TK64 strain (1), however, made such a study feasible, and it also raised the question of how such a genetically engineered bacterium might effect the soil carbon cycling if it were released into the environment. The objectives of this past year's reseach were to elucidate the genetic and chemical bases for the carbon mineralization effect, using the recombinant *S. lividans* strains [strain TK64.1 expressing lignin peroxidase gene ALip-P3 in plasmid pIJ702.LP (2)]. TK64.1 was inoculated into soil, and its effects on carbon turnover were monitored and compared to control soils and soils inoculated with the nonrecombinant parental *S. lividans* TK64 strain. In addition to monitoring carbon mineralization effects, we also examined how release of the recombinant strain affected the rate of incorporation of lignocellulosic carbon into soil humus fractions.

METHODS

The parental *S. lividans* TK64 and recombinant *S. lividans* TK64.1 strain expressing lignin peroxidase-encoding plasmid pIJ702.LP have been described previously (1,2). A gene encoding the ALiP-P lignin peroxidase of *S. viridosporus* (2) was cloned on a 4.1 kb fragment of chromosomal DNA into multi-copy vector PIJ702 to construct pIJ702.LP (1).

Peroxidase activity and H_2O_2 concentrations were measured as described by Ramachandra *et al.* (2). Plasmid isolations and characterizations, Southern hybridizations, and other molecular analyses of DNA preparations were performed as described by Wang *et al.* (3,4). Growth of wild-type and recombinant *Streptomyces* strains in soil, *Streptomyces* inoculation procedures, selective enumeration procedures, measurement of carbon mineralization, and the Palous silt loam soil used in the study have been described by Wang *et al.* (3,4). Preparation of ^{14}C-lignin and ^{14}C-cellulose labeled lignocelluloses was as previously (5,6), and incorporated of ^{14}C into soil humic and fulvic acids was monitored by extraction of the acids from soil and analysis by liquid scintillation counting after combustion to CO_2.

RESULTS

When recombinant strain *S. lividans* TK64.1, which expresses a lignin peroxidase gene in plasmid pIJ702.LP (1), was released into a rich organic soil in flask scale laboratory experiments, the rate of CO_2 evolution from the soil was temporarily enhanced (4). The enhancement was shown to be lignin peroxidase specific, and significant only in nonsterile soils still containing an active microbial flora. The increase in carbon mineralization resulted from increased production of lignin peroxidase by the recombinant strain in soil. This resulted in a transitory increase in the rate of lignin depolymerization, which in turn temporarily stimulated lignocellulose degradation by the native soil microflora. The affected lignin was likely the relatively undegraded lignin that had been recently deposited in the soil. In effect, the recombinant strain transiently relieved a rate-limiting step in carbon cycling in soil, that is lignin depolymerization. The increase in lignin depolymerization rate in turn temporarily made more cellulosic polysaccharides available to the celluloytic, non-ligninolytic soil microbial population. This resulted in a temporary spike in the metabolic activity of the microflora. While the effect was transitory, this is the first report of a genetically engineered microorganism (GEM) having a measurable effect on a soil biogeochemical cycle. Studies of the incorporation of carbon into the humus fractions of the soil showed that the GEM-caused enhancement of soil organic carbon mineralization probably did not significantly effect the soil humification process. The rate of incorporation of organic carbon into soil humic and fulvic acids was not significantly altered by introduction of the GEM. This conclusion was based upon results showing that when a rich organic soil inoculated with the recombinant strain was amended with ^{14}C-lignin or ^{14}C-

cellulose labeled lignocellulose, incorporation of ^{14}C into soil humic and fulvic acid fractions over a 60 day incubation period was not significantly different as compared to control soils or those inoculated with the non-recombinant parental strain. Thus, the overall effect of this GEM on the soil ecosystem, at the level of carbon turnover, appears to be measurable, but short term. The observed temporary spike in carbon mineralization probably has little if any long term effect on soil humification or fertility.

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ANTICIPATED FUTURE RESEARCH

It is not currently anticipated that additional research will be carried out on this project. It would be of interest to study the long term (e.g., a year) fate of the plasmid bearing recombinant *S. lividans* TK64.1 in soil, particularly with regard to maintenance and expression of the lignin peroxidase-encoding plasmid. It would also be valuable to extend the humification effects study out to a year or more. Humification is a relatively slow process, and statistically significant effects of the GEM might not be observed over the short periods of time covered in the present research.

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LABORATORY OBSERVATIONS ON THE INHIBITION OF SOIL FUNGI BY MPCAs OF THE GENUS *Pseudomonas*

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INTRODUCTION

Biotechnological methods have stimulated interest in the use of microorganisms to ease the environmental impact posed by anthropogenic toxic chemicals. One aspect of the studies conducted in our laboratory over the last several years, involved an examination of the genetics, expression, and metabolic controls of 2,4-D and phenoxyacetic acid degradation in fluorescent pseudomonads. In one xenobiotic model system, the use of a deregulated operon (continuously expresses the ability to biodegrade) has been proposed to stimulate the bioremediation of phenoxyacetic acid and 2,4-Dichlorophenoxyacetic acid (2,4-D). However, before these kinds of modified strains (genetically engineered microorganisms) can be field tested, it has been proposed that a series of test methodologies be developed to evaluate the ecological consequences of their releases. Such methodologies need to be accurate, reliable, economical and easily applicable for appropriate risk assessment studies.

In a series of ecological studies designed to evaluate the consequences of 2,4-D biodegradation on microbial ecosystems, we demonstrated an unexpected accumulation of a metabolic intermediate (2,4-dichlorophenol) that reduced cell numbers of indigenous soil fungi. Similarly, in control experiments where 2,4-D amendments were not made, we found evidence that the fluorescent pseudomonad, *Pseudomonas putida* PPO301 also caused a decline in the number of fungal propagules present in nonsterile soil. Although fluorescent pseudomonads are important (agronomically) in the control of fungal plant diseases such as damping off of cotton and take all of wheat, very few attempts have been noted to study the effects of these bacteria on beneficial fungi.

The present study represents a follow up of our initial observations and involves a more general assessment of the interactions between bacteria and a variety of fungi, including beneficial fungi of economic importance. The initial series of fungal inhibition assay experiments have been conducted using agar plates and sterile soil. In the latter case, bacteria-fungi antagonism in the sterile soil was assessed by replica plating onto agar plates.

METHODS

Bacterial Cultures and Media

Cultures of *P. putida* PPO301, with and without plasmids, *P. fluorescens* Pf-5 and VT21(TR21), and *P.*

aeruginosa PA01C, with and without plasmids, were used in this study. Luria-Bertani (LB) agar was used for plate counts. Liquid cultures were grown in Brain Heart Infusion (BHI) broth. Broth cultures were propagated on a rotary shaker at 200 rpm. Appropriate antibiotics were added when selective media were needed. Unless otherwise stated, cultures were incubated at 30°C.

Fungal Cultures and Media

Thirty nine fungal isolates representing free-living soil forms, mycorrhizae, microbial pest control agents, and biodegraders of xenobiotics were used in this study. Except for the ectomycorrhizae where MMN slants were used, all fungal cultures were maintained on potato-dextrose agar slants at 4°C. Fungal liquid cultures were grown in MMN broth on a rotary shaker (200 rpm) at 25-30°C.

Fungal Inhibition Assays

In vitro bacteria-fungus plate assays were performed on agar plates consisting of media of different compositions and included potato dextrose agar (PDA) (Difco Laboratories, Detroit, Mich.), MMN, medium 523 and Kanner's medium. Fluorescent pseudomonads were patched along the perimeter of the agar plates and incubated overnight at 30°C. Ten μl of fungal suspension or a plug from the leading edge of a 5 day old fungal culture, grown on MMN broth or PDA, was placed at the center of the agar plate previously patched with the test bacteria. The plates were then incubated at 25-30°C for 5 to 14 days. For mycorrhizal fungi, longer incubation time (30-40 days) was necessary before scoring for inhibition could be made. The degree of fungal inhibition by fluorescent pseudomonads was expressed relative to a control (fungus grown in the absence of fluorescent pseudomonads) by measuring the diameter of fungal growth over time.

Assay of Fungal Inhibition in Sterile Soil

Sixty five grams of Willamette River bottom soil was placed in a glass petri dish and autoclaved three times (by tyndallization) each for 2 hours at 121 C and 15 psi. The sterile soil was inoculated with 20 ml of a washed cell suspension (24-48 old culture) such that the final bacterial density was 10^8 CFU/gram of sterile soil. Controls were not inoculated with the test bacteria. A free-living fungal (S4) inoculum was prepared by culturing in MMN broth and incubating for 5 days at 30°C and 200 rpm on a rotary shaker. After the bacteria were inoculated into sterile soil in petri dishes and were incubated for 60-75 hours at 30°C, an aliquot of 0.1 ml of 8.4×10^3 CFU/ml of the fungus S4 was centrally inoculated per petri dish. Each treatment was replicated at least in triplicate. Fungal spread or growth was monitored by replica plating onto Martins medium agar plates. Bacterial growth in the soil was assessed by serial dilution plating on selective LB agar plates.

RESULTS AND DISCUSSION

All 39 isolates of fungi representing diverse origins and properties were evaluated for potential sensitivity (in plate assays) to eight strains of Gram negative bacteria. A scoring system of minus (-) to four plus (4+) range was used to denote the extent of inhibition. A complete inhibition was assigned a four plus where as insensitivity was denoted with a minus sign.

A large variation was noted in the extent of fungal inhibition induced by the various bacterial strains and different media. However, on medium 523, virtually all bacterial strains exhibited maximum inhibition (4+) of fungal growth, i.e., no fungal growth occurred over the 7-14 days of incubation. The antagonism was due to diffusible substances expressed in both the presence and absence of added ferric chloride.

Susceptibility of ectomycorrhizae to the bacteria was evaluated on medium MMN. Great differences were noted in the patterns of inhibition. In general, Pf-5 and PA013 demonstrated the greatest levels of inhibitory effects. *P. fluorescens* VT21(TR21) was the least inhibitory. This diversity in the extent of inhibitory activities suggests that the different bacterial strains produce different type(s) of inhibitory substances.

Antifungal activity was detected in sterile soil inoculated with the free-living soil fungus (S4) and various strains of *P. fluorescens* and *P. aeruginosa*. Several significant features were derived from these experiments. *P. aeruginosa* strongly inhibited the growth of the fungus in sterile soil medium. Growth inhibition was noted over the 25 day observation period. Maximum growth of the fungus occurred in the control soil (without the bacterium) and reached the perimeter of the plate within 8 days of incubation. PA013 (pRO103) exhibited significantly less fungal inhibition implying the plasmid somehow interfered with production of the inhibitory substance(s). Numbers of the Pf-5 inoculum declined 10-fold by day 15. This decline in bacterial numbers coincided with slightly greater spread of the S4 fungus. On the other hand, S4 growth in the presence of VT21 (TR21) was only slowed and by day 21 covered as much of the soil as the uninoculated control following a 50-fold reduction of bacterial numbers in the soil.

FUTURE WORK

Studies are underway to develop assays using nonsterile soil and plants to evaluate the interaction of pseudomonads with indigenous soil microflora, including fungi. Studies will be conducted to ascertain the type(s) of chemicals produced in the rhizosphere environment that alter the composition of the ecosystem. Fungus-bacterium antagonism and its autecological significance will be evaluated using fungus and fluorescent pseudomonads both marked with stably expressed antibiotic labels.

PUBLICATIONS

This study was initiated this fiscal year and there are no publications at this time.

EFFECTS OF ENVIRONMENTAL FACTORS ON BACTERIAL CONJUGATION

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INTRODUCTION

The intentional release of bacteria to foreign habitats is common. The agricultural use of bacteria, *e.g.*, *Rhizobia* to enhance nitrogen fixation, has resulted in the introduction of high concentrations of reactor grown bacteria directly into the natural environment. The enhancement of bioremediation capability of indigenous bacteria by augmentation with bacteria containing specific gene sequences has been proposed. Pathogens contained within the effluents from sewage treatment facilities are used for soil emendation. There are many proposals for, and have been some tests of, the release of bacteria modified by genetic engineering techniques. In addition to scheduled releases of bacteria, many possibilities exist for the unscheduled release resulting from breaches in industrial reactor vessels or transport from release sites by foraging animals.

Many bacteria from scheduled or unscheduled releases will end up in either agricultural or industrial wastewater. Wastewater also is a likely place for genetic interactions to take place because it contains a high bacterial titer, and, during the treatment process, the bacteria will be exposed to different environmental conditions, including aerobic, anaerobic, etc., which may enhance or curtail gene transfer. Conjugation may occur using wastewater-resident conjugative plasmids; it is also possible for non-conjugative plasmids to participate in conjugal transfer by the mobilization process.

Conjugation requires the presence of specific genes, e.g., the *tra* genes of conjugative plasmids and the *mob* genes of mobilizable non-conjugative plasmids. In addition to the presence of these genes needed for conjugation or mobilization, bacteria must be able to produce functioning gene products in their environment. In order to predict the possible escape of gene sequences from released bacteria to indigenous populations it is necessary to know how specific components of an environment affect the transcriptional (and translational) control mechanisms for the production of these products. We report here the effect of different factors on transcription of *tra* and *mob* as might occur in wastewater.

METHODS

Bacteria and Plasmids

E. coli chi1784 that contains the plasmid R100-1 was used to determine inhibition of conjugation by exogenous compounds, with *E. coli* chi1997, which is plasmidless, serving as the recipient. *E. coli* HB101 was used as host for plasmids F, R100-1, RP4, R6K, N3, RA1, R144, R27, and colEI-amp.

E. coli DH5alpha was used as host of plasmid pTK31. This reporter plasmid contained *tra*P promoter sequences of plasmid pFL114 fused to the *lac*Z of plasmid pMC1403. This construct enabled us to monitor the effect on the transcription through the *tra*P promotor of chemicals coincubated with the bacteria.

The bacteria were grown on primarily on L-broth supplemented with appropriate antibiotics. Some conjugation experiments were performed in sterile synthetic wastewater. Compounds tested for their effect on conjugation or beta-galactosidase activity were added to actively growing cultures. Beta-galactosidase activity was monitored by method of Miller (1972) in which cells were permeabilized, then induced with *o*-nitrophenol-beta-D-galactoside (ONPG). The developing yellow color was monitored at A_{420}.

Transcription of the *mob* Genes

In order to determine if the mobilization specific *mob* genes were being transcribed, total RNA was extracted from the cells and probed by slot blot with oligonucleotides specific for either the *mob*2 or the *mob*3 (and *mob*5) gene sequences. Comparisons were made of extracts from bacteria containing either a conjugative plasmid (F, R100-1, RP4, R6K, N3, R144, RA1 or R27), the colEI-amp plasmid, or both of these plasmids. Prior to binding to the membrane, extracts treated with either DNase or RNase to destroy one nucleic acid or the other. The absence of DNA or RNA was confirmed by gel electrophoresis.

RESULTS AND DISCUSSION

Effect of Environmental Factors on Conjugation

The chemical composition of wastewater can have significant impact on the probability of gene transfer by conjugation. Significant inhibition (>3 logs) was exhibited in the presence of millimolar concentrations of Zn^{2+}, Fe^{3+}, and SDS, which are relatively common (especially of Fe^{3+}, which is often added to enhance precipitation). It has been proposed that the effect of zinc is directly on the receptor of the pilus while SDS affects the structural integrity of the pilus. Significant inhibition, but at much higher concentrations, was exhibited by Ca^{2+}, Mg^{2+}, Na^{+} and K^{+}. No inhibition was observed with Triton X-100.

To see if any of these inhibitors specifically affected the transcription of *tra*P, the major regulatory gene of the *tra* operon of the derepressed IncFII plasmid R100-1, a fusion was made of the promotor of *tra*P and the *lac*Z beta-galactosidase reporter gene. In this way the effect of external compounds on the transcription of *tra*P was monitored. Comparisons were made between the effect of compounds on *lac*Z genes located on this fusion plasmid with the *lac*Z located other plasmids or on the chromosome (using ONPG as an inducer). All constructs showed similar inhibition of beta-galactosidase transcription as a function of temperature and Zn^{2+} concentration. Fe^{3+}, however, showed decreased induction of beta-galactosidase from the *tra*P fusion, but an increase in the amount of enzyme

induced when *lac*Z was under its natural promoter. Therefore, at least part of the decrease in conjugation frequency which was observed when ferric ions were present was probably due to the inhibition of *tra*P transcription.

Induction of *mob* Genes

Some of the dissemination of genes in natural environments is due to the mobilization of non-conjugative plasmids. It is possible that some environmental elements will specifically affect mobilization of these non-conjugative plasmids, but not the functions on conjugative plasmids. We used ColEI, a common non-conjugative plasmid, to analyze these interactions. Because this naturally-occurring plasmid had no easily selectable gene, we used a modified ColEI that contains the beta-lactamase gene of plasmid pBR322. This ColEI-amp plasmid is mobilizable because it contains *mob* genes as well as a *bom* site, the origin of transfer during transfer to a recipient cell. In particular, ColEI has the *mob*2, 3 and 5 genes. The *mob*5 gene is wholly contained within the *mob*3 gene sequences. Under conditions in which ColEI is the sole plasmid in a strain grown in monoculture, there was only trace synthesis of RNA from the *mob* sequences. When a conjugative plasmid, however, was introduced into this cell containing the *mob* sequences, transcription initiated. This transcription depended solely on the presence of the conjugative plasmid and was not dependent on the presence of a potential recipient cell. The induction was found to occur with several plasmids from different incompatibility groups (F [IncF], N3 [IncN], RA1 [IncA/C], RP4 [IncP]I, R6K [IncX], R27 [IncH], R100-1 [IncFII], R144 [Inclalpha]). The induction by such a variety of conjugative plasmids suggests that there is a common, and perhaps universal, product of the conjugative plasmids which induces the synthesis of the *mob* gene products.

FUTURE WORK

Effect of Environmental Factors on *mob* Induction

In a manner similar to the effect of Fe^{3+} on transcription at the *tra*P promoter, it is possible that various environmental components would have an effect on induction of *mob*. We will analyze the effect of various compounds common to wastewater on the mobilization of ColEI and on *mob* transcription. This would aid in predicting probability of conjugative plasmids mobilizing non-conjugative plasmids.

Isolation and Characterization of *mob* Effector

Although eight conjugative plasmids have shown the capability of inducing *mob* function, the universality of this induction by plasmids from different incompatibility groups has yet to be completely determined. We will analyze frequency of colEI mobilization for conjugative plasmids studied and correlate this mobilization with the inducton of *mob* gene transcription.

The induction of *mob* genes by plasmids from several different

incompatibility groups suggest that there may be a common effector molecule. It would be of great interest, therefore, to know whether there is similarity in sequence between all such inducing plasmids. A common sequence for such a regulator gene would be of interest to (a) quantitatively and specifically monitor by hybridization analysis the presence of the total population of conjugative plasmids without knowing which plasmids were present and (b) assess the amount of conjugative activity which is going on in wastewater and other natural environments by the amount of active product (RNA or protein) which cells are synthesizing *in situ*.

Evaluation of Metabolic State Necessary for Conjugation

The synthesis of proteins necessary for conjugation may be a function of the metabolic state of the cell. It is unknown whether all conjugation occurs in the initial period following release. It may be possible that following 24 hours in wastewater no more conjugation will occur. In this case the risk of release is immediate, but has a short half life. Alternatively the intention of releasing plasmid containing bacteria with the possibility of the gene transfer into the native population would be limited.

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TEST PROCEDURES FOR ASSESSING HAZARDS OF MICROBIAL PEST CONTROL AGENTS TO FRESHWATER FISH

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INTRODUCTION

Widespread concern over the environmental safety of chemical pesticides has stimulated interest in the development and use of microbial pest control agents (MPCAs). Selected because of their host specificity, MPCAs (bacteria, fungi, viruses, and protozoa) are generally regarded as safer alternatives to some environmentally-hazardouschemicalpesticides. However, as living organisms, MPCAs pose concerns not shared by chemicals, ie., their survival, multiplication, and dissemination in the environment and the potential to cause infection or disease in nontarget species.

As mandated under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), EPA requires testing as described in Subdivision M of the Pesticide Assessment Guidelines to address potential ecological and human health effects prior to the registration of a MPCA. These requirements include determining the safety of MPCAs to nontarget aquatic species, including fish.

While test protocols are available for assessing effects of microbial agents on fish, basic information is needed on the factors influencing the extent and kinds of interactions between microbes and fish to better understand and predict potential effects and environmental exposure of nontarget fish populations. The studies presented here investigated the interactions between fish and the registered MPCA, *Bacillus thuringiensis subsp. israelensis (Bti)* under laboratory conditions.

METHODS

Single species exposures were conducted with two commercial *Bti* spore-crystal formulations, Vectobac-G (Abbott Laboratories) and Mosquito Attack (Reuter Laboratories). Exposure concentrations used ranged from 10^4 to 10^6 CFU/ml, based on spore counts, and were at and above recommended field application rates.

Four species of freshwater fish were used: fathead minnows, *Pimephales promelas*, bluegill sunfish, *Lepomis macrochirus*, and two salmonids, rainbow trout, *Oncorhynchus mykiss*, and brook trout, *Salvelinus fontinalis*.

Bti formulation was added to the water and fish were exposed under static or static renewal conditions for periods of from one hour to 30 days. Following exposure fish were removed, rinsed in clean water, and sampled to determine the extent of *Bti* accumulation or transferrred to clean water to study clearance of *Bti*.

Fish samples, either whole body (WB) or dissected tissues, were homogenized, generally pasteurized (65°C for 20 min.) to reduce background vegetative cells, and plated into tryptic soy agar (TSA). Typical *Bti* colonies were counted following a 24 hour incubation at 32°C.

To determine if germination and colonization occurred within the fish, homogenates were subdivided and plated with and without prior pasteurization onto a selective, differential media, PEMBAC (developed by Dr. Allan Yousten, VA Polytechnic Institute and State Univ., Blacksburg, VA). Comparison of total *Bti* (non-pasteurized samples) and *Bti* spore (pasteurized) counts was used to determine if germination occurred.

RESULTS AND DISCUSSION

Concentration-dependent dissolved oxygen depletion with both formulations, believed to result from stimulation of indigenous aquatic bacteria by nutrients in the formulations, resulted in mortality of all fish within 24 hours at and above water concentrations of $2\text{-}3 \times 10^6$ CFU/ml; partial mortality of trout occurred at 5×10^5 CFU/ml. No direct adverse effects of *Bti* on fish were observed.

Rapid accumulation of *Bti* spores by all fish tested was measured following addition of either formulation to their water. No differences in responses were detected to the two *Bti* formulations. Plate counts on dissected guts were nearly identical to WB counts in all species showing that ingestion was the major exposure route. Skin swabs and gills had counts orders of magnitude lower than gut samples. For example, fathead minnows whole body and gut samples contained 10^5 to 10^7 CFU/fish while gill samples were 10^2 to 10^3 CFU/gill.

Differences in relative accumulation of *Bti* spores by species was related to feeding habits and food size preferences. The omnivorous fathead minnows actively ingested the small formulation particles resulting in WB counts that were two to four orders of magnitude higher than counts in the bluegill and trout.

Upon transfer to clean water, rapid elimination of *Bti* spores occurred through fecal elimination. Maximum loss occurred within the first 24 hours after transfer; near complete elimination occurred in 4 to 10 days if reingestion did not occur.

A decrease in WB spore counts with increasing exposure time and the presence of spores in feces for over a week after spore counts on tissue homogenates were negative, led to studies to investigate germination within the fish. Comparisons of total *Bti* counts and spore counts of subsamples of tissue homogenates using PEMBAC agar demonstrated that germination of spores occurred within the fish gut. Attempts to identify vegetative cells within these samples were not successful. It may be that even though spores germinated (as shown by loss of heat-resistance), they did not continue through outgrowth to form vegetative cells but formed spores in feces via the process of microcycle sporulation.

No evidence of increases in total counts with time were shown suggesting that colonization of the fish guts did

not occur. Results of these studies showing rapid accumulation of spores directly from the water and short-term retention of *Bti* suggests that fish may have signficant infuence of the persistance and dissemination of *Bti*, and possibly other microbes, within an aquatic ecoysystem.

FUTURE WORK

We are planning additional laboratory studies on fish-MPCA interactions, including work with fungus (possibly *Lagenidiumgiganteum*) and other bacterial species, including non-sporeformers (possibly with several species of *Pseudomonas*). Plans are underway to evaluate additional endpoints (histological and physiological/immunological) for our effects testing procedures. Studies comparing laboratory-derived data with that from small scale field studies will also be conducted in conjunction with other members of ERL-Duluth's research program.

In addition, studies will be begun to develop procedures to study the influence of stressors of environmental and physiological origin on the susceptibility of fish to microbial exposure. As part of these procedures, positive controls for infectivity/ pathogenicity endpoints will be developed by including exposures to known fish pathogens.

PUBLICATIONS

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INTERACTIONS OF NON-TARGET INVERTEBRATES WITH *Bacillus thuringiensis var. israelensis* IN NATURAL PONDS

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INTRODUCTION

A goal of the MPCA research program at ERL-D is to improve the confidence of predicting effects in natural systems using laboratory derived data. Our research has three projects. Each of these is represented in this section. One project is to develop and evaluate single species test systems. Research has been conducted with fish and invertebrates exposed to either fungi or bacteria spore-based microbial pest control agents (MPCAs). The second project is to develop and evaluate multiple species/microcosm tests. The third project is to conduct fate and effect studies in natural systems treated with the MPCA. The natural system study is the topic of this presentation.

Few studies exist which are specifically designed to evaluate non-lethal interactions of non-target invertebrates with MPCAs in natural aquatic systems. Most studies were designed to measure only effects on either target or non-target animals. We propose to develop general information on how an added microorganism interacts with both the target and non-target animals. The objective of this project was to determine, in temporary pools treated to control mosquitoes, whether or not non-target animals accumulated and retained the spores of *Bti*.

METHODS

Site and Application

The study was conducted in three pools located in a wooded area near Duluth, MN. Two pools were located in an open grassy area, while the third pool was more enclosed by trees. The area contains many small depressions (2-10 m in diameter) that fill with snow melt and rain in the spring and usually remain wet for up to 2 months. During the summer the pools were occasionally filled with run-off from rain. All pools contained emergent cattails and all were heavy mosquito producers.

Three pools were divided with Scrimweave, a rip-stop woven polyolefin material. Wooden walkways were constructed to provide access to inner pool areas to enable sampling without disturbing the pool sediments.

One side of each pond was treated with Vectobac, a formulation consisting of corncob particles covered with

Bacillus thuringiensis var israelensis (Bti). The amount added to each pond was specifically calculated for each site with a target of 10^4 *Bti* spores per ml of pond water. The formulation is designed to disperse the agent throughout the water. The particles float for a short time before sinking.

Sampling

Samples of water and invertebrates were collected from both sections of each pool. The aquatic animal samples were collected by sweeping the water with a fine mesh net and transferring the animals to a sterile glass jar to be transferred to the laboratory for analysis. Emerging insects were captured and transferred to the laboratory in sterile glass jars.

At the laboratory, invertebrates from the aquatic samples were removed from the jar and separate species were placed in sterile glass test-tubes. The animals were washed to remove any loosely held *Bti* by removing the pool water with a pipet, then rinsed with sterile water. Each sample was rinsed twice, after the water had been removed. A sample may consist of a single large individual, or for smaller animals such as daphnids, a pool of up to 10 animals. After rinsing twice, all samples were frozen in 5 ml of sterile water. In all cases, the values were adjusted to reflect the number of *Bti* spores (as Colony Forming Units, CFU) per individual.

Each sample to be processed was thawed and poured into a sterile plastic bag. The sample tube was rinsed with 5 ml of sterile water which was added to the bag and the sample was blended in a Stomacher™. The sample was removed from the bag with a sterile pipet, heat shocked, cooled and pour-plated at dilutions of 10^{-2} and 10^{-4} with Tryptic Soy Agar.

The *Bti* content of emerged insects was measured after returning the insects to the laboratory. Individuals are placed in a Stomacher bag, frozen, thawed and blended in sterile water with the Stomacher. The blended sample is heat-shocked and pour plated on Tryptic Soy Agar.

RESULTS AND DISCUSSION

The concentration of *Bti* in samples of water, and in insects, zooplankton, snails and emergent insects were measured. Six taxa of insects, 3 taxa of zooplankton and 1 taxon of snails were collected with sufficient frequency to provide data throughout the summer. Four hundred and forty-nine samples were processed. Of that total, 104 samples were adult insects. Accumulation differed for each taxa and the extent of the accumulation might have been affected by pool characteristics.

Insects

Uptake of *Bti* is rapid in insects. Insects sampled soon after the application showed the highest concentrations. Within several days, many of the insect taxa had lost most of their accumulated *Bti*. However, there were some samples that measured uncharacteristically higher than samples taken before or after that specific sample. The insects collected from the pools were predaceous, therefore ingestion of prey that

contained *Bti* might account for the higher values.

Zooplankton

Zooplankton also rapidly accumulated *Bti*. The highest concentration in any of the animal samples was found in *Lynceus*, (*Conchostraca*: clam shrimp) which rapidly accumulated up to 30,000 CFU/ animal. The daphnids in pool 1 contained only a small amount of *Bti* when the four-hour sample was taken but by 24 hours the average count was about 2000 spores per animal. The amount of *Bti* in the daphnids decreased through the next two sampling periods until at day 7 the content was about 300 spores per animal. At 14 days post-treatment the count had decreased to about 100 spores per animal. The pool dried up before the next scheduled sampling date.

Pool 3 was deeper and larger than pool 1 and samples could be taken throughout the season. The rate and extent of uptake by daphnids in this pool was much different than was measured in pool 1. The accumulation did not show a peak but remained flat throughout the monitoring period. The content never did exceed 300 - 400 spores per animal in this pool.

The differences in uptake between daphnids in each pool may be due to pool structure. Pool 1 was shallow, less than 1 m in depth, while pool 3 was deeper and contained about 3-times as much water volume. In both pools, although the *Bti* was measured in the water it ultimately accumulated on the surface of the sediment. In the shallower pool the animals would have been exposed both in the water and by contact with the sediment. In the deeper pool, those daphnids in the water column would have less contact with the higher concentrations associated with the sediment. The consistency in values after 7 days may indicate a minimum value for zooplankton which accumulated *Bti*.

Lynceus and other zooplankton are detritus or filter feeders. These forms would easily collect *Bti* either from the water or from feeding on the surface of the sediment. Within 6 to 8 days most zooplankton had lost over 80% of the accumulated *Bti*. A low concentration of *Bti* was found in these animals the remainder of the season.

The last of the aquatic forms analyzed in this study were *Planoribidae* snails. The concentration of *Bti* found in these animals did not increase rapidly soon after the application and did not decrease as was demonstrated in other animals. This may be related to feeding practices or contact with sediment surfaces. If feeding was on or near a cob particle, high *Bti* concentrations would be expected.

Adult Insect

Adult insects were also collected and analyzed. Early in the season, the samples were largely chironomids and mosquitoes. Later in the season, most adults were odonates, either dragonflies or damselflies. A few caddisflies were also collected and analyzed. Only one chironomid sample contained *Bti* and, as was expected, none of the mosquitoes contained *Bti*. *Bti* was found in a few damselfly adults but the greatest concentrations were found in

dragonflies. Dragonfly content ranged from less than 100 to more than 1000 CFU per individual. Most of the samples contained between 100 and 500 CFU per individual. The highest concentrations were found in dragonfly exuviae which were up to 10 times higher than in the emergent adult.

The adult insect collections show that the *Bti* spore could move from the site of application either in or on the bodies of some emerging insects. The data also show that the amount carried by each insect is highly variable and that not all emerging insects contain the spore. Odonates may contain *Bti* because of their predatory feeding habits. Direct ingestion of particles may also result in *Bti* accumulation. A study this year will begin to evaluate some of these issues.

FUTURE

An integral part of the risk assessment of microbial pest control agents must be to evaluate the predictive value of laboratory data. This data begins to describe how invertebrates can affect distribution of an MPCA in natural systems. This year's uptake and distribution research will include another application to the ponds. Special emphasis will be placed on the movement of the spore out of the system by emerging insects. Long-term plans are to use the divided pond method to evaluate other MPCAs and to understand how the invertebrate and vertebrate populations affect the MPCA survival and distribution.

PUBLICATIONS

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FIELD VALIDATION OF LABORATORY MICROCOSMS USING *Bacillus thuringiensis var. israelensis*

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INTRODUCTION

The potential for negative environmental effects resulting from the release of genetically engineered organisms (GEMs) is a source of concern for both regulators and the public. Unfortunately, it is not yet possible to predict the survival or ecological effects of an introduced microorganism released into the environment. Single species laboratory exposures can measure direct toxicity or pathogenicity of a new microorganism toward a given species, but they provide little information on the critical questions of survival and ecological effects. More complex tests are needed to predict effects at the community and ecosystem level.

Microcosms, contained model ecosystems, should be important tools for answering these ecological concerns. A variety of terrestrial and aquatic microcosms have been used in chemical testing but their use for evaluating GEMs has been limited. This reflects a shortage of validated protocols. Although many microcosm procedures have been proposed by various investigators, lack of information about sensitivity, repeatability, and similarity to the "real world" has precluded their widespread use. Indeed, none of these procedures should be used until we can demonstrate that (1) they are sensitive enough to detect effects, (2) they give the same answer twice, and (3) they accurately mirror events in natural systems.

We have developed two aquatic microcosm protocols of differing complexity. Both have performed well in lab tests with *Bacillus thuringiensis var. israelensis (Bti)*. We are now in the process of duplicating lab tests in the field. Preliminary analyses of field data are encouraging. Although the two microcosms showed differences in their ability to model the natural ponds (calibration), both correctly predicted that *Bti* would survive for months, would kill target organisms (mosquito larvae) but would cause little ecological impact.

METHODS

Microcosms

MFC microcosms: Our original microcosms were modifications of systems originally developed for chemical testing by John Leffler. After extensive testing of these "mixed flask culture" (MFC) microcosms we developed a protocol for evaluating

survival and ecological effects of microorganisms added to aquatic communities. These microcosms are small (1-Liter), easily maintained systems that can support a variety of bacteria, protozoa, algae, zooplankton and insect species. The biotic community in these systems is a species assemblage collected from the wild and allowed to co-adapt in the laboratory.

Core microcosms: While the MFC protocol performed adequately in tests with *Bti*, it was not a close surrogate model of a natural pond. Among other differences it lacks a complex organic sediment, is highly eutrophic and wholly aerobic.

As an alternative, we constructed new microcosms from intact sediment cores. Cores were collected from a dry temporary pond known to contain large populations of zooplankton, insects, and molluscs. We anticipated that resting stages of most of these organisms would be present in the dry cores. Cores 75mm in diameter and 40 mm deep were removed with a PVC corer and transferred to wide-mouthed glass canning jars (Ball Corp.). The rehydrated jars were held in a Percivaltm Growth chamber at 20°C on a 12/12 hour - light/dark cycle.

Field Sites

Field tests were conducted at a remote location owned by the Duluth, MN International Airport. Three temporary ponds (vernal pools) were used in the field tests. These three were selected on the basis of their similar water chemistries and populations of zooplankton, protozoans, snails, and insects (including mosquitos). Each pond was subdivided with a 1-M high barrier constructed of Scrimweave™, a rip-stop woven polyolefin material. Wooden walkways were constructed over each pond to provide better access and permit sampling without disturbing the sediments of the pond. One side of each divided pond was treated with Vectobactm (corn cob grits treated with *Bti*) to yield a water concentration of approximately 10^4 *Bti* spores per ml. This material was broadcast across the pond with a spreader constructed from a one-gallon plastic bottle.

Test Endpoints

The same test endpoints were used in both lab and field tests.

Fate of *Bti*

Survival of *Bti* was monitored daily for the first two weeks after application, and weekly thereafter. *Bti* density was determined in both water and sediment by heat-shocking 65°C and plating appropriate dilutions on Tryptic Soy Agar (TSA). Both total colonies and typical *Bti* colonies were counted. Plates that had suspicious *Bti* colonies were bioassayed after the plates were exposed at 25°C for three days to allow germination of spores.

In addition to water and sediment samples, animals were periodically collected and analyzed for *Bti* content. For large organisms (e.g. tadpoles, insects, snails) only one individual was used per sample. Smaller organisms (*e.g.*, zooplankters) were analyzed in groups of five, so that *Bti* concen-

trations would be sufficient for detection. These groups were washed by suspending them in distilled water, shaking vigorously and filtering through a sterile membrane filter. Tadpoles and snails were then homogenized in 5 ml of sterile water using a Tissumizer (Tekmar Corp., Cincinnati, OH). Other organisms were ground in 5ml of water using a Ten Broek manual tissue grinder. All ground samples were then pasteurized for 20 minutes at 65°C. Cooled samples were then diluted, on TSA agar and incubated overnight at 30°C.

The duration of *Bti* toxicity to mosquito larvae was determined with daily bioassays on water samples collected from microcosms and ponds. Bioassays were done in the lab in a series of small glass vials containing 5 ml sample and 5 *Aedes atropalpus* larvae. The bioassays were read at 18 hours and held for another 24 hours to observe any delayed killing activity. Bioassays for each pond were run a week prior to treatment and daily until the samples were no longer toxic.

Ecological Effects

A suite of physical, chemical and biological measurements were made both to characterize the systems and to detect effects. These are grouped into functional (related to processes within an ecosystem) and structural (populations) variables. Functional variables included the following measurements:

Carbon Cycling
- Dissolved Organic Carbon (DOC)
- Total Organic Carbon (TOC)

Nitrogen Cycling
- Ammonia (NH3)
- Nitrate + Nitrite
- Total Nitrogen

Phosphorus Cycling
- Ortho-Phosphate
- Total Phosphate

Primary Production
- Diel Oxygen Gain

Community Respiration
- Diel Oxygen Loss

Structural variables included measurements of populations of bacteria, protozoa, rotifers, algae, cladocerans, copepods, ostracods, and macroinvertebrates. Temperature and pH were also measured routinely.

RESULTS AND DISCUSSION

Calibration

MFC vs Core Microcosms: The core microcosms developed into fully functioning, biologically diverse systems. They showed several differences from typical MFC microcosms. For comparative purposes we contrasted events in the developing cores to events in a typical MFC microcosm experiment. The MFC nutrient data are from experiments run at ERL-D by Frank Stay.

Communities were slower to develop for cores than in MFC microcosms, probably due to the lower initial nutrient levels. The MFC protocol used a nutrient medium to "jump-start" the systems, while in the cores, nutrients were initially tied up in organic matter

and gradually released by decomposition processes. Nitrates were reduced to near zero in MFC systems within four weeks, but phosphates remained high. Primary production was initially very high in MFC systems and decreased over time, while primary production in the cores continued to increase over time. In response to the initial algal bloom in MFC systems a large zooplankton community developed but later crashed. The zooplankton community developed much more slowly in core microcosms. As organisms died off there was an increase in ammonia in MFC systems that did not occur in the cores. Finally, the pH of the two systems was quite different, ranging between 8 and 9 in MFC systems and between 5 and 6 in core systems.

Laboratory vs. Field: Temporary pools are biologically and physically more variable habitats than laboratory microcosms. Species composition, seasonal changes and recurring dry periods are some obvious differences. The water levels in these pools are often low and are sensitive to daily and diel temperature changes. At all sites, daily low water temperatures were within a few degrees of 10°C while high temperatures ranged from 15°C to 32°C depending on the air temperature. This is in contrast to laboratory microcosms where water level is carefully controlled and water temperature is maintained at 20°C.

Despite these fluctuations, pH in the pools showed little day-to-day change. Since the diel change was so small we measured only morning pH in 1990, and found a mean value of 6.4. This compares with mean pH values of 8.75 and 9.6 for morning and afternoon, respectively, in MFC microcosms. Core microcosms behave much more like the ponds and showed little difference between morning and evening.

Core microcosms were much closer surrogates of natural ponds than MFC systems. Nutrient levels, primary production, and community respiration, and the diversity of invertebrate biota in core systems were close to those seen in natural ponds. Although the temporary pond fauna were far more diverse than those of the microcosm communities, the major taxa were similar in both. The zooplankton community of the microcosms and field sites contained cladocerans, *cyclopoid* and *harpacticoid* copepods, and ostracods. Both microcosms and field sites also supported several species of coleopterans, chironomids, and aquatic oligochates, as well as snails (*Lymnaea sp.*) and clams (*Sphaeriidae*). Some predaceous insect taxa (e.g., *Dytiscid* beetle larvae and *Chaoborus* larvae) were deliberately removed from microcosms to prevent excessive predation on the zooplankton community. Other insect taxa (e.g., dragon fly nymphs), *Eubranchipoda (conchostracans)* and tadpoles (*Hyla crucifer*) were present in ponds but not in microcosms.

Validation

Bti Survival: *Bti* spores survived for extended periods in both laboratory microcosms and field sites. While most spores disappeared from the water column within a few days, levels in the sediments remained stable for most of each test.

Although spores persisted, toxicity rapidly disappeared in both lab and field. In the field, sediment samples lost toxicity to mosquito larvae between 9 and 12 days after application. Similarly samples from both microcosms lost toxicity between 7 and 12 days after application. This loss of larvacidal activity is due to the inactivation or microbial degradation of the parasporal protein crystal associated with *Bti* spores. Despite the loss of toxicity, spores from microcosms and field sites could produce new toxic crystals when cultured on TSA agar.

The patterns of *Bti* uptake by invertebrates were similar in lab and field. The amount of *Bti* accumulated varied among the organisms tested. Filter feeding planktonic organisms accumulated the smallest amounts while the bottom-feeding snails accumulated very large amounts. While there were some differences in rates of uptake and losses between lab and field, the most important finding is that we could confirm in both lab and field that the organisms had been exposed to the test agent.

Ecological Effects: The effects on the target organism (mosquitoes) were obvious in both lab and field tests. In each case they were killed within 24 hours after application. The effects on non-target organisms and functional variables were not so obvious. In fact, our first task in comparing effects on microcosm and field communities has been to develop appropriate statistical procedures for analysis of field data.

We detected no significant ecological effects in either lab or field. We did become more aware of the difficulty of detecting effects in the field. In the laboratory we are can control environmental conditions and manipulate microcosms to reduce variability. Since this is not possible in the field, replicate field sites show much greater variability than replicate microcosms. This makes it much more difficult to detect effects in the field. We are currently investigating several procedures for analyzing such data.

In an attempt to deal with this high variability we investigated the use of an analysis of covariance (ANCOVA). In this procedure we adjusted experimental means for the influence of the covariate factors. In this case, depth of the pond and water temperature were the most significant covariates. We conducted ANCOVAs for each day of the exposure, and found no consistent effects that could be attributed to *Bti* treatment.

We have not yet completed all summation and analysis of data collected during the 1990 pond exposures. All but one set of ponds dried up by mid-July. With heavy rains in August and September all the ponds re-filled, however, and we were able to collect additional samples in October. We are still analyzing these and a few earlier samples so our conclusions remain incomplete at this time. At this point we do not see any significant effects caused by *Bti* in the field.

SUMMARY

Microcosm tests provide fate, direct effects and ecological data that can be used as a surrogate for outdoor testing. In this report we have discussed the strength of the link

between microcosms and field performance. On the basis of current data we can draw several conclusions:

1. Both the MFC and the core microcosm model events were observed in natural systems. The core microcosm is more closely correlated with the field than the MFC.

2. We were able to monitor the fate and effects of *Bti* in both lab and field and recorded similar results:

a. *Bti* spores persisted for the duration of the test.

b. *Bti* toxicity persisted for 7 to 12 days.

c. Non-target animals accumulated *Bti*. The extent of the accumulation and rate of loss of *Bti* were species-specific.

d. Target animals were killed in both systems.

e. Non-target animals were not sensitive in either system.

Tests in a single laboratory or field ecosystem will not provide definitive answers to the questions of survival and ecological effects of a particular introduced microorganism. Survival and effects are determined by a complex series of abiotic (*e.g.*, pH, temperature, redox potential, structure and composition of the substrate) and biotic factors (*e.g.*, the presence or absence of competitors, predators, prey or host species). Clearly, tests in one type of ecosystem cannot provide results generalizable to all ecosystems. This presents a dilemma, since it is obviously not possible to run all the tests needed to evaluate a micro-organism under every combination of abiotic and biotic factors.

The core procedure may provide an alternative to running multiple tests. The system is flexible enough to use biota and sediment from any local site. Survival and effects questions could then be focused on a particular ecosystem of concern, rather than on a generalized generic aquatic system. The questions of field calibration and validation will be further clarified as we complete additional tests.

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CURRENT INVESTIGATIONS ON MICROSPORIDIAN TEST SYSTEMS

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INTRODUCTION

Since 1982, Gulf Breeze Environmental Research Laboratory (GB/ERL) has supported a request by the Office of Pesticide Programs to develop testing protocols for fate and effects testing of microbial pest control agents (MPCAs). This has included description of single and multi-species test systems and toxicity and pathogenicity testing protocols for freshwater and marine nontarget organisms.

It was recognized that MPCA test systems must be capable of containing the MPCA and providing reproducible data, must be reasonable in cost and size, must maintain nontarget test organisms from a variety of phyletic groups, and should represent as closely as possible the conditions of a natural system. Additionally, it should have the capacity for testing all microbial groups of MPCAs, including viruses, bacteria, protozoa and fungi. Multi-species test systems have the advantage of testing several species with one exposure and may demonstrate interactions among nontarget test species.

Single and multi- species test systems employed at GB/ERL quickly evolved into the present totally-enclosed aquarium that incorporates an undergravel filter system using artificial substratum and an external ultraviolet light. Several different marine and freshwater nontarget organisms, including fishes, grass shrimp, bivalve molluscs and aquatic plants, have been held in these systems and challenged with a variety of MPCAs. Representatives of all four MPCA groups were initially tested, including a baculovirus *Autographa californica*, a bacterium *Bacillus thuringiensis*, a protozoan *Nosema cuneatum* and a fungus *Lagenidium giganteum*. Positive controls demonstrated characteristic infections and pathogenicity in target species; however, no signs of infection or pathogenicity were found in nontarget species using histology, serology, and electron microscopy.

More recently, GB/ERL research has focused on microsporidian MPCAs. Microsporidians are small unicellular organisms, all of which are obligate intracellular parasites with a unique mode of infecting host cells. Currently, there is one registered microsporidian, *Nosema locustae*, developed for control of rangeland grasshoppers and other requests have been received by the Office of Pesticides Programs. Future registration requests for microsporidians will probably increase as their pesticidal capabilities become better known. There is a relative abundance of new information on the biology of microsporidians: Many are now known to have indirect life cycles, multiple sporulation sequences, vertical as well as horizontal transmission and a

broader host specificity than previously believed.

One of the first microsporidians tested at Gulf Breeze was the mosquito pathogen *Nosema algerae*, which has a relatively simple direct life cycle. Development of *N. algerae* as an MPCA was originated in the 1970's when limited field tests were performed for it's use as a mosquito control agent. Currently, *Edhazardia aedis* is being investigated in collaboration with the USDA's Insects Affecting Man and Animals Research Laboratory for possible use in the control of container-breeding mosquitos. This microsporidian is an interesting model because it has a more complex life cycle than *N. algerae*, exhibiting multiple sporulation sequences and the capability of both vertical and horizontal transmission. Nontarget testing for this MPCA has been expanded to include fish embryo/larval assays and mysid shrimp life-cycle assays.

METHODS

Nosema algerae

A series of single species assays were conducted on freshwater and estuarine grass shrimp (*Palaemonetes kodiakensis* and *P. pugio*), marine rotifers (*Brachionus plicatilis)*, and inland silversides (*Menidia beryllina*) employing the aquarium system described above. Exposure was water-borne except for grass shrimp, which were also exposed by intrahemocoelic injection or gavage.

Edhazardia aedis

Single species tests with grass shrimp, *P. pugio*, and mosquito fish, *Gambusia affinis,* were conducted in the aquarium systems described above. Grass shrimp were exposed via water or gavage. *Gambusia affinis* were fed infected or uninfected mosquito larvae once a day for 7 days then uninfected artificial food for 7 days. Samples were taken eight hours after the first feeding and then at the end of the experiment to examine gill, liver, stomach and reproductive tissues for presence of *E. aedis*.

Exposure systems were developed to test estuarine mysids *Mysidopsis bahia* to *E. aedis* through an entire life cycle. Newly released young mysids were exposed to *E. aedis* spores (1000 mL^{-1}) at optimal (20 ppt) salinity for 1 week and monitored for survival. Adult mysids were exposed for 4 weeks under low-salinity (7 ppt) test conditions and monitored for survival and reproductive effort.

Fish embryo/larval toxicity assays were adapted to MPCA testing for water-borne exposures. Embryonic inland silversides, *Menidia beryllina*, were exposed to *E. aedis* spores in moderately-hard freshwater (hardness 100mg/L as CaCo3). Single blastula-stage fish embryos were placed in each of 30 individual tissue culture tubes with spore concentrations of 0, 10, 100 and 1000 mL^{-1}. Tubes were sealed with teflon lined caps and embryos incubated at 25°C with a 14:10 photoperiod until death or hatching occurred.

RESULTS AND DISCUSSION

Nosema algerae

Infections did not develop in gavaged grass shrimp, but did develop in those that received intrahemocoelic injections. Infected tissues included the gills, antennal gland, eyes, skeletal muscle, heart, and gonads. Proof of infection was demonstrated ultrastructurally by the presence of mature spores and developmental stages in infected tissues. Infections did not occur in the marine rotifer after ingestion of spores or in inland silversides fed marine rotifers containing ingested spores. Due to the extreme exposure measures, infection of nontarget organisms in nature is not indicated by these results; however they do demonstrate that infections can be identified and characterized using the diagnostic techniques available.

Edhazardia aedis

Exposure of *E. aedis* spores to grass shrimp did not cause any mortalities nor were any microsporidian life stages detected in sampled tissues. Eight hours after feeding of infected larvae, *E. aedis* was found in the guts of fish, *G. affinis*, demonstrating ingestion with the infected mosquito larvae. However, none were detected at the end of the experiment, one week after fish were removed from the mosquito larvae diet. This indicated that colonization and/or infection of mosquito fish did not occur.

Initial exposures of newly released young mysids to *E. aedis* spores indicated no mortality (> 90% survival) after 1 week under optimal salinity and temperature conditions. Exposures of adult mysids for four weeks under conditions of low salinity stress (7 ppt) showed reduced mysid survival (from 80% to 54%) and inhibited reproduction (the total young produced by three females was reduced from 9 to 3). Based on these results, a series of studies have been initiated to examine the interaction of stress conditions (10 ppt salinity and temperatures of 20, 25, and 30°C) survival and reproductive capacity of *M. bahia* exposed through an entire life cycle.

Results from a preliminary test with *M. beryllina* indicated that *E. aedis* spores cause an increase in embryo mortality (3% control vs. 27% exposed) and deformed larvae at hatching. This effect did not appear to be concentration dependent; rather, all exposure levels of *E. aedis* showed approximately the same result. Preliminary observations of exposed embryos indicated that the chorion of developing embryos may serve as an attachment surface for *E. aedis* spores. The mode of pathogenicity/toxicity remains unknown.

FUTURE WORK

Research at GB/ERL on microsporidians will continue to expand the number of MPCAs tested and determine toxicity, infectivity and pathogenicity on nontarget aquatic organisms. Emphasis will be placed on microsporidian species with different and more complex life cycles, such as *Amblyospora* and *Parathelohania*, which have intermediate hosts in their life cycles.

The results obtained with *E. aedis* on fish larvae will be pursued and

mechanisms of toxicity or pathogenicity will be elucidated. The adaptation of this fish embryo/larval toxicity assay to MPCA testing appears extremely productive and other MPCAs will be examined using this procedure.

Development of the mysid test system is highly appropriate for MPCAs developed for mosquito control. *Mysidopsis bahia* is an estuarine arthropod that is closely related to mosquitos and can be reared through it's entire life cycle in the laboratory. It is a representative of the dominant aquatic, non-insect arthropods (the Crustacea), and plays a crucial role in the trophodynamics of the highly productive estuarine ecosystem.

Mysids are the first nontarget test organism to be used at GB/ERL to explore the effects of environmental stress on pathogenicity and toxicity of MPCAs. Eventually, exposure conditions will be altered to determine MPCA effects under stressful environments for several nontarget species. Stressors will include both natural environmental factors (salinity and temperature) and chemical pollutants. It is expected that these types of stress are prevalent in nature and more accurately reflect conditions of susceptibility.

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DEVELOPMENT OF TEST METHODS TO ASSESS FATE OF MICROBIAL PEST CONTROL AGENTS AND THEIR EFFECTS ON NONTARGET AQUATIC ORGANISMS

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INTRODUCTION

Microbial pest control agents (MPCAs) are regulated by the Environmental Protection Agency. One responsibility of EPA is to prescribe tests for determining the potential ecological risks of MPCAs to nontarget organisms.

To fulfill the Agency's need for ecosystem effects testing of MPCAs, a fully contained, single species test system was developed to determine whether exposure of a nontarget aquatic invertebrate to an MPCA will result in infectivity, toxicity or pathogenicity. This system was field calibrated to assess whether MPCAs will colonize, persist or germinate in the animal. MPCAs representing a vegetative bacterial cell, *Pseudomonas fluorescens*; and both a fungal, *Colletotrichum gloeosporioides* (*Cga*), and a bacterial spore, *Bacillus sphaericus*, were used for field calibration.

In addition, we have successfully adapted standard chemical toxicity test methods for assessing nontarget effects by exposing the estuarine crustacean, *Mysidopsis bahia*, and embryos of the inland silverside fish, *Menidia beryllina* to *Beauveria bassiana*, an entomopathogenic fungus.

METHODS

Single Species Test System

American oysters, *Crassostrea virginica*, were exposed in enclosed 57 liter aquaria filled with filtered (2 μm) seawater from Santa Rosa Sound. Oysters were fed with a mixture of 3 marine algae 3 times a week.

To field calibrate the elimination rates of MPCAs, exposed oysters were thoroughly cleaned and divided into 2 groups. One group was placed in wire cages suspended in 6-8' of water (6 inches above the bottom) in Santa Rosa Sound. The remaining oysters were placed in an enclosed 57 liter aquarium equipped with aeration, and a pump driven, recirculating system (1 ℓ/min) which allowed water to pass first through an external cartridge filter of crushed coral then through a UV sterilization unit. Both the exposure and UV-clearance tanks were sealed with a plexiglas lid. Sampling ports and inlets for air and water were sealed with silicon stoppers.

Viable counts of the MPCAs were performed on aquaria water and oyster samples at regular intervals. Sections of whole oysters were taken for histological examination. Tissue samples

were fixed in Bouin's solution, dehydrated, embedded in paraffin, and sectioned at 6 μm. Sections were stained with Harris' hematoxylin and eosin, Brown and Brenn tissue Gram stain or periodic acid shift.

Nontarget Effects

ASTM recommendations for acute exposures were used in toxicity tests exposing *M. bahia* with viable spores of *B. bassiana*. Spores were obtained from cadavers of infected *Heliothis zea* to assure pathogenicity. Inoculum was prepared by scraping conidiospores from the surface of 11 day old plates. Spores were either treated with Triton X-100 (0.03%) or left untreated before exposure of the animals. Animals (10 per bowl, 2 bowls per spore density) were exposed for 96 hours at spore densities of 2×10^6, 1×10^6, 1×10^5, 1×10^4, 1×10^3 per ml. Autoclaved controls treatments were also performed. Beauvericin (Sigma), an secondary metabolite produced by this fungus, was also tested with *M. bahia*.

Single blastula stage embryos of the inland silverside, *M. beryllina*, were placed in each of 120 Leighton tissue culture tubes and exposed to *B. bassiana* at densities of 1×10^6, 1×10^5 and 1×10^4 conidiospores per ml. Embryos were incubated at 25 C under a 14L:10D photoperiod. Observations for effects were made daily.

RESULTS AND DISCUSSION

Single Species Test System

Neither toxicity, pathogenicity, or infectivity was observed in oysters exposed to *B. sphaericus*, *Cga* or *P. fluorescens*. Germination of *B. sphaericus* or *Cga* spores was not observed in the oyster tissue. The rates of clearance of these MPCAs from the oyster were compared in exposed oysters relayed in the UV-tank or in Santa Rosa Sound. With all MPCAs tested the rates of clearance from oysters in the sound versus the UV-clearance tank were similar.

The rates of clearance, however, between MPCAs varied widely. Oysters were exposed to either *B. sphaericus* and *P. fluorescens* for 2 weeks. *B. sphaericus* spores were undetectable in oyster tissue at 5 weeks. In contrast, *P. fluorescens* was still detectable in oyster tissue at 49 days. Oysters, exposed to *Cga* spores for 3 days, contained 1.7×10^5 spores per gram dry wt. These spores were rapidly cleared from the oyster and were undetectable in oyster tissue at 9 days of clearance.

Exposure plates, placed in various locations during the laboratory tests, indicated a completely sealed system. The clearance rates of MPCAs from oysters in the fully contained system were similar to the clearance rates in the field. Thus, this system will be of value before an environmental release has been deemed safe in determining whether, or how long, genetically engineered MPCAs or other micro-organisms will persist in nontarget aquatic invertebrates and whether exposure will result in toxicity, infectivity or pathogenicity.

Nontarget Effects

In an initial toxicity test with *M.*

bahia, *B. bassiana* spores were treated with Triton X-100 to aid in dispersal. This test demonstrated no toxicity of the spores up to and including 1 X 10^6 per ml test water. However, a subsequent exposure of test animal to these spores without the wetting agent indicated that with this particular strain, mortality was associated with high particulate density of the spores (as low as 1 X 10^6 per ml) an not with spore viability. Testing of beauvericin, an antibiotic produced by some other strains of this fungus, has indicated some toxicity.

While responses of the inland silverside embryos to *B. bassiana* has been variable among the three tests performed to date, in all instances pathogenicity has been observed in the form of rupture of the chorion of developing embryos followed by death.

FUTURE WORK

The fully contained exposure and clearance system will continue to be field calibrated using a viral and protozoan MPCA. Testing *B. bassiana*, its secondary metabolites, and other MPCAs will proceed using *M. bahia*. Additional exposures of inland silverside embryos are planned to determine the reason(s) for variability of responses among tests.

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FATE OF *Bacillus sphaericus* MICROBIAL PEST CONTROL AGENT IN THE ENVIRONMENT

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INTRODUCTION

Some strains of the bacterium *Bacillus sphaericus* have been found to be pathogenic for mosquito larvae. This pathogenicity is caused by the production by the bacteria of protein toxin which is lethal upon ingestion by larvae of many species of mosquito. Unlike the toxin produced by *Bacillus thuringiensis subsp. israelensis* (*Bti*), the toxin of *B. sphaericus* is not active against black fly larvae. The gene for the *B. sphaericus* toxin has been cloned and sequenced from several strains of the bacteria and has been found to differ among strains by only a few amino acids. However, the strains do differ somewhat in their toxicity for different species of mosquitoes and these differences probably lie in the slight differences in amino acid sequence in the toxins. The toxin is synthesized in the bacterial cell at the time of sporulation and it accumulates in the cell as a parasporal inclusion body or "crystal". The inclusion body contains primarily two proteins, and both of these are required for toxicity to larvae. Thus, the toxin of *B. sphaericus* is a "binary toxin".

In its role as a mosquito larvicide, viable *B. sphaericus* spores along with the attached parasporal bodies will be delivered into the aquatic environment. Since the metabolism of *B. sphaericus* is different than that of *Bti*, the ecological niche in which it may survive or grow in the aquatic environment may also differ. One salient feature that has attracted attention is the apparent ability of *B. sphaericus* to persist in the mosquito larval feeding zone for longer than *Bti*. This effect has not been observed in all field trials, but it has been reported often enough to make this bacterium an attractive larvicide in situations where frequent application is not practical. The reasons for this larvicidal persistence are unclear.

Our studies are examining fate of the bacterial spores during their interaction with the aquatic environment as well as the fate of spores ingested by certain non-target invertebrates.

METHODS

Bacterial Growth

B. sphaericus 2362-7, a spontaneous rifampicin-resistant mutant of *B. sphaericus* was used in all experiments. Spores were produced by growth in nutrient broth-0.05% yeast extract-mineral salts (Mn^{2+}, Mg^{2+}, Ca^{2+}) broth (NYSM) with shaking at 30°C for 48 hours. Spores were washed and held as a distilled water suspension at 4°C. Spore numbers were determined by heating 2 ml of a

sample at 80°C for 12 min. and plating on NYSM agar containing 50 μg/ml rifampicin and 0.002% cycloheximide.

Midge Larvae

Seventy five, fourth instar *Chironomus riparius* larvae were placed into 200 ml sterile distilled water in a 23 cm. X 23 cm. dish at 23°C. Three-tenths ml Tetramin suspension was added along with *B. sphaericus* 2362-7 spores at a final concentration of 9.6 X 10^6/ml. After 5 hours of feeding, the larvae were rinsed 3 times in 100 ml sterile distilled water. Five larvae were weighed, homogenized in a ground glass tissue grinder, and a spore count performed as described. Forty five spore-fed larvae were placed into 200 ml water containing Tetramin but without spores. Groups of five were homogenized at intervals to obtain spore counts.

To collect larval feces, twenty five spore-fed larvae were placed into a funnel blocked at the narrow end with screen. The funnel outlet led into a latex rubber tube which dipped into an ice water bath. A small amount of Tetramin was added to the sterile distilled water in the funnel. After 17 hours, the 50 ml of water in the funnel and tube were removed and the funnel and tube were rinsed with 50 ml water. The combined volumes were centrifuged (27,200 x g, 15 min), the resulting pellet containing 2362-7 spores suspended in sterile distilled water, and a spore count performed.

Snails

Thirty-one snails, *Helisoma trivolvis*, were placed into 200 ml sterile distilled water in a 23 cm. X 23 cm. dish at 23°C. *B. sphaericus* 2362-7 spores were added to a final concentration of 9.6 X 10^6/ml. After grazing for 5 hours, the snails were rinsed 3 times with 50 ml sterile water and placed into 300 ml sterile water containing a piece of lettuce. Two snails were immediately removed, the bodies separated from the shell, the foot dissected away, and the remaining tissue homogenized. A spore count was performed on the homogenate. The remaining snails were placed in fresh sterile water daily for the first three days after feeding on spores and twice weekly thereafter. Lettuce was provided as food throughout the experimental period. Three of the spore-fed snails were placed in a funnel apparatus described above for fecal collection. These snails were also moved to fresh water daily for three days and twice weekly thereafter to prevent reingestion of feces that might not pass through the screen at the bottom of the funnel. Feces were collected for 17 hours, the collection water centrifuged (27,200 x g, 15 min.), and the resulting pellet suspended in sterile distilled water for spore counts and bioassay.

Oysters

Four, 112-liter glass aquaria were used for spore feeding tests; two for inoculation and two for controls. Aquaria were equipped with under-gravel filters covered with crushed coral and filled with artificial seawater at a salinity of 10 parts per thousand. Sixty cleaned oysters were placed into each test aquarium. *B. sphaericus* spores

were added and the aquaria sealed. Oysters were fed with a mixture of 3 marine algae every third day. To determine the spore burden in oysters, samples of digestive, gill and remaining whole oyster tissues were homogenized as previously described.

To determine spore elimination rates, 20 oysters were removed from each inoculated aquarium after 28 days, cleaned, and placed into a flow-through seawater tank. Four oysters were removed every week for 5 weeks and a spore count was performed on the whole oyster tissue. Fecal material was obtained directly from the shell surface. When recognizable, fecal material was collected from the tank bottom using a pipette. Fecal material was placed into a test tube, and the excess liquid was removed from the surface. The tube was centrifuged (7,700 X g, 10 min.), and the supernatant discarded.

Toxicity of Snail and Oyster Feces

Feces from snails and oysters were collected as described above. Fecal suspensions were bioassayed for toxicity to second instar larvae of *Culex quinquefasciatus*.

Stonefly Larvae

Paragnetina media (Walker) larvae were placed individually into chambers containing 400ml tap water at 20°C. Midge larvae were fed on/ *B. sphaericus* spores overnight, rinsed 3X, and 3 larvae placed into each stonefly chamber. Only stoneflies that had eaten 3 midges within 4 hours were used in the depuration experiment. Immediately after feeding on midges and at intervals thereafter, the stonefly larvae were rinsed, appendages dissected away, bodies weighed, and homogenized. Homogenates were plated for spore counts. Beginning 24 h after having ingested spore-laden midges, the water in each stonefly chamber was changed and this was repeated on alternate days. On the days on which the water was not changed, each stonefly larva was fed 1 midge larva (not containing spores). The water changes alternating with midge feeding were continued throughout the experiment.

Crane Fly Larvae

Tipula abdominalis larvae held individually in 400 ml chambers in the dark at 13°C were each fed 1 yellow poplar conditioned leaf disc that had been soaked in *B. sphaericus* spore suspension. Each disc had approximately 5×10^6 spores adhering to it. Twenty four hours after consuming the spore-laden disc, the water in each larval chamber was changed and a fresh leaf disc lacking spores was fed. The water was changed and a leaf disc fed at 48 h intervals thereafter for the duration of the experiment. At intervals groups of 3 larvae were removed, the guts dissected out, weighed, homogenized, and spore counts performed on the homogenates.

RESULTS AND DISCUSSION

Indigenous Microflora

None of the animals or their feces used in this study contained microflora

which survived heating at 80°C and grew on NYSMRC selective medium in numbers which interfered with enumeration of *B. sphaericus*.

Midge Larvae

Elimination (depuration) of spores from fourth instar larvae was followed for 4 days following ingestion of spores. Larvae examined immediately after feeding averaged 1.2×10^6 spores/mg larval dry weight. This number declined to 7.7×10^2 spores/mg after 4 days at which time the larvae began to pupate. The total viable bacterial count on the fourth day was approximately equal to the spore count. The spore content of feces declined in parallel to body spore content.

Larvae that were reared, allowed to pupate, and emerge as adults in water initially containing 1×10^7 or 4.3×10^3 spores/ml, had an average of 8.2×10^3 and 68 spores in/on the bodies (15 and 18 flies, respectively). At the conclusion of the experiments, the culture water contained 9.5×10^6 and 8.4×10^2 spores/ml. Control flies reared in water lacking added spores averaged 4 spores/fly (14 animals) and no spores/fly (10 animals), respectively.

Snails

Depuration of spores from snails was followed for 49 days. There was a rapid decline (about 2 logs) in the body burden of spores during the first 7 days followed by an approximately constant number of spores (between 2×10^1 and 2×10^2/mg) for an extended period. At 49 days the body spore content declined to less than 100 spores/mg for the first time. As with midge larvae, the total viable bacterial count approximated the spore count. The spore content of feces declined even more rapidly than that of the body, but it also leveled out/and feces containing low numbers of spores continued to be produced for the remainder of the experiment.

To eliminate the possibility that the persistence of spores in snails was a result of reingestion of feces containing spores, snails were allowed to feed in 9.6×10^6 spores/ml for 23 hours which resulted in a body content of 4.9×10^6 spores/snail. These snails were placed in a sterile fecal collection funnel which was drained and rinsed with sterile water twice daily before being refilled with sterile water. After 7 and 14 days, the snails were transferred to another sterile funnel. After 21 days, the feces from the snails were collected and the body burden of spores determined. The snails averaged 1.7×10^4 spores/snail and the feces contained 2.3×10^3 spores/snail.

The LC50 of the spore/toxin preparation fed to snails was 1.2×10^2 spores/ml. This declined 66% to 3.6×10^2 spores/ml in feces.

Oysters

Oysters placed in spore-containing water and then transferred to flowing seawater, decreased in spore content from 2.5×10^6 spores/g dry tissue to 5.0×10^3 in 21 days and had eliminated the spores in 37 days. Histological examination showed no indication of spore germination or of vegetative bacilli in the gut. Oyster feces were

non-toxic when fed to mosquito larvae, apparently having been detoxified by passage through the oyster.

Stonefly Larvae

Ingestion and clearance of spores in a predator-prey relationship was examined with stonefly larvae (predator) and spore-carrying midge larvae (prey). The midges were rapidly consumed by the stoneflies and *B. sphaericus* spores were found to be present in the stoneflies at levels approximately equal to the sum of the spores present in the midges. The spore number declined rapidly and spores were below the level of detection by 7 days.

Crane Fly Larvae

In contrast to rapid depuration seen in midge and stonefly larvae, crane fly larvae retained a large number of spores for an extended period of time. The animals had a mean number of 4.5×10^5 spores/gut immediately after eating a leaf disc. This number decreased to 8.5×10^4 after 4 weeks. This was the highest level of spore retention observed among any of the animals studied. Spores were present in the feces for the entire period, but the content dropped more rapidly than did the spore content of the animal gut. Total viable plate counts done at 1 day and 2 weeks after feeding on spores approximately equaled the spore count. This suggests that at least on those times there was not a large population of vegetative cells growing in the animal. Such a population could have continuously produced spores to replace those lost in the feces. We cannot exclude the presence of a small vegetative population approximately equal to the spore population that would not be detected given the sensitivity of plate counts. The crane fly has an unusually alkaline foregut and a possible relationship between this phenomenon and spore retention or production is being investigated. Also, more a exact location of the spores in this animal is being determined.

Summary

Spores of the MPCA, *B. sphaericus*, were consumed but largely or completely eliminated from the gut of 2 insects (midge, stonefly) and 1 mollusk (oyster). A second mollusk (snail) eliminated most of the spores but retained a low number for an extended period of time. A third insect, crane fly, retained a significant number of spores for up to I month. All animals tested passed viable spores in their feces. Midges reared in spore-containing water emerged as adults carrying spores in/on their bodies. The mosquitocidal toxin of *B. sphaericus* was partially destroyed by passage through the snail and completely (or nearly completely) destroyed by passage through the oyster.

FUTURE WORK

We will continue to examine the effect of different gut conditions on survival of *B. sphaericus* spores. This will focus on the highly alkaline gut of the crane fly where extended survival was observed and move to another leaf shredder, *Pteronarcys proteus*, which

has a slightly acidic gut. We will also examine a microcrustacean, *Daphnia*, for the first time. The possible proliferation of *B. sphaericus* in nontarget cadavers produced when the animals died of other (non *B. sphaericus* toxin) causes will be investigated. These interactions of spores with nontarget animals will be in addition to other studies of the interaction of spores with physical factors in the aquatic environment.

PUBLICATIONS

Yousten, A.A., E.F. Benfield, R.P. Campbell, S.S. Foss, and F.J. Genthner. Fate of *Bacillus sphaericus* 2362 spores following ingestion by nontarget invertebrates. J. Invertebr. Pathol. (in press).

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PROTOCOL DEVELOPMENT FOR TESTING THE EFFECTS OF BACTERIAL PESTICIDES ON BENEFICIAL BEETLES

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INTRODUCTION

Microbial pest control agents (MPCAs) are increasing in use because of their specificity and low mammalian toxicity and pathogenicity; however, they are a potential hazard to non-target, beneficial insects. MPCAs could be used more effectively in integrated pest management if their impact on non-target insects was better understood. Non-target insects are ecologically and economically important in a variety of ways, including: as natural predators and parasites of pests, as introduced biological pest control agents of both insect and plant pests, as food for wildlife, and as decomposers. Subdivision M of the Pesticide Testing Guidelines calls for assessing the potential hazard of MPCAs on several orders of terrestrial non-target insects including *Coleoptera* (beetles). Such tests need to be standardized because the results of the tests may be affected by the conditions of the bioassay. To develop a standard assay for testing the effects of bacterial pesticides on beneficial insects, the effect of temperature and dietary stress, and of larval instar on the susceptibility of *Hippodamia convergens* (the convergent lady beetle) to a weak bacterial pathogen was tested. *H. convergens* is a major predator of aphids and occurs throughout most of the United States and as such is an important biological control agent.

METHODS

H. convergens was reared on pea aphids, *Acyrthosipon pisum*, at 25°C, 70% RH. *Pseudomonas fluorescens* was used as the pathogen (i.e. the surrogate MPCA). It is a bacterium that was isolated from the haemolymph of dead adult beetles during a time when there was high morality in the laboratory colony. The relative susceptibility of different larval instars of *H. convergens* to *P. fluorescens* was compared by determining the dose response of each instar at 25°C on an aphid diet. First instar larvae were used to determine the effects of diet and temperature stress on susceptibility. Since temperature and the insect's diet can potentially affect the bacterial virulence as well as insect susceptibility, the treatments were given to the insects in 24 hour pulses before the insects were exposed to the pathogen. Three different diets (water, 5% sucrose, and aphids) were used at each of two temperatures (25° and 30°C), thus there were six treatments.

The experiment was set up to determine the probit dose response of the pathogen on the beetle. Insects

were exposed to the pathogen by dipping them in the appropriate dose of the bacteria. Five or six doses were used per treatment with twenty insects per dose for a total of 100-120 insects per treatment. There were two replicates of each treatment. After insects were dosed, they were maintained at 25°C, 70% RH, 14:10 hours light:dark. Mortality was recorded on day six. Both replicates were combined for the probit analysis of mortality.

RESULTS AND DISCUSSION

The mean LC_{50} for the first, second and third instars was 7.9×10^9 cfu/ml. There were no significant differences in the slopes or intercepts of the probit lines for these age groups. However, the last instar was 40 times less susceptible with an LC_{50} of 3.2×10^{11} cfu/ml. The slope of the probit line was not significantly different from the other instars but the intercept was ($p \leq 0.001$). The effect of age is probably independent of size since there is a continuum in size as the insect goes from one instar to the next, but there is not a continuum in susceptibility.

In the larval stress tests, at 25°C, the highest susceptibility was in the aphid diet treatment with an LC_{50} of 4.8×10^9 cfu/ml, and the probit line was significantly different ($p \leq 0.001$) from both the sucrose and water diets at 25°C. Insects given an aphid diet were 151 times more susceptible than those given the water diet at 25°C. The addition of sugar to the starvation diet did not significantly affect susceptibility as the probit lines of the sucrose and water diets at 25°C were not significantly different from each other.

With the 30°C treatment, the variance in the responses increased and none of the slopes or intercepts of the probit lines were significantly different for the three diets; however, the relative order of the LC_{50}s was the reverse order of that found at 25°C. When a comparison was made between the two temperature treatments for each diet, the effect was not significant for the aphid and sucrose diet treatments. In the water diet treatment, the 30°C temperature significantly increased the intercept of the probit line ($p \leq 0.01$), indicating an increase in susceptibility. The results of the stress experiment indicate an interactive effect between temperature and diet on susceptibility, since the relative effect of one factor depends on the other factor.

Even though *P. fluorescens* is a very weak pathogen of *H. convergens*, our results indicate that environmental and dietary regimes can affect the results of tests used to determine the effects of MPCAs on beneficial insects. It is important to note that the most stressful diet does not necessarily result in the highest susceptibility and that stress conditions can increase the variability in the test results. A happy medium was sought to create a bioassay that was both sensitive and reliable. The conditions selected for the test protocols was a 25°C incubation temperature with an aphid diet.

FUTURE WORK

In the future, we look to produce laboratory tested protocols for the effects of fungal MPCAs on *H.*

convergens, and to develop protocols for beneficial *Diptera*, such as the *Tachinidae*. The methods used for laboratory testing of the protocols will be similar to that used for this protocol. However, future work is planned for laboratory and field studies used to determine the appropriateness of the LC_{50} endpoint. Some of the questions we would like to address are: What does a laboratory LC_{50} mean when it comes to field applications? Might some other endpoint be more appropriate? We would also like to monitor the effects of specific MPCAs on non-target insects under field conditions. The results of laboratory and field tests could then be incorporated into a model for predictive value.

PUBLICATIONS

James, R.R. and Lighthart, B. 1990. Bioassay for testing the lethal effects of bacterial pathogens on the predatory beetle *Hippodamia convergens* Gue. (*Coleoptera: Coccinellidae*). National Technical Information Service publication no. PB91-127795. Springfield, VA.

ASSESSING HOST SPECIFICITY OF FUNGAL MPCAs TO THE BENEFICIAL WASP *Trichogramma pretiosum*

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INTRODUCTION

The registration process for Microbiological Pest Control Agents (MPCA's) requires data on nontarget effects to representatives of key beneficial insect species, *e.g.*, *Trichogramma pretiosum Riley*, a lepidopteran egg parasite. *Trichogramma spp.* are recognized as important, both as naturally occurring and commercially released natural enemies of pest *Lepidoptera*. The laboratory evaluation of MPCAs for nontarget effects is generally recognized as the first step in determining the risk a pest control agent may pose to natural enemies. Although methods have been developed to evaluate chemical pesticide effects on *Trichogramma spp.*, bioassay's evaluating entomogenous fungal pathogenicity to *Trichogramma spp.* have reported only negative effects.

This study was undertaken to develop bioassay procedures to evaluate pathogenicity of entomogenous fungi, compare specific and nonspecific fungi, and evaluate factors that may affect the expression of fungal virulence to *T. pretiosum* or the susceptibility of *T. pretiosum* to fungal pathogens.

METHODS

Insects Used

A colony of *T. pretiosum* was maintained on U-V killed Cabbage Looper eggs, *Trichoplusia ni* (Hübner), at 25 ± 1C°, 75% RH and with continuous light (40w fluorescent).

Fungi Tested

The life form of the fungi tested was conidia. Pure stocks of *Beauveria bassiana* and *Metarhizium anisopliae* conidia, without mycelia or agar, were obtained by inoculating Sabouraud Maltose Agar + 1 % yeast extract (SMA +Y) plates, lined with a sterile disc of 200 mesh nylon screening. After 21 days incubation, plates were dried and conidia were scraped from the screening with a sterile spatula and stored dry (20-40mg portions) at -70°C until needed. *B. bassiana* was passed through *T. pretiosum* by inoculating ten wasps with enough conidia (10^8/ml) to ensure 100% mortality by 72 hours when incubated at 100% RH and 25°C. As wasps died, they were moved to a fresh vial, incubated until covered with conidia, seven days, and ten new wasps were added to the vial.

These steps were repeated five times. Conidia on cadavers from the last set were washed with 1.0% T-80, and a stock of conidial inoculum was generated as described above. To ensure maximum pathogenicity of the host specific fungus *Nomureae rileyi*, conidia were collected directly from infected *T. ni* cadavers and used immediately.

Inoculum Preparation

Approximately 20mg of conidia were suspended in 3 ml of sterile 0.10% (v/v) Tween 80 (T-80) with an all glass tissue grinder and processed until clumps of conidia were no longer visible. A dilution sequence was prepared with a final T-80 concentration = 0.01%. Concentrations of conidia were based on viable conidia and estimated with drop plate counts.

Exposure to pathogen

Because of the small size and relative delicateness of *Trichogramma spp.* special techniques were developed to handle and expose wasps to pathogens which minimized risk of pathogen escape while allowing efficient handling of the large numbers of *T. pretiosum* needed for dose response tests. To sort and handle, wasps were anesthetized on a cold plate (3-10°C). Medium to large females (0.5-0.75mm long) were used for the bioassay. Wasps were transferred in groups of 75 to confinement chambers of an immersion apparatus. Wasps were exposed to pathogens by filling the immersion apparatuses with conidial suspensions. Apparatuses containing wasps were then dried on a vacuum manifold with down stream air filtered to remove entrained conidia. Wasps from each apparatus were divided into three groups of 20 wasps each, transferred to clear one ounce plastic cups supplied with a streak of 50% fructose syrup, sealed with snap over plastic lids, and incubated inverted in humidity chambers. To evaluate the effect of relative humidity on the expression of virulence by *B. bassiana* and *M. anisopliae*, cups with wasps were either supplied with four droplets (0.010 ml ea.) of distilled water and incubated in a humidity chamber with saturated KH_2PO_4 (= rapid saturated air) or water droplets were omitted and cups were incubated in chambers with distilled water (= delayed saturated air).

Mortality was evaluated on days 3 and 4 after exposure to fungi. Estimates of the reported dose response parameters and pairwise comparisons of dose response slope functions were made with probit software (SAS Version 6.06).

Conidia adhering to wasps were enumerated by using drop plate counts on SMA + Y plates. Counts were prepared using ten wasps from each dilution in a ten-fold dilution sequence and homogenized in 1 ml of 0.1% T-80 with tissue grinders. Duplicate plates were prepared directly from each dilution with no further dilution. Plates were counted at 48 hours for *B. bassiana* and at 72 hours for *N. rileyi*.

RESULTS AND DISCUSSION

The bioassays differentiated between host specific and nonspecific pathogenic fungi. Of the three species of fungi with which *T. pretiosum* was challenged, only *N. rileyi* failed to produced mortality. *B. bassiana* levels of virulence were significantly different for the isolates tested. Virulence of a given isolate of *B. bassiana* maintained continuously on artificial media was significantly increased when passed through *T. pretiosum*. Estimates of conidia adhering to wasps after immersion indicated that the lack of *N. rileyi* pathogenicity to *T. pretiosum* was not due to rate of conidia attachment to wasp cuticle but to other factor(s). *M. anisopliae* and *B. bassiana* while both pathogenic to *T. pretiosum*, expressed different levels of virulence to *T. pretiosum* when saturation of air in the test vials was rapid versus delayed. When saturation of air was delayed the virulence of these fungi were nearly identical. When saturation of air was rapid the virulence (LC_{50}) of both pathogens increased (= decrease in LC_{50}) with *M. anisopliae* having a significantly lower LC_{50} (=more virulent). However, the dose response slope function for *B. bassiana* increased by 64% while the slope for *M. anisopliae* decreased by 74%. *T. pretiosum* longevity and susceptibility to *B. bassiana* was not affected by withholding food, withholding water, or by exposure to temperature extremes (5° or 30°C for 24 hours). These results indicate that the nontarget effects of entomogenous fungi on *T. pretiosum* are largely affected by innate differences of the fungi tested and the environmental factors, relative humidity and temperature, which affect the expression of virulence by the fungus. For quantitative comparisons of different species, isolates, or strains of entomopathogenic fungi the culture history, original isolate source and environmental conditions adequate for expression of the pathogen's virulence must be rigorously documented and controlled. The shifts and differences in the dose response slope functions observed when saturation of air was achieved rapidly suggest that comparisons between pathogens for relative virulence and host susceptibility must go beyond the classic point comparisons (LC_{50} fiducial limits) and include the other dose response functions *e.g.*, slope. This bioassay will discern between host specific and nonspecific pathogenic fungi and produce quantitative results to compare nonspecific fungal MPCAs for pathogenicity to *T. pretiosum*.

FUTURE WORK

Future research is to be focused on developing test procedures to evaluate pathogenicity of MPCAs to adult and larval honey bees (*Apis mellifera L.*) and effects on colony health and productivity. Our goals are to (1) develop a microbiologically defined test system using microcosms and mini-hives to evaluate the effects of MPCA's on specific life stages, e.g., workers versus larvae, and the colony and (2) evaluate the effects of biotic and abiotic environmental factors on bee susceptibility. Parameters proposed to evaluate effects include, but are not limited to, worker longevity, larval survivorship,

food consumption and storage, and the growth rate of the colony. Pending development of the microcosm, the system will be validated for detecting effects using known life stage specific honey bee pathogens. This will be followed by evaluations of environmental factors on susceptibility of bee life stages to pathogens.

PUBLICATION

Sewall, D.K. and B. Lighthart. 1990. Standard practice for conducting fungal pathogenicity tests on the Lepidopteran egg parasite *Trichogramma pretiosum* (*Hymenoptera:Trichogrammatidae*). NTIS Acces. # PB90-263849/AS. 40 pp.

AN OVERVIEW OF PROTOCOL DEVELOPMENT FOR AVIAN PATHOGENICITY TESTS

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INTRODUCTION

Microbial pest control agents (MPCAs) are microorganisms applied to agricultural and silvicultural environments to control proliferation and dissemination of insect or plant pests. During the past four years, we have been working to develop a set of standardized protocols that can be used to determine the pathogenicity of microorganisms in nontarget avian species. The mallard (*Anas platyrhynchos*) and the northern bobwhite (*Colinus virginianus*) were chosen as the test species since both are required by the U.S. Environmental Protection Agency for routine wildlife toxicity testing during the registration process of chemical pesticides. These protocols are used primarily by the Office of Pesticide Programs when prospective registrants propose new microbial pest control agents (MPCAs). The protocols are written such that could be used to determine pathogenicity of any microorganism, either wildtype or genetically engineered; however, they lack requirements for determining potential gene exchange between the introduced microbe and endogenous gut microflora.

Three routes of exposure of the birds to MPCAs were evaluated: oral, intravenous (I.V.), and respiratory. Three classes of microorganisms, a virus, fungus, and bacterium, were used to test protocols developed for oral and I.V. exposure tests. A bacterium and a virus were used to evaluate the respiratory protocol. This report reviews our development of the protocols with emphasis on respiratory protocols developed during the last year and a half and a study by our collaborator, Dr. Matsumoto, at Oregon State Universtity to develop a sensitive method for determining if birds are being exposed to *Bacillus thuringinensis*.

METHODS

Oral and Intravenous Protocols

Ten-day-old chicks and ducklings were used in the tests because they lack full immunocompetency until 20-25 days of age, and in order to maximize the number of replicates. The test microbes used were *Autographa californica* nuclear polyhedrosis virus as the viral agent; *Metarrhizium anisopliae* as the fungal agent; *Salmonella pullorum* as the bacterial agent. For the oral exposure route, birds were given a single oral inoculation by insertion of a gavage needle down their esophagus, and injecting a known volume and concentration of the organism. The

intravenous route is similar, with the microbial injection inoculated into the right jugular vein. The dose administered to the birds was 10^4 times the insect LC_{50} which is 100 times the commercial application rate and represents a potential worst case scenario. Forty birds were inoculated with live test pathogen, forty with inactivated pathogen, and another forty with diluent only. Birds were monitored for 28 days post inoculation with weekly recording of body weights, weekly collection of blood samples for serum chemistry analyses and antibody response assays, collection of fecal samples at specified intervals to determine if organism is shed in the feces, and histopathological examination of all tissues showing gross lesions or that are collected from birds that die during the experiment.

Salmonella pullorum is known to be pathogenic in poultry and was chosen as a positive control. LD_{50} studies were conducted for both species.

Respiratory Protocol

In collaboration Dr. Crystal Driver and colleagues at Battelle Pacific Northwest Laboratories, a head-only exposure chamber was developed for aerosol exposures of 10-day-old quail. An initial study was conducted to determine the deposition of particles in the respiratory tract of quail after intratracheal instillation and aerosol exposure of polystyrene beads ranging in size from 0.4 to 20 microns. Subsequent tests were conducted to test the procedures for either intratracheal (I.T.) or aerosol exposure routes. A non-motile, non-pathogenic *E. coli* was chosen to represent the bacterial agent. Fifteen minutes after exposure, birds were euthanized and bacterial reisolation attempted from nasal turbinates, trachea, lungs, syrinxes, and air sacs. A similar study was conducted with *Autographa californica* NPV, but technical problems with bacterial contamination interfered with attempts to reisolate the organism from tissues.

ELISA Development For Mallard Antibodies

A commercial suspension of *Bacillus thuringiensis var. israelensis* (*Bti*) was purchased and the toxin proteins were purified. Bacteria were sonicated and incubated with trypsin and proteinase K to solubilize the proteins. The supernatant was washed twice with ammonium sulfate and purified on a Sephadex G150 column. Four protein peaks were produced. Peak 4 was purified on DEAE Bio-Gel A, and separated on SDS-PAGE electrophoresis into three peaks: A 58 KiloDalton (KD) peak, and 19 or 20 KD peak, and another 19 KD peak. The 19 KD peak, previously shown to cause hemolysis of mouse erythrocytes, was used for the ELISA and coated onto the plates at 0.2 µg/ml concentration.

Experimental mallards were exposed to *Bti* by aerosolization. Birds were placed inside the carrier crate and covered with a plastic bag. The bacteria was nebulized into the bag for one hour which resulted in 145 ml of the suspension being used. Ten birds received a 1:10 dilution of the commercial preparation, ten birds received a 1:100 dilution, and five birds

served as negative controls. Birds were allowed to inhale the contaminated air for 45 minutes. They were exposed six times at 14-day intervals with blood samples collected every 28 days during this period. Antibodies to the *Bti* toxin were measured by the ELISA.

RESULTS AND DISCUSSION

Oral and Intravenous Protocols

The baculovirus, *Autographa californica* NPV, and the fungus, *Metarrhizium anisolpiae,* were nonpathogenic to the birds when administered orally or I.V. inoculation. They caused no detectable alterations in the physiological parameters measured. The studies with *S. pullorum* showed that 10-day-old quail are very susceptible to pullorum disease, with 65-100% mortality; however, no mallards died or exhibited signs of morbidity. Blood chemistry values and antibody response assays of the quail were inconclusive due to high mortality rate, small volumes of serum obtained and few birds that survived long enough to seroconvert. Mallards inoculated orally had significant alterations in serum chemistry values; antibody titers were detected as early as 1 week post inoculation. Bacteria were isolated from all tissue of quail at necropsy and from feces collected 6 days post inoculation. Attempts to isolate the organism from mallard fecal samples were unsuccessful and bacteria was isolated only from liver tissue of four birds. Tissue samples examined histopathologically showed the quail to be severely affected. Gross and histopathological examination of mallard tissues were normal. Use of *S. pullorum* as a positive control demonstrated that: 1) the protocols are written such that lethal and sublethal effects can be detected; 2) there can be significant differences in susceptibility between mallards and bobwhite; 3) *S. pullorum* is an effective positive control for bacterial tests with bobwhite but not for mallards.

Respiratory Protocol

Particle size distribution in the respiratory tract of young quail differed significantly between I.T. and aerosol inoculation methods. The I.T. route deposited almost twice as many particles in the respiratory tract compared to the aerosol exposure, and particles were much larger in size. No particles greater than 0.8 microns lodged in any air sacs following aerosol exposure whereas I.T. instillation deposited 10 micron particles in the clavicular air sac and 5 micron particles in the thoracic air sacs. The intratracheal inoculation method caused sneezing and coughing by the birds which resulted in contamination of the workers and the exterior of the bird. From these preliminary studies, it was concluded that aerosol exposure is a safer, more realistic exposure route than intratracheal instillation.

ELISA Development for Mallard Antibodies

Antibody production was observed when antibodies were measured against the 19 KD protein but the test lacked the required specificity. Preinoculation

samples and control birds also showed positive antibody response. When an ELISA was developed using the 58 KD protein as the antigen, the specificity increased significantly. Antibody titers decreased rapidly following a single exposure to *Bti.* These experiments demonstrate the potential for use of the ELISA as a tool to monitor sentinel mallards exposed to *Bti* applications.

FUTURE STUDIES

The oral and intravenous exposure protocols require testing with a protozoan to determine if and where difficulties might arise when working with this class of microorganism. In addition, we plan to develop a positive control system with a protozoan that is pathogenic in birds.

The respiratory protocol requires further testing with a pathogenic bacterium (*e.g. S. pullorum*), a virus, fungus, and protozoan. Mallards should be incorporated into the respiratory tests to determine if there are differences in particle deposition patterns due to anatomical/physiological differences in respiratory tract.

Work will continue with the mallard ELISA test. It is anticipated to employ this test with free-ranging birds this summer or fall once an area is established where *Bti* is being applied.

The oral, intravenous, and respiratory protocols developed test for lethality and pathogenicity. The next step is to develop protocols that test whether an MPCA would cause reproductive impairment. The studies will include a chronic feeding exposure study, and evaluation of contamination of embryonated eggs via eggshell penetration of microorganisms. Fertility, teratogenesis, hatchabiltiy, and egg shell quality will be major endpoints measured.

PUBLICATIONS

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ROUTE SPECIFICITY OF THE TOXICITY OF THE *Bacillus thuringiensis subsp. israelensis* 28 KILODALTON PROTEIN

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INTRODUCTION

Repeated human exposure to elevated levels of natural and genetically altered microbial pesticides and biotechnology agents increases the probability of occurrence of detrimental human health effects. This is particularly true of agents that are broadcast or applied on a large scale in the environment. Thus, agents or factors with such potential must be investigated to aid in the risk assessment process.

The polypeptides of the parasporal crystals of *Bacillus thuringiensis subsp. israelensis* (*Bti*) have been reported to produce a variety of undesirable biological effects such as cytolysis, hemolysis, neurotoxicity, etc., as well as mammalian toxicity. Our previous studies implicated the solubilized 28 kDa polypeptide of the parasporal crystal as the cause of mammalian effects including toxicity and hemolysis of various mammalian erythrocytes (Mayes et al., 1989). Monoclonal antibody affinity-chromatography purified 28 kDa polypeptide was demonstrated to cause a profound and lethal hypothermia and bradycardia in mice challenged intraperitoneally with this protein. The 28 kDa polypeptide was found to possess minimal insecticidal activity (Held et al., 1986).

To more fully assess the potential for detrimental effects in humans, studies were carried out to ascertain the effects of different routes of challenge with the solubilized *Bti* parasporal crystal 28 kDa polypeptide in rats. Additionally, changes in serum parameters after intraperitoneal challenge with the protein mixture were monitored. Results are presented in this report.

METHODS

Rats were challenged intraperitoneally (IP) and intratracheally (IT) as described for mice by Mayes et al. (1989) and Sherwood et al. (1988), respectively. Sprague Dawley rats were dosed intravenously (IV) by injection into a lateral tail vein and subcutaneously (SC) into the area of the back. Peroral treatment (PO) was accomplished with the use of a gavage needle. A range of dosages were utilized for the various routes. Mice were observed for 14 days for clinical signs and mortality.

Studies of the effects of solubilized *Bti* parasporal crystal polypeptides on blood parameters were undertaken. One hundred gram rats were challenged IP with an 8 μg/g mixture of crystal polypeptides and, two hours later, blood was collected by cardiac

puncture. Serum was obtained by allowing the blood to clot in Eppendorf tubes for 20 min. at 0°C and then centrifuged. Serum was analyzed with a Centrifichem 400 automated analyzer.

RESULTS

Results showing the effects of the maximum administered dose by various routes (Table 1) indicate that only IP injection of rats caused mortality. An IP injection LD50 of 1.95 μg *Bti* solublized parasporal crystal protein/g body weight was estimated from the complete data set. Preliminary results with a limited number of animals challenged IV, IT, SC and PO indicate that there was no mortality. SC injection, however, caused localized cutaneous necrosis at the site of administration. The area of skin involved was proportional to the magnitude of the dose.

Table 1. Toxicity to rats of solubilized *Bacillus thuringiensis subsp. israelensis* crystal polypeptides by different routes of administration.

Route of Administration	Maximum Dose	Mortality
intratracheal	10 μg/g	0/6
intravenous	21 μg/g	0/6
per os	9 μg/g	0/6
subcutaneous[a]	9 μg/g	0/12
intraperitoneal	9 μg/g	21/39[b]

[a] An increased lesion size was correlated with increased dosage.
[b] LD50 = 1.95 0/17 μg/g (p,0.05).

The changes in blood parameters indicate effects in numerous organ systems (Table 2). Serum levels of albumin, blood urea nitrogen, creatinine, creatinine phosphokinase, glucose, and phosphate were significantly elevated in rats challenged IP with *Bti*. Serum levels of sodium were sigificantly decreased in the same rats. Parameters which were not significantly affected were: alkaline phosphatase, alanine transaminase, total bilirubin, total protein, amylase, total CO2 , lactate dehydrogenase, albumin/globulin, anion gap, calcium, potassium, and chloride.

DISCUSSION

The 28 kDa protein of the *Bti* parasporal crystal is toxic IP to a number of vertebrate species (Mayes, et al., 1990). Consequently, it is all the more surprising that the present results indicate that it is lethal to rats only when administered IP. The estimated median lethal dose of 1.95 μg/g by this route is close to that reported for Swiss Webster mice (2.3 μg/g) (Mayes et al., 1989). SC injection, however, produces localized cutaneous necrosis at the site of administration. The lack of effects by the IV, IT and PO routes

Table 2. Changes of serum chemistry parameters in rats injected intraperitoneally with solubilized polypeptides from *Bacillus thuringiensis subsp. israelensis*

	Target Organ System			
Parameter	Heart	Kidney	Pancreas	Other
Albumin		↑		
BUN	↑			
Creatinine		↑		
CPK	↑			
Glucose			↑	
Phosphate				↑
Sodium				↓

remain unexplained. It is interesting to note that although *Bti* produces dramatic hemolysis and cytolysis *in vitro*, preliminary evidence in our studies show no measurable hemolysis *in vivo* after IP challenge (Mayes et al., 1989).

FUTURE WORK

Future work will attempt to explain the mechanisms involved in the route specific toxicity of *Bti*. Factors that may effect the activity of the 28 kDa protein when challenged by routes other than IP will be investigated. This should help to clarify the risk to human health of the *Bti* 28 kDa protein.

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CLEARANCE OF ENVIRONMENTAL PSEUDOMONADS FROM CD-1 MICE FOLLOWING INTRANASAL EXPOSURE

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INTRODUCTION

Even though environmentally released microorganisms are considered safe from a health standpoint, there is always a potential for the occurrence of adverse health effects. Exposure may occur through ingestion, inhalation, or dermal contact with the microbial product. Previous research in our laboratory has investigated the ability of engineered microorganisms to survive in the intestinal tract and compete with the normal flora of mice. Translocation of these microbes to the spleen and liver, which can be indicative of a possible systemic infection, has also been examined. In these previous studies, the microorganisms were introduced by gavage.

The current study investigates potential health effects associated with intranasal (i.n.) exposure to engineered microorganisms. Effects monitored include a) morbidity, b) mortality, and c) survival of the dosed strains in the lungs, nasal washing, and intestinal tract.

METHODS

Bacterial Strains and Preparation of Dosing Suspension

Overnight (16-hour) yeast extract-tryptone cultures of *Pseudomonas aeruginosa* strain AC869 (Chatterjee et al., 1982, Mol. Gen. Genet., 188,279) and *P. cepacia* strain AC1100 (Kilbane et al., 1982, Appl. Environ. Microbiol., 44,72) were concentrated in phosphate buffered saline (PBS). Subsequent dilutions were made in PBS where indicated.

Intranasal Exposure

Fasting (16 hour) strain CD-1 male mice were anesthetized with methoxyfluorane and dosed i.n. with 50 μl of the microbial suspension. At 3 hours, and 1, 2, 5, 7, 10, and 14 days after treatment, animals were sacrificed and the lungs and intestinal tract (small and large intestines, cecum) removed and placed in buffer. The nasal cavity was washed with PBS. Animals and tissues were concurrently weighed.

Enumeration of Dosed Strains

Homogenized tissues were diluted and plated onto *Pseudomonas* isolation agar (PIA) with (AC869) or without (AC1100) 50 μg/ml kanamycin. Plates were incubated at 30°C for 48 (AC869) or 72 (AC1100) hours. Colony forming units (CFU) were determined and representative colony types isolated for antibiotic sensitivity determinations to insure source identification.

Statistical Analysis

A two-way analysis of variance was performed to determine what effect the treatment had on lung and animal weights. A value was considered significant if $P<0.05$.

RESULTS AND DISCUSSION

Morbidity and Mortality

P. aeruginosa strain AC869 had an LD50 of 2.7×10^7 CFU. When mice were dosed with $\sim 10^9$ CFU of strain AC869, mortality occurred within 24 to 36 hours. Slight morbidity occurred in animals treated with $\sim 10^7$ CFU. These animals had ruffled fur, weight loss, and were lethargic. A statistically significant ($P<0.05$) increase in lung weights occurred in these animals as well. No effects were observed when mice were administered $\sim 10^3$ CFU.

When mice were treated with $\sim 10^8$ CFU of *P. cepacia* strain AC1100, slight morbidity was observed. The decrease in body weights was accompanied with an increase in lung weights. Animals also were lethargic and had ruffled fur but recovered within 5 days after treatment.

The slight morbidity observed in treated animals is probably due to endotoxin (lipopolysaccharide), a component of gram negative bacterial cells. Mortality in strain AC869-treated animals may occur through endotoxin shock possibly coupled with pathogenicity factor(s). Strain AC869 also may have more endotoxin than strain AC1100.

Pulmonary Survival

Strain AC869 was detectable at high levels in the nasal wash and lungs of animals treated with $\sim 10^9$ CFU prior to animal death. When treated with $\sim 10^7$ and $\sim 10^3$ CFU, clearance of the dosed strain was dose dependent. When administered $\sim 10^3$ CFU, strain AC869 was not detectable in the lungs after 3 hours, but was present in the nasal washings intermittently for 14 days. Even though there are no overt health effects associated with this dose, the nasal cavity may serve as a reservoir for future intestinal or pulmonary inoculations. The $\sim 10^7$ CFU dose was cleared from the lungs after day 7 but was evident in the nasal washings at day 14.

Unlike strain AC869, the $\sim 10^8$ CFU dose of *P. cepacia* strain AC1100 was cleared from the lung after day 7 and from the nasal cavity after day 2. Even though strain AC1100 is not detectable in the nasal wash, it may still be present at numbers below the detection limit of the method used.

Intestinal Survival

Strain AC869 was easily detected in the GI tract within 3 hours of treatment. In the mice treated with the highest dose ($\sim 10^9$ CFU), recovery values remained constant until 100% mortality occurred. At the end of 14 days, strain AC869 was still detectable in mice treated with $\sim 10^7$ CFU in all 3 sections of the GI tract. However, the strain was cleared from the cecum and large intestine after 3 hours and never

detected in the small intestine when mice were administered ~10^3 CFU.

P. cepacia strain AC1100 was detectable in all three regions of the GI tract at 1 day post treatment, cleared from the small intestine by day 2, and not detectable in the cecum and large intestine after day 2. When dosed by gavage, strain AC1100 is not recovered from the GI tract even at 3 hours after treatment. When dosed i.n., mucus may coat the bacteria and protect it from adverse conditions in the stomach and small intestine.

The GI tract is a reservoir for potential pathogens. Endotoxin can promote translocation from the GI tract to the mesenteric lymph nodes, spleen, and liver, causing a systemic infection. The treated strains may be harbored in the GI tract or alter the normal flora producing future adverse health effects.

FUTURE WORK

Intranasal exposure to other biotechnology agents will be investigated in endotoxin sensitive and resistant mice. Translocation to the mesenteric lymph nodes, spleen, and liver will be determined. Alterations in normal flora will be examined. Morbidity, mortality, clearance, and translocation in antibiotic-treated animals also will be investigated. Pathogenicity factors associated with *Pseudomonas spp.* will be studied.

PUBLICATIONS

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EFFECT OF ORGANOCHLORINE COMPOUNDS ON THE BIOACTIVATION OF 2,6-DINITROTOLUENE IN FISCHER-344 RATS

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INTRODUCTION

Exposure to environmentally released microorganisns may occur during production, application, or use of biotechnology products. There also is a potential for exposure to the target environmental contaminant and its microbial metabolites that arise during bioremdiation. Therefore, it is important to understand the effects of exposure to these hazardous chemicals and their metabolites in conjunction with microorganism use.

The intestinal tract contains enzymes that can transform pro-mutagens and procarcinogens to their mutagenic and carcinogenic metabolites. Key enzymes involved in this process include nitroreductase and β-glucuronidase. For example, 2,6-dinitrotoluene (DNT), a hepato-carcinogen, has been reported to require GI tract bioactivation to express genotoxicity.

In this study, Fischer 344 rats were orally treated with the organo-chlorine compounds, pentachlorophenol (PCP), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), or Aroclor 1254. 2,6-DNT-induced urine mutagenicity, DNA adduct formation, and key GI tract enzymes were assayed to determine if alterations in GI tract flora and/or metabolism occur due to exposure of these compounds.

METHODS

Animal Exposure

Weanling male Fischer 344 rats were dosed p.o. with 20 mg/kg PCP, 54 mg/kg 2,4,5-T, or 25 mg/kg Aroclor 1254 daily for 5 weeks. At 1,2,4, and 5 weeks of treatment, rats were administered 75 mg/kg 2,6-DNT and placed into metabolism cages for urine collection.

Urine Collection, Preparation, and Mutagenicity Bioassay

Twenty-four-hour urine samples from animals treated with the peanut oil vehicle, the organochlorine compound, 2,6-DNT plus peanut oil, or the organo-chlorine compound plus 2,6-DNT were collected over dry ice. Urine was treated with β-glucuronidase and sulfatase and then concentrated on C-18 columns. The resulting methanol extract was dried under N2 and stored at -80°C until bioassay. Samples were diluted in DMSO and tested for mutagenicity in a microsuspension bioassay using *Salmonella typhimurium* strain TA98 without metabolic activation. At week 5, urine was fractionated by HPLC (C-18) and mutagenicity bioassay performed directly on the fractions that were solvent exchanged into DMSO.

GI Tract Enzyme Analysis

Six rats treated with the organochlorine compound and 6 rats that received the peanut oil vehicle were sacrificed at 2 and 4 weeks of treatment and the small intestine, cecum, and large intestine were removed and placed into reduced buffer. The tissues were homogenated under CO_2 and placed into an anaerobic chamber where aliquots were pipetted into the reaction vessels. The reaction vials were stoppered, removed from the anaerobic chamber, and 25 μl of the enzyme substrate mixture (in DMSO, 20 mg/ml *p*-nitrophenyl-β-D-glucuronide, 3,4-dichloronitrobenzene)added. The reaction mixtures were incubated for 1 hour at 37°C with gentle mixing. The reaction was terminated and products (*p*-nitrophenol, β-glucuronidase; 3,4-dichloro-aniline, nitroreductase) extracted, derivatized, and analyzed.

DNA Adduct Analysis

DNA was isolated from frozen livers and digested to mono-nucleotides with micrococcal endonuclease and spleen phospho-diesterase. P1 nuclease was used to select for adducted nucleotides. The DNA was labeled with (γ-^{32}P) ATP and T4 polynucleotide kinase. The labeled DNA adducts were resolved on PEI-cellulose TLC plates.

RESULTS AND DISCUSSION

Pentachlorophenol Effects

By week 4 of PCP treatment, a decrease in nitroreductase activity coincided with toxic urine in the bioassay. Five weeks after PCP treatment, urine again was collected and fractionated by HPLC to separate the toxic components from the mutagens. Animals that received both 2,6-DNT and PCP had more mutagenic urine than animals that received 2,6-DNT alone. Peanut oil controls and PCP only treated rats voided non-mutagenic urine. PCP potentiated the formation of 2,6-DNT-derived DNA adducts whereas none were observed in peanut oil controls or rats treated with PCP only.

Arochlor 1254 Effects

2,6-DNT induced urine genotoxicity was elevated in Aroclor-treated rats at 1,2,4, and 5 weeks after treatment. This finding corresponded with either a decrease in small intestinal nitroreductase activity or an elevation in small intestinal β-glucuronidase activity. Again, at week 5, elevated DNA adduct formation corresponded with urine genotoxicity.

2,4,5-Trichlorophenoxyacetic Acid Effects

In contrast to PCP and Aroclor 1254 effects, 2,4,5-T treatment reduced urine 2,6-DNT-induced genotoxicity 1 week after treatment with levels returning to control values at weeks 2 and 4. No differences in DNA adducts were observed after 5 weeks but small intestinal nitro-reductase activity was decreased at week 1 and β-glucuronidase activity reduced at week 2. Unlike PCP and Aroclor 1254, 2,4,5-T is not an inducer of the hepatic

P450 monooxygenase enzymes. From our studies, it appears that the following criteria must be met to elevate 2,6-DNT-induced urine genotoxicity and hepatic DNA adduct formation: a) small intestinal nitroreductase activity reduced and/or β-glucuronidase activity elevated, and b) the compound must induce hepatic monooxygenase enzymes.

FUTURE WORK

Future studies include examining the effects of a biodegradative micro-organism with its target compound on the key GI tract enzymes. Similar studies involving microbial metabolites of the compound of interest may be included.

Other future work includes evaluating the effect of cresote and petroleum products on 2,6-DNT-induced urine genotoxicity, GI enzymes, and hepatic DNA adduct formation. Genotoxic metabolite identification will be done as well.

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ENVIRONMENTALLY RELEASED MICROORGANISMS AND THE ENTERIC MICROFLORA

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INTRODUCTION

The release of natural occurring or genetically engineered microorganisms for bioremediational purposes has prompted questions concerning their effects on the environment and human health. The Health Effects Research Laboratory (HERL) of the Environmental Protection Agency (EPA) is responding to these questions. The long term objective of our collaborative research in this regard is to determine what effects bioremediational microorganisms (pseudomonads in particular) have on the "normal microflora." of the mouse. If bioremediational microorganisms are able to disrupt the "normal microflora" to any significant extent, they could compromise it's regulatory and protective role in the gastrointestinal tract, thereby enhancing the host animal's susceptibility to an assortment of diseases.

Attempts will be made to induce pseudomembraneous colitis in CD-1 mice by oral administration of *Clostridium difficile* coincident with alterning their protective "normal microflora" by antibiotic tratments. Using this *in vivo* model as a guideline, we will determine if the protective effects of the "normal microflora" can be altered or disrupted by oral administration of bioremediational microorganisms.

The first phase of this research has involved development of appropriate analytical systems andmethodologies. Described in this report are (a) our attempts to determine if an Oxyrase™ technology for anaerobic incubations can be use instead of anaerobic hoods, (b) development of culture media for enumeration of *C. difficle* and *C. perfringens* using the Oxyrase™ system, and (c) isolation and enumeration of *Lactobacillus reuteri* (purportedly a member of the protective "normal microflora") in the mouse GI tract.

METHODS

Growing Enteric Anaerobes Using Oxyrase™

Oxyrase is a partially purified, sterilized suspension (0.2 microns or smaller) of a microbial (*E. coli*) membrane system that, in the presence of a suitable hydrogen donor, removes dissolved oxygen rapidly and completely from aqueous and semisolid

environments. It is commercially available from OXYRASE, INC (Ashland, OH 44805) and was used according to the manufacuter's instruction. Broth cultures were supplemented with 0.1 unit of Oxyrase ml^{-1} and 30 mM sodium lactate. Agar pour plates were supplemented with 0.3 units ml^{-1} of Oxyrase, 30 mM lactate and overlayed with 15-20 ml of 2% agar to slow diffusion of oxygen in the air to the nutrient layer containing the culture. For agar spread plates the 2% overlay agar contained 0.3 units ml^{-1} of Oxyrase, 30 mM lactate, and 20 mM phosphate buffer at pH of 7.5. All incubations were carried out at 37°C in a conventinal incubator.

Growth of *Clostridium difficile* (Strains 7698 and 10463) and *C. perfringens* (NCTC 8798)

Stock cultures wee maintained in Cooked Meat Medium and inocula prepared using BHI broth. *C. perfringens* was enumerated using Trypticase Soy Cycloserine (TSC) agar counting black colonies after 8 h at 37°C. *C. difficile* was enumerated using TSC agar supplemented with Cefoxitin (16 μg ml^{-1}) and counting black colonies after 18 h at 37°C. *C. perfringens* does not grow in the latter medium, and *C. difficile* colonies do not appear in 8 h in the former medium.

Determination of Total Anaerobes in the Mouse Gastrointestinal (GI) Tract

Tissue extracts were macerated and diluted in a sterile gelatin-salts solution. The preparations were then spread on Brucella Agar (supplemented with sheep blood, vitamin K, and hemin) for incubation in the anaerobic hood, or pour-plated in the same medium when using the Oxyrase.

Isolation and Enumeration of *Lactobacillus reuteri*

Lactobacillus Selection (LBS) agar medium (pH = 5.4) was used to enumerate the total gut population of lactobacilli. The sub-population of *L. reuteri* colonies on appropriately diluted plates is idenified and enumerated based on the ability of *L. reuteri* colonies to convert glycerol under anaerobic conditions to a metabolic intermediate, 3-hydroxypropion-aldehyde, which has potent anti-microbial activity.

RESULTS AND DISCUSSION

Results obtained to date indicate that Oxyrase can be used as an alternative methodology to isolate and grow enteric anaerobes. Pure cultures of a relatively oxygen-tolerant anaerobe (*C. perfringens*) and an oxygen-intolerant anaerobe (*C. difficile*) were observed to grow in comparable manner using either the Oxyrase™ method an anaerobic chamber. This was observed for both liquid and agar cultures.

Similar results were obtained when the two methods were used to determine the total number of anaerobes present in (a) the entire mouse (GI) tract or (b) the cecum/colon segments of the tract. Also, we were able to determine that the CD-1 mice which will be used later in this research have very low background populations

of either *C. difficile*-like or *C. perfringens*-like bacteria resident in their GI tract.

L. reuteri has been found in the GI tract of all animals examined to date. This species is believed to play an important role as a member of the regulatory and protective "normal microflora." Our initial attempts to isolate this species from CD-1 mice have been unsuccessful. An examination of "pet store purchased" mice, on the other had revealed the expected complement of *L. reuteri* cells in their GI tract. These results indicate, not surprisingly, that rearing methods can effect the composition of an animals "normal microflora."

FUTURE WORK

The Oxyrase anaerobic technology will be applied toward accomplishing the goals of the proposed research which are:

(a) to develop an *in vivo* model disease system, (i) to study and monitor the protective role of the "normal microflora" against disease, and (ii) to evaluate the influence of environmentally released microorganisms on this protective role.

(b) to determine if an *in vitro* model can be developed and used for these purposes.

In Vitro SURVIVAL AND COMPETITION OF ENVIRONMENTAL PSEUDOMONADS WITHIN A HUMAN FECAL FLORA CULTURE

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INTRODUCTION

Investigation of potential adverse health effects due to the environmental release of microorganisms is needed. Previous work in this laboratory has examined the colonization and competition potentials of environmental pseudomonads with the intestinal flora of CD-1 mice both *in vivo* and *in vitro*. The current research is a complementary study investigating the ability of some of these same *Pseudomonas* strains to survive and compete in culture with human fecal microbiota.

METHODS

Veal infusion broth enriched with yeast extract, vitamin K1, and hemin served as the *in vitro* culture medium. Human fecal flora, obtained using Anaerobic Culturette Collection and Transport Systems (Becton Dickinson Microbiology Systems), were grown overnight in enriched veal infusion broth and used as the culture inoculum. *Pseudomonas aeruginosa* strains BC16 (isolated from a product for PCB-degradation), PAMG (a mouse intestinal isolate), and AC869 (a 3,5-dichlorobenzoate degrader) were grown overnight in yeast extract-tryptone broth and concentrated in VPI dilution buffer for culture inoculation. A 0.1 ml aliquot of one competitor strain was used to inoculate the culture.

At 12 hours, a 0.06 ml aliquot of the culture was removed and used to inoculate a new culture. The new culture was incubated for 12 hours and the process repeated for a total of 4 transfers (5 cultures). The competitor strain and fecal microbiota populations from the first, third, and fifth cultures were enumerated by spread-plating 0.1 ml of the appropriate dilutions onto selective media at pre-selected time intervals.

RESULTS AND DISCUSSION

All three competitor strains grew anaerobically in pure culture to a level of 10^8-10^9 organisms per milliliter. Although *Pseudomonas spp.* are aerobes, they can use nitrate as a terminal electron acceptor. In culture with human fecal flora, all three strains declined in number, but were still present in low numbers in culture 5. *P. aeruginosa* AC869 appeared to be the best survivor of the strains tested. Survival of the competitor strains was comparable in culture with human or mouse flora.

Differences between the human and mouse enumerated flora populations were minimal. The total aerobic and anaerobic counts were higher in

mouse fecal cultures than in the corresponding human fecal cultures for the 6 hour timepoint, perhaps indicating faster growth of the mouse cultures.

No effect of strain addition on the human fecal flora populations was detected for strain BC16. Statistical tests have not yet been completed to determine if strains PAMG or AC869 alter the balance of the flora populations.

FUTURE WORK

Flora populations and enzyme activities for single-stage, 2-stage, and 2-stage with cell-recycling continuous cultures will be compared to determine which most closely mimics the *in vivo* situation. Survival and competition of environmentally relevant strains will then be tested in continuous culture. Rodent and human *in vitro* results then can be combined with rodent *in vivo* results in a parallelogram approach for extrapolation of potential human health effects.

PUBLICATIONS

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SESSION III
RISK CONTROL RESEARCH

Decontamination and Mitigation

Investigators are exploring a variety of risk reduction strategies, including the development of genetically altered strains for conditional lethal control of survival and gene exchange and procedures for physically and chemically decontaminating field sites. These procedures will be evaluated under a variety of environmental conditions.

Field Releases

ORD scientists develop criteria for evaluating containment and monitoring strategies. Test methods are based on the actual application of recombinant and surrogate organisms to specially designed experimental release sites representing different environmental habitats. Methods for detection, monitoring distribution, dispersal, and dislodging characteristics, and for proper containment of microorganisms during field releases are being evaluated.

Process Containment

Researchers produce the appropriate scientific information to develop protocols for evaluating the causes of emissions from biological process facilities, and control and reduction of risks associated with these emissions. Engineering and cost models for assessing risks associated with the manufacture, and use in manufacture of genetically engineered microorganisms in large-scale fermentation facilities will be produced. Issues dealing with process equipment design, decontamination technology, worker exposure and protection, and loss prevention techniques will be considered.

THE USE OF LETHAL BACTERIAL GENES TO LIMIT THE SURVIVAL OF INTENTIONALLY RELEASED GENETICALLY ENGINEERED MICROORGANISMS

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INTRODUCTION

Concerns about the release of genetically engineered microorganisms to the environment may be assuaged if the released strains contain a mechanism whereby they may be killed after completion of their appointed task(s). This mechanism should, ideally, be contained within the organism and should not hinder the survival of the released strain prior to the application of the lethal signal, i.e. the strains should have conditional lethality. A potentially lethal gene putatively involved with plasmid maintenance, *kilA* from plasmid RK2, has had its regulation altered so that expression is dependent on the presence of an added compound. The native upstream regulatory sequences were removed from *kilA* and replaced with the TOL plasmid lower pathway operator-promoter OP2 to form plasmid pEPA88. Cells containing this plasmid and an appropriate regulatory gene should be killed in the presence of an added OP2 inducer such as benzoic acid.

This report contains the results of tests on *Pseudomonas aeruginosa* PAO1 containing pEPA88. Effects on growth, lethality, and the previously uncharacterized mechanism of action of *kilA* were examined.

METHODS

P. aeruginosa PAO1 was transformed with pEPA88 and incubated in a minimal salts medium in the presence and absence of benzoate. Several parameters were examined including increases in culture turbidity, cell survival (CFU's), ^{3}H-glutamate uptake, and the differences in these parameters in different cell size fractions. A second lethal plasmid was constructed which placed *kilA* down stream of the inducible *tac* promoter (pWHJ63). This plasmid was transformed into *E. coli* JM109 and the effect of *kilA* expression on DNA synthesis was determined.

RESULTS

Microscopic examination of PAO1 (pEPA88) cultures incubated in benzoate minimal medium showed two distinct morphological forms. Numerous long, filamentous cells were observed as well as a significant number of normal size cells. Filaments appeared after a 2 hour incubation with benzoate and remained until the termination of the experiments at 24 hours. Filaments were not formed in samples which did not contain benzoate. Filaments could be separated from single cells by differential filtration

through 3 μm pore-size filters. Microautoradiography of cells labelled with ^{3}H-glutamate indicated that filaments were metabolically active. After 4 hours, 5 % of the biomass (μg protein/ml) was in the greater than 3 μm fraction (filaments). By 8 hours and 12 hours, 60% and 70% of the biomass, respectively, was in the greater than 3 μm fraction. By 24 hours, 50% of the biomass was filaments. At 4 hours, 17% of the ^{3}H-glutamate was taken up by the greater than 3 μm fraction. At 8 hours, 45% of the uptake was by filaments. By 12 hours, 87% of the uptake was in the larger fraction and 83% was taken up by filaments after 24 hours exposure to benzoate. Biomass specific rates of glutamate uptake were 3 to 5 times greater for filaments at all time points. In contrast to metabolic activity measurements, all of the colony forming organisms were found to be in the less than 3 μm fraction. This indicates that while the filaments were more active, they were incapable of cell division and subsequent colony formation.

Filaments and single cells were also observed in *E. coli* cells containing pWHJ63 after induction of *kilA* by IPTG. Filaments began forming within an hour. Microscopic examination indicated that the number of filaments remained constant after a 2 hour exposure to IPTG. ^{3}H-Thymidine incorporation by these cells indicated that filaments continued to synthesize DNA.

DISCUSSION

The use of *kilA* has proven inappropriate as a biological control agent for *Pseudomonas aeruginosa* PAO1. Two types of response have been observed in cells containing induced *kilA* genes. The first is a biostatic effect characterized by normal sized cells which, while not actively growing, remain metabolically active. The addition of an alternative carbon source provides growth substrate and these cells begin to grow and divide and will form colonies when plated on nutrient agar. The second lethal response is noted by the production of long filamentous cells. Metabolic activity remains very high in these cells, yet these cells do not grow and form colonies when presented with alternative growth substrates. The lethal mechanism of *kilA* has not been previously characterized. Our results indicate that lethality is not caused by an inhibition of protein nor DNA synthesis. The production of filaments and the inability of them to divide and form colonies indicates that *kilA* functions by inhibition of daughter cell separation. The reasons for the two types of response are not known. Plasmid minipreps indicate that those cells which do grow maintain intact pEPA88 plasmids so that loss of the plasmid construct cannot account for loss of activity.

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FIELD RELEASE OF A GENETICALLY-ALTERED BACULOVIRUS WITH A LIMITED SURVIVAL CAPACITY

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INTRODUCTION

Although baculovirus epizootics are often the major factor in natural control of lepidopterous insects, there has been limited commercial usage of these viral insecticides. One of the primary problems with using baculoviruses as microbial pesticides is that target insects are not killed quickly and continue to damage crops for 3 to 10 days. To overcome this problem baculoviruses can now be genetically engineered to enhance their pathogenicity. New genes can be inserted into viral genomes which will effect a fast death.

The insertion of new genes into baculoviruses is technically easy, and many commercial and academic laboratories are currently conducting programs aimed at producing genetically-enhanced viral pesticides. However, a major problem which must be addressed prior to the release of these products is the environmental consequences, *e.g.*, persistence, spread, genetic stability, gene transfer potential, and displacement of natural virus populations (Wood, in press; Wood and Granados, in press - see publications list). In order to address these issues, we have developed and tested a release strategy by which the engineered baculovirus pesticides could have *enhanced pathogenicity* but *low survival and displacement potential* in nature.

The strategy for this construction is to delete the polyhedrin gene and replace it with a foreign gene whose product will enhance pathogenicity. The polyhedrin gene product is required for occlusion of virus particles within polyhedra and is therefore essential for survival and transmission in nature. Therefore, the infectivity of the altered virus is stabilized by occlusion within polyhedra produced by wild-type virus. This is accomplished by co-infection of cells with both the wild-type and altered virus. Late in the replication cycle, the polyhedrin produced by the wild-type virus occludes both virus types. Persistence of the altered virus in the virus population is determined by subsequent levels of co-infection and co-occlusion.

This field release project is being conducted to test our laboratory based model under natural conditions. The release was conducted with an altered *Autographa californica* nuclear polyhedrosis virus (AcMNPV) which had a deleted polyhedrin gene but *no foreign gene inserted*. The altered virus was co-occluded with the wild-type AcMNPV. Investigations are being performed to evaluate the spread of virus in nature and the survival potential of the altered virus within the wild-type virus population.

METHODS AND RESULTS

During the field application phase of this project (1989), three applications of polyhedra containing 49% genetically-altered and 51% wild-type *Autographa californica* nuclear polyhedrosis virus (AcMNPV) particles were made on a quarter acre cabbage plot in Geneva, NY. The cabbage plants were seeded with a total of 4500 *Trichoplusia ni* (cabbage looper) larvae which were all infected and killed by the virus. Polyhedra samples were purified from individual larvae. The viral DNA was purified, digested with the restriction enzyme *Eco*R1 and fractionated by agarose gel electrophoresis. Based on molar ratios of the *Eco*R1 I fragments (altered virus genome has a smaller I fragment), it was determined that the progeny virus produced in these larvae contained 42% genetically-altered virus. In addition, it was estimated that approximately 1×10^{14} progeny polyhedra were deposited in the field.

The questions asked in 1990 were: 1) how much of biologically-active virus (which contained 42% altered virus) was present in the soil in the spring of 1990, 2) with this as the starting inoculum how much occluded, altered virus would be produced and 3) what was the distribution of virus in the field plot. The two approaches taken to answer these questionswere soil sampling and larval seeding.

In 1990, the test site was replanted with cabbage in precisely the same location and manner as in 1989. The cabbage plants were seeded with *T. ni* larvae 4 times during the growing season. Several thousand larvae were inspected in the field without evidence of viral infections. When half of the larvae started to pupate, a total of 1600 larvae were brought into the laboratory and examined for virus infections. Less than 1% of the test larvae became infected, indicating that only a small amount of virus in the soil was deposited on the plant tissues.

As a second evaluation procedure, soil samples were taken from the same 97 locations in the application area as were sampled in the spring of 1989 prior to the release. In order to quantitate the amount of virus in the test site, polyhedra were extracted from the soil samples using a combination of differential centrifugation and detergent treatments. The amount of biologically active virus was determined by feeding one-gram soil extracts to each of 30 larvae from each location (2910 samples). Based on control data, the soil extraction efficiency was 25%. Therefore each insect was fed the amount of virus present in 0.25 grams of soil. The percent death from these bioassays was used to determine the number of biologically-active polyhedra per gram of soil.

Based on infectivity of the soil extracts, the average number of polyhedra detected in soil samples from the quarter acre application area was 1219 ± 2269 polyhedra per gram of soil. The average number of polyhedra in the soil samples from the buffer zone (1.75 acre area surrounding the application area) was 274 ± 404. The high standard deviations are the result of high variation between samples not within replicated samples.

Based on laboratory studies, it was predicted that the biological stability of

polyhedra containing wild-type and altered virions would be equal to polyhedra containing only the wild-type virus. In order to evaluate this, soil samples were taken from a control plot which had been treated identically in all respects to the application site except that the polyhedral inocula contained only wild-type virions. The average number of polyhedra in this control site in the spring of 1990 was 1151 $\pm$ 1942 polyhedra per gram of soil. This value is very close to the number of active polyhedra found in the application area. It is, therefore, concluded that occlusion of altered virions does not effect the stability of polyhedra under laboratory or field conditions.

Having established the level of active virus deposition in the field and the percentage of altered virus (based on introduction data), we are now determining how much altered virus replication has resulted from this level and type of inoculum. To this end, infected larvae from the quantitation procedure discussed above are being further analyzed. The polyhedra from the individual larvae have been purified and the percentage of occluded, altered virus is being determined by DNA restriction analysis. As previously demonstrated, the concentration of inocula as well as the percent altered virus present in the inocula determine the amount of altered virus in progeny polyhedra. It is considered that larvae feeding on leaf tissue contaminated with soil would ingest less than 0.25 grams of soil, and therefore the inoculum level in this test can be considered high in terms of natural contamination processes.

We are now in the process of completing our analysis of the amount of altered virus present in the polyhedra isolated from the individual larval samples. The present data show that approximately one-third of the polyhedra samples contain altered virus. The range in concentration is 12-57% altered virus. The data set is incomplete at present, however the average amount of occluded, altered virus is approximately 20%.

FUTURE INVESTIGATIONS

Questions for 1991:

A. How many biologically-active polyhedra remain in the test site after one year?

B. Using soil extracts as inocula, what quantity of altered virus will be occluded in progeny polyhedra?

The original prediction was that from 1990 to 1991 there would be a decrease in both values. If a decrease occurs, then the co-infection/co-occlusion process used in this study would be of use to ensure that a released engineered baculovirus would slowly be removed from natural virus populations (nonpersistent). Accordingly, the engineered virus could not displace natural virus populations based on its competitive disadvantage.

If the number of biologically-active polyhedra do not decline between 1990 and 1991 and the amount of occluded, altered virus remains the same as in 1990, the co-infection/co-occlusion strategy does not provide the environmental attributes for which it was

designed. Accordingly, new approaches will be needed to ensure environmental safety following releases of genetically-enhanced viral pesticides. In the absence of biological containment, engineered baculoviruses may pose a multitude of potential problems.

In the spring of 1991, we will again take soil samples from the same 97 test site locations as used in 1989 and 1990. As performed in 1990, thirty larvae will be fed with extracts from one-gram soil samples (1/4 gram based on extraction efficiency) from each site. Based on the percent infection of larvae, the concentration of biologically-active polyhedra will be determined and compared with the 1990 data.

The dead larvae from the soil assays will then be processed to determine the amount of occluded, altered virus present in polyhedra. The polyhedra from each larva will be processed and analyzed separately. The percent altered virus and standard deviation will be compared with the 1990 data.

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EPIPHYTIC FITNESS GENES AND PHENOTYPIC ADAPTATION

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INTRODUCTION

The fate and effects of microorganisms introduced into the open environment are usually predicted from an understanding of their biology and ecology studied under controlled conditions, usually in culture. Studies we have made of introduced recombinant bacteria reintroduced into field plots indicated that the strains we used exhibited considerable phenotypic plasticity. That is, the behavior of Ice⁻ *Pseudomonas syringae* strains grew and survived with different efficiencies depending on the conditions under which they had originated. For example, we had observed that cells harvested from broth cultures were less capable of surviving on leaves surfaces after inoculation than were cells grown under solid agar conditions. If we are to accurately predict the growth and survival of bacteria under natural conditions we must be aware of the factors such as phenotypic plasticity which may effect the behavior of cells while in nature. Similarly, we need to know more of the nature of the habitats occupied by bacteria after introduction into the open environment such as plants since the growth and survival of the introduced bacteria will be dictated by how well their adaptations match the physical and chemical environment presented to them by the plant. We have therefore initiated studies to determine the range of differences in epiphytic fitness exhibited by a given genotype of bacterium that is exposed to different environments prior to inoculation onto plant surfaces. Since bacteria that occupy specific habitats such as leaf surfaces are rather unique and exhibit superior ability to grow and survive in such habitats we expect that they posses unique traits that allow them to exploit such habitats. Little is known of the fitness traits that bacteria employ to exploit natural habitats such as leaf surfaces. If such fitness traits were better known, it should be possible to better define the range of habitats that an introduced bacterium would colonize based on its possession such attributes. Similarly, it should be possible to reduce the fitness of introduced organisms by eliminating habitat-specific fitness traits. We have therefore initiated studies to determine novel fitness determinants in the epiphytic bacterium, *Pseudomonas syringae*.

METHODS

Phenotypic Plasticity and Nature of Epiphytic Habitats

Pseudomonas syringae strain MF714 was grown for 12 hours either in Kings Medium B (KB) broth or agar at 28°C and harvested or inoculated onto bean plants 24 hours prior to use. Bacterial

cells were removed from the surface of plant by sonication for 5 minutes in distilled water and concentrated by centrifugation. Bacterial cells form each of these sources were suspended in distilled water at a concentration of 10^7 cells/ml and sprayed onto bean plants under field conditions or in a growth chamber with different temperature and relative humidity conditions. Bacterial populations were determined every 2 to 8 hours for up to 4 days after inoculation. Viable bacterial populations were determined by dilution plating onto KB containing 100 μg/ml rifampicin. The proportion of the viable cells that were culturable was determined by microscopic examination of cells recovered form leaf surfaces at different times that were exposed for 12 hours to solutions of dilute yeast extract containing 50 μg/ml nalidixic acid. The relative exposure of bacteria of different genotypes and of cells of a given genotypes exposed to different environmental conditions on leaf surfaces was determine by quantifying the ratio of cells surviving exposure to a topical treatment of 15% hydrogen peroxide or exposed to a flux of UV irradiation of 2000 erg/mm^2.

Determination of Epiphytic Fitness Determinants

Erwinia herbicola strain BRT89 was subjected to transposon mutagenesis with Tn5 delivered on the plasmid PDGS61 for which negative selection could be imposed. This plasmid consisted on pLAFR3 containing Tn5 and the levan sucrase gene from *Bacillus subtilis*. Strains containing the levan sucrase gene are killed when grown on media containing 5% sucrose. Tn-5 mutants were then inoculated onto bean plants and subjected to alternating periods of wetness and drying. The final population size of individual mutants on leaves after 73 hours of alternating environmental conditions was estimated by a measurement of the freezing temperature of individual leaves cooled slowly after immersion in tubes of ice nucleus-free water. The relationship between population size of Ice$^+$ strain BRT98 and freezing temperature of leaves was determined. Genomic regions of *Pseudomonas syringae* strain B728a identified by insertion of Tn-5 that confer epiphytic fitness were identified in a library of genomic clones of this strain using the flanking regions of cloned DNA containing Tn-5 from the mutants. Subcloning experiments on the genomic clones were followed by complementation studies to determine the minimum size of DNA necessary to confer the stress tolerance phenotype identified using these mutants.

RESULTS AND DISCUSSION

Pseudomonas syringae strains differed greatly in their behavior after application onto leaf surfaces as a function of the physiological condition of the cells prior to inoculation. Cells harvested from broth cultures died much more rapidly upon application to plant surfaces than cells harvested from agar plates. Broth-grown cells also required up to twice as long to resume growth after inoculation on leaf surfaces. In contrast, cells of *P. syringae* that were recovered from the

surface of bean leaves exhibited up to 1000-fold greater survival than cells harvested from broth culture after application to leaves. Cells recovered from leaves also resumed growth on leaf surfaces as much as twice as fast as even cells harvested from agar surfaces. These results indicate that bacteria exhibit considerable phenotypic plasticity. It is likely the pattern of gene expression in cells in natural habitats such as leaves are much different than that in culture. Predictions of microbial behavior upon field releases must take into account the great deal of phenotypic plasticity that microbes appear to possess.

Bacterial survival in natural habitats such as leaf surfaces appears to be greatly influenced by their modification of the habitat. The fraction of cells of *P. syringae* that survived on leaf surfaces was directly proportional to the concentration of cells initially applied to the leaf. While up to 10% of the cells applied as a suspension of 10^9 cells/ml survived during the first 48 hours after inoculation on leaves, only about 0.005 of the cells applied as a suspension of 10^3 cells/ml survived during this period. Application of 10^9 dead cells in conjunction with 10^3 live cells increased the survival of the 10^3 live cells to about 10%. The rate of immigration of cells from sites of field releases thus could greatly effect the efficiency with which they survive the transport from the field site and become established in an adjacent site.

The natural habitats of similar bacteria appear to differ greatly. For example, the ability of cells of different *P. syringae* strains to become established in "protected sites" on leaves differed greatly and differed greatly form other phylloplane residents such as *Erwinia herbicola* and *Xanthomonas maltiphilia*. The fraction of *P. syringae* cells that could not be killed by surface sterilization of leaves ranged from less than 0.2% to as much as 24% of the total bacterial microflora on a leaf. Most non-pathogenic bacterial species such as *E. herbicola* and *X. maltiphilia* did not exhibit more than 1% of the cells in such "protected sites". These great differences in habitat preference are unexpected and indicate that such preference should be carefully established for recombinant organisms to be released into the open environment for which a prediction of survival and subsequent behavior is desired. The eradication of bacteria having greatly different habitat preferences also may differ greatly and should be further investigated.

A novel leaf freezing assay is being used to assess the epiphytic fitness of Tn-5 -induced mutants of *E. herbicola*. Bean plants are being inoculated with individual transposon mutants of this species and subjected to alternating period of wetness and dryness. The final population size of the Ice^+ *E. herbicola* strain used in this study is proportional to the freezing temperature of leaves colonized by this strain. Mutants deficient in their ability to grow or survive on leaf surfaces are being identified by a reduction in the temperature at which leaves colonized by such a mutant strain freeze.

Transposon mutants of *P. syringae* which exhibit a reduced ability to survive the stresses of drying on leaf surfaces have been identified and are being characterized. Most of these

mutants do not exhibit a deficiency in any phenotype such as osmosensitivity that can be associated with stress tolerance. The genomic determinants of one such mutant that conditions the ability to survive on leaves as surface moisture drys from the leaf has been localized to a 6.5kb region. This region will compliment the reduced survival exhibited by the mutant strain. Fusions of this gene with a promoterless ice nucleation gene are being made to further localize the position and size of the genes involved in stress tolerance and to permit the assessment of the transcriptional activity of this region under natural conditions and under conditions of specific environmental stresses. The identification of such novel stress-tolerance genes should permit their identification in other bacterial to be released into the open environment and thus to indicate their potential for tolerating the stresses at field sites. Alternatively, such genes could be specifically inactivated to biologically contains organisms at field sites by making them incapable of surviving specific environmental stresses.

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COMPARISON OF SURVIVAL ON THE PHYLLOPLANE OF BACTERIA RELEASED IN GREENHOUSE AND FIELD ENVIRONMENTS

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INTRODUCTION

Because of current concerns regarding the release of genetically engineered microorganisms (GEMs) into the environment, the fate, survival, and effects of many GEMs will need to be evaluated in small-scale releases performed in controlled, contained environments. In our research, the use of greenhouses for predicting the results of field releases, and the influence of bacterial species, plant species and environmental conditions on bacterial survival in the greenhouse and the field were investigated.

Small-scale releases in controlled environments have been recommended to evaluate the effectiveness of genetically engineered microorganisms (GEMs) and to detect any potential adverse effects prior to their release into the environment. Microcosms have been used as controlled environments in which to conduct small-scale releases for the evaluation of the fate, survival and effects of GEMs in aquatic and terrestrial ecosystems. The use of microcosms in such studies has been criticized, however, because of their limited size and lack of biological, chemical and physical complexity relative to the natural environment.

Greenhouses can provide an environment intermediate between the microcosm and the field for studying the survival and effects of GEMs prior to field release. Greenhouses allow a larger scale release into a more complex environment than a microcosm yet retain some of the containment and control advantages of a microcosm.

As has been done with microcosm studies, greenhouse releases need to be evaluated for their ability to predict the results of field releases. In addition, the influence of bacterial species and plant species, and also meteorological conditions, on the ability of greenhouse releases to simulate field releases needs to be investigated. The goal of our research was to address these issues.

METHODS

Plant Growth Conditions and Experimental Design

The experiment was conducted once in the greenhouse and twice in the field. In each trial, *Erwinia herbicola* (NalR), *Klebsiella planticola* (RifR), and *Pseudomonas syringae* (RifR) were sprayed on bean plants (*Phaseolus vulgaris*) and on oat plants (*Avena sativa*) for a total of six plant-bacterium

treatments. Treatments were arranged in a split-strip plot experimental design. Five replicate plots of beans and of oats were randomly assigned and planted in the greenhouse and in the field. In the greenhouse, seeds were planted directly in the soil floor. Each oat and bean replicate plot was divided in thirds and randomly assigned a bacterial treatment for a total of five plot replicates per plant-bacterium treatment. The area of each treatment plot that was sprayed with a bacterial species was 5 x 10 ft in the field and 2 x 5 ft in the greenhouse. Unsprayed areas were maintained between plant-bacterium treatment plots to prevent cross-contamination.

Application of Bacteria to Plants

All bacterial suspensions for greenhouse and field sprays were prepared from 18 h cultures grown in Luria-Bertani (LB) broth with the appropriate antibiotics. The cells were washed 3 times in 0.01 M phosphate buffer (pH 7.2) by centrifuging for 10 min. at 5000 rpm and resuspended in sterile water to ca. 2×10^7 CFU/ml. Bacteria were applied when bean plants were beginning to flower and oat plants were forming seed head. Plants were uniformly sprayed until run-off with a CO_2 pressurized backpack sprayer.

Sample Collection and Processing

Plant samples were collected in sterile bags on days 1, 3, 7, 10, 14 and 21 post-application. In the greenhouse and field II experiments, samples were also collected two hours post-application. Bean samples consisted of 3 leaves per plot that were collected at low, middle and high levels of the plant canopy. Oat samples consisted of 3 plants per plot, with seed heads and roots removed. A total of 15 samples per plant-bacterium treatment was collected per sample day. Samples were immediately taken to the laboratory where they were weighed and processed by adding 20 ml of 0.01 M phosphate buffer (pH 7.2) and treating 1 minute in a Stomacher Lab-Blender. Samples were diluted in 0.01 M phosphate buffer (pH 7.2) and plated on LB agar containing 500 μg/ml nalidixic acid or 100 μg/ml rifampicin and 100 μg/ml cycloheximide and incubated at 30°C for 24 hours.

Monitoring Environmental Conditions

Meteorological data were collected during the greenhouse and field experiments with a 21x Micrologger. Temperature, relative humidity, and incident light were measured every minute and averaged per hour.

Data Analysis

Plate counts were converted to logarithmic colony forming units per gram of plant tissue (log CFU/g) and used in the Statistical Analysis System (SAS Institute) in a stepwise backward elimination regression procedure. Separate regression curves for each experiment with each bacterial species were fit in the same model by using classification variables. Patterns of bacterial survival were compared by testing the hypothesis that the intercept

and slope of the regression line for each bacterial genus was equal for the greenhouse, field 1, and field 2 experiments.

RESULTS AND DISCUSSION

Despite the application of equivalent bacterial concentrations in the trials, bacterial populations after only one day post-application were significantly lower in the field 1 and field 2 trials as compared to the greenhouse trial. In the greenhouse and field 2 trials, two hour post-application samples were also collected; bacterial populations in the field were on the average 10-fold lower than in the greenhouse. Substantially lower light levels occurred in the greenhouse trial than in the field trials. The initial large decrease in bacterial populations in the field 1 and field 2 experiments may have been caused by exposure to ultra-violet light. Solar radiation is an important environmental factor in the field that is not accurately represented in most greenhouse and microcosm simulations of field releases.

Because of the greater decline during the first day in bacterial levels in the field trials as compared to the greenhouse trial, the intercepts of the regression lines for the greenhouse trial were all significantly greater than those of the field trials. The slopes of the regression lines, however, were not statistically different between the greenhouse trial and field trial 1 for the oat *P. syringae* treatment and the oat and bean *E. herbicola* treatments.

Survival was very different among the three bacterial species. In addition, the extent of agreement in results between the greenhouse and field trials was influenced by bacterial species. The *K. planticola* treatment always showed a decline in population and reached the lowest bacterial level, whether on oat or bean plants or in the greenhouse or field. In contrast, the *P. syringae* and *E. herbicola* treatments displayed variable responses. Because *E. herbicola* and *P. syringae* commonly occur on the oat and bean phylloplane, unlike *K. planticola* which is primarily a soil inhabitant, their potential for survival and growth was higher than *K. planticola* but more variable due to a greater influence from meteorological conditions.

In the greenhouse experiment, populations for all three bacterial species were significantly greater on oat plants than on bean plants. In the field 1 and field 2 experiments, however, no significant differences in bacterial survival between plant species were observed. These results emphasize the need in simulated releases to evaluate survival of a bacterial species on the particular plant species on which it will be released in the field. Due to the initial decrease in bacterial levels in the field trials, the greenhouse experiment accounted for the maximum bacterial population levels observed in the field experiments in all but one treatment. Our results suggest that greenhouse experiments will be more successful in predicting trends in bacterial populations for field releases than actual population levels and that the predictive success of greenhouse experiments will vary with plant species, bacterial species, and environmental conditions.

FUTURE WORK

Our results indicate that meteorological conditions significantly influence the survival of bacteria applied to plants in field environments. Consequently, the use of environmental chambers or "mesocosms" that successfully reproduce the different meteorological factors of field environments will be required for accurate prediction of field releases.

Additional research evaluating the individual and collective influence of such variables as temperature, relative humidity, incident radiation, leaf moisture and rain fall on survival of applied microorganisms will be necessary. A statistical and experimental assessment of conducting such experiments is under discussion.

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NATIONAL ECOLOGICAL FIELD RESEARCH STATION

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The U.S. Environmental Protection Agency environmental biotechnology research program is charged with the development of methods to measure the impact of microorganisms released to the environment. The techniques and data generated by this research program are used by the Office of Toxic Substances and Office of Pesticide Programs in making decisions on the human and ecological safety of microorganisms under consideration for release into the environment. EPA sponsored biotechnology research programs have focused on the detection, fate, transport, survival, genetic exchange, effects, and monitoring methodology and design for field-released microorganisms in terrestrial ecosystems. The techniques and methods developed, to date however, are products of experiments conducted in laboratory, greenhouse, and microcosm environments. Many of the methods now being proposed for use in tracking and enumerating microorganisms such as DNA-hybridization (probes), polymerase chain reaction (PCR), and bulk recovery of DNA directly from soil samples, have yet to be tested over long periods of time under field conditions. While useful to the program offices and the biotechnology industry, none of the results have been validated under field conditions. The lack of a field research facility has rendered it impossible to integrate the many facets involved in the survival of microorganisms in the environment and has hindered the development of predictive risk assessment expertise. The development of permanent National Ecological Field Research Stations at which long-term experiments could be conducted would be a direct benefit to EPA and would be useful to EPA-funded researchers and biotechnology industry research scientists as well.

A potential site for a pilot field research facility is available at the EPA experimental farm on the northeast corner of the Nevada Test Site (NTS) in southern Nevada. The NTS is approximately 15,000 square miles of dedicated land used by the Department of Energy located 90 miles north of Las Vegas with a commute from the Environmental Monitoring Systems Laboratory (EMSL-LV) on U.S. Highway 95 of under two hours. The farm was originally used for radiation monitoring studies on plants and cattle but has been idle for several years. The farm site is comprised of 16 acres of flat agricultural fields with a gentle slope to the south. Electrical power, telephone service, and a well are in operation and only one small laboratory space is currently used on an infrequent basis by the Nuclear Radiation Division of the EMSL-LV for monitoring of range cattle and deer. The fields, remaining building space, and hookup site for research trailers would be available to biotechnology researchers. The farm

offers a secure site, removed from encroachment by development or intrusion by vandals. Low cost living and dining facilities, machine shop, and field engineering by the NTS on-site contractor are available within 15 miles of the farm. Extensive meteorological data are available indicating a climate with a full range of seasons. The maximum summer daytime temperature is customarily 95-96°F (approximately 10-15°F cooler than Las Vegas). Light snows are not uncommon in the winter although the average maximum daytime high temperature is above 60°F. High winds may blow across the site during the year but calm periods are common in the early morning.

Initial experiments at this pilot facility could include field validation of laboratory, greenhouse, and microcosm studies using non-recombinant bacteria and fungi. Such experiments would provide useful data to the program offices on the survival, dispersal, and fate of field-applied microorganisms and on the effectiveness of field monitoring methods. These experiments at a pilot site would also illustrate the advantages of the field research site concept.

While no one site could be expected to meet every possible environmental conditions, the NTS farm offers the unique opportunity of an existing EPA facility located in a remote yet accessible area within easy commuting distance to an EPA monitoring laboratory. The facilities at the farm are both generic and flexible to accommodate a wide range of testing parameters and conditions. This pilot facility would provide a baseline from which additional field research stations could be patterned.

OVERVIEW OF BIOTECHNOLOGY ENGINEERING RISK MANAGEMENT PROGRAM

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BACKGROUND

The biotechnology engineering subprogram is a small component of the Agency's Biotechnology Risk Assessment Research Program, which seeks to characterize risk through methods development, data collection and analyses, and mathematical modeling. It is comprised of research projects in three broad categories: environmental exposure, environmental effects, and risk control. The engineering risk management research activities, a major component of risk control, addresses three major concerns:

* Mechanisms of accidental or deliberate release of the modified genome or organism from the site of production (e.g., as in spills or effluents),

* Availability and effectiveness of containment controls or destruction techniques, and

* Worker exposure, particularly due to aerosols.

Potential commercial applications of biotechnology are divided into two loosely defined categories; (1) contained product manufacturing processes and (2) uncontained and semi-contained processes. Our approach has been to address these two applications individually, with our first and primary efforts devoted to contained product manufacturing as requested by the OTS.

In 1988, the first phase of the biotechnology assessment effort was completed, providing a state-of-the-art review entitled, "Final Report on Biosafety in Large-Scale rDNA Processing Facilities." This effort was undertaken to document existing information regarding (1) the potential source of hazards associated with large-scale fermentation facilities based on recombinant DNA, and (2) to identify biosafety practices to manage such risks. The report consists of four volumes which deal with the following subjects: a summary of applicable regulations, a review of biological characteristics of a large variety of microorganisms, unit operations and equipment used in production facilities, releases and containment, and methodologies for assessing worker exposure.

CURRENT PROJECTS

Assessment of Filter Containment of Bioaerosols

K. Willeke, Aerosol Research and Respiratory Protection Laboratory, Department of Environmental Health, University of Cincinnati; Cincinnati, OH, M. Lehtimaki, Technical Research Center of Finland; R. Wyza, Battelle Memorial Institute, OH.

The OTS has requested field verification of information used to estimate the effectiveness of filters to remove bioaerosols from fermenter off-gas. Bioaerosols are generated in significant numbers in fermenters by the combination of rheological properties of fermenter broths, mechanical agitation, and introduction of air/oxygen for growth and CO_2 by microbial activity. Techniques are being developed to employ off-the-shelf equipment to characterize the particle size distribution (by number frequency) using light-scattering. When operation is satisfactory, the instrumentation will be used in conjunction with the previous project in the field to: (1) assess the concentration of bioaerosol in the fermenter headspace and attempt to relate the concentration to operating conditions; (2) assess the amount of aerosol challenging the control filter; (3) determine if the apparatus has sufficient sensitivity to directly measure the filter efficiency; and (4) test the apparatus for its ability to identify sources of bioaerosol leakage into the workplace.

Containment Technology and Validation Techniques

G. Leaver, G.A.L. DeCosemo, and I.W. Stewart, Warren Springs Laboratory, U.K.; R.H. Cumming, Teeside Polytechnic; A.N. Cottam, Health and Safety Executive, U.K.; Al-Hassan, Birmingham University, Birmingham, Alabama.

In the United Kingdom, the Warren Spring Laboratory under the sponsorship of the Department of Trade and Industry, is developing techniques to evaluate process equipment containment. We have completed an agreement to support this work which addresses the following areas:

a. Development and Validation of Biological Fugitive Emission Measurement. This project investigates techniques for sampling, storage, and rapid assay of samples for biologically active materials. Procedures are being developed for optimum positioning and frequency of sampling for several techniques.

b. Containment Aspects of Bioprocess Equipment. Research is being conducted to develop standard methods for measuring containment of bioprocess unit operations and equipment components using various leak detection techniques so that a breach of containment can be detected before the introduction of viable biological materials. Laboratory studies are being conducted to evaluate specific methodologies and equipment. In-plant studies will be used to evaluate applicability to real work-place situations.

Evaluation of Alternative Methods of Assessment of Bioaerosols in Large-Scale rDNA Manufacturing Facilities

K. Martinez and P. Jensen, National Institutes of Occupational Safety and Health, Cincinnati, OH.

In preparation for conducting in-plant tests, we have evaluated commercial bioaerosol samplers designed for workplace sampling. This effort has provided the information needed to select the most cost-effective sampling system and design of a quality assurance program. This work is being performed for EPA by NIOSH experts who have conducted laboratory experiments on a variety of commercial samplers to determine sampling efficiency and influence on bacterial viability during sampling.

Evaluation of the Release of Bioaerosol from a Fermenter in a Large-Scale rDNA Processing Facility

K. Willike, Kettering Institute, University of Cincinnati, OH.

Bioaerosols were sampled at the gas off-take in the top of a fermenter to determine approximate particle size distribution and droplet concentrations exiting the fermenter.

FUTURE ACTIVITIES

Formation of Bioaerosols

Research into the formation of bioaerosols is a continuing need. The investigation of the formation of bioaerosols needs to be extended to the formation during separation processes to describe the formation of bioaerosols in the fermenter headspace based on typical operating conditions.

Efficiencies of Processes to Inactive Microorganisms

Research into the kill/inactivation efficiencies of various physical and chemical treatments commonly used for both sporeforming and nonsporeforming microorganisms is needed. Although limited data on inactivation efficiencies are available, experiments were conducted in demand-free water and do not represent the typical conditions in fermenters.

Release Applications of Microorganisms

As more biotechnology PMN's are reviewed, information concerning release scenarios for field tests and other non-contained uses covered by TSCA will be needed. An investigation of the potential use scenarios for release-applications of microorganisms should focus on the method of application, potential occupational exposures, protocols for monitoring workers during non-contained uses, and efficiencies of methods of controlling releases to minimize release to non-target areas of the environment.

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BIOTECHNOLOGY QUALITY ASSURANCE GUIDELINES MANUAL

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The Environmental Protection Agency requires every monitoring and measurement project to have a written and approved quality assurance (QA) plan. The purpose of the QA plan requirement is to ensure that the data will yield scientifically sound and unbiased conclusions related to the projects principle hypothesis. Quality assurance principles and practice have been previously published and guidance documents have been written to assist the physical science researcher in preparation of QA project plans but these publications were written with the emphasis on chemical research projects and do not specifically address many of the QA issues unique to biological and biotechnology-based research projects. Sampling, measurement, and quality control methods for biological and biotechnological parameters become increasingly more complex due to concerns for the viability of the organisms under study and biological/ biotechnological scientists are often confused by the terminology used in QA discussions owing to their unfamiliarity with this emerging area.

The purpose of this document is to describe and clarify QA and quality control (QC) responsibilities prescribed by the EPA for biotechnology researchers. This document is intended to be used as a guidance document and is not to serve as a template by the research scientist. Nor is this document intended to be an exclusive source of information on quality assurance considerations for biotechnology researchers. It is designed to provide a logical connection between EPA QA policy requirements and the research scientist focusing on biotechnological projects. It is intended to assist the scientist in achieving an understanding of QA and successfully implementing a quality assurance project plan (QAPP).

This manual will include discussion of the elements of a QAPP with particular emphasis on issues encountered with GEMs monitoring inthe environment and incorporating biological and biotechnolgical research activities not discussed in the available QA guidance literature. Comprehensive disucssions of QA principles, practical applications, and definitions are also included.

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