Environmental Risk Assessment of a Genetically-Engineered Microorganism: *Erwinia carotovora*

by

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(ABSTRACT)

Environmental use of genetically-engineered microorganisms (GEMs) has raised concerns over potential ecological impact. Development of microcosm systems useful in preliminary testing for risk assessment will provide useful information for predicting potential structural, functional, and genetic effects of GEM release. This study was executed to develop techniques that may be useful in risk assessment and microbial ecology, to ascertain which parameters are useful in determining risk and to predict risk from releasing an engineered strain of *Erwinia carotovora*.

A terrestrial microcosm system for use in GEM risk assessment studies was developed for use in assessing alterations of microbial structure and function that may be caused by introducing the engineered strain of *E. carotovora*. This strain is being developed for use as a biological control agent for plant soft rot. Parameters that were monitored included survival and intraspecific competition of *E. carotovora*, structural effects upon both total bacterial populations and numbers of selected bacterial genera, effects upon activities of dehydrogenase and alkaline phosphatase, effects upon soil nutrients, and potential for gene transfer into or out of the engineered strain.

No significant difference was found in survival of the engineered strain as compared to its wildtype parent. Both strains survived for over two months in microcosms. The effects of both strains upon populations of total bacteria and se-

lected bacterial genera were determined; while some effects upon community structure were observed, they were not significant.

The engineered strain was not found to be a superior competitor compared to its parent; three different doses of engineered and wildtype strains were used. In addition, neither strain affected activities of dehydrogenase or alkaline phosphatase in soil. Likewise, no effects were observed upon the nutrients monitored.

However, transfer of the kanamycin resistance gene that had been inserted into the engineered *E. carotovora* strain may have occurred. Five species of indigenous bacteria displayed kanamycin resistance 15 days after being exposed to the engineered *Erwinia*. DNA from these strains was isolated, purified, and hybridization experiments executed to determine if any homology existed between these DNAs and the kanamycin resistance gene that had been inserted into *E. carotovora*. Using biotin-labeled probes and low-stringency washing conditions, homology was observed. However, before gene transfer can be proven, additional studies, including amplification and sequencing, may be required.

Although a simple microcosm design was employed, it yielded sufficient information to conclude that release of the engineered *Erwinia carotovora* will not affect any of the microbial measures of integrity that were studied in a manner different from that of the wildtype. Effects upon plant material and other higher taxa will be the focus of future studies.

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PROLOGUE

During the 1970's, several techniques were developed that allowed the *in vitro* fragmentation and rejoining of molecules of deoxyribonucleic acid (DNA) and the placement of these molecules into living cells. Use of these recombinant DNA techniques led to the development of biotechnology, broadly defined as the application of biological organisms and their products to commercial processes (Office of Technology Assessment, 1988). Among types of organisms now being used for biotechnological applications, bacteria have been used more than others because of their short generation times, lack of a defined nucleus, and relatively 'simple' genetics as compared to eukaryotes. Genetic engineering of microorganisms is used to develop organisms capable of performing specific commercially-desired tasks. These applications include producing pesticides and pharmaceuticals, fixing nitrogen, leaching ores, recovering oil, degrading hazardous wastes, and constructing biological control agents for pests or diseases. Biotechnology products will, one hopes, benefit human society.

However, environmental applications of genetically engineered microorganisms (GEMs) has been limited to small-scale trials because of (1) a lack of knowledge about their impact on ecosystems and (2) an increased public awareness of concern over the release of novel organisms. Some ecologists, citing releases of starlings, gypsy moths, the Chestnut blight pathogen, and the Dutch elm disease pathogen, predict an "inevitable environmental disaster" while other scientists, especially molecular biologists, believe the chance of such an event is remote (Brill, 1988). Comparison of exotic species release to GEM release may not be valid since exotic species represent an exotic genome while GEMs may represent indigenous genomes altered only to a small extent (Office of Technology Assessment, 1988). To date, no adverse consequences have resulted from the small-scale release of

GEMs (Office of Technology Assessment, 1988); however, all of the releases at this time have entailed the use of microbes that have had parts of their genome removed or have had innocuous insertions that pose no obvious environmental threats. At some point, however, GEMs with capabilities of altering basic ecosystem processes, such as nitrogen fixation and nutrient cycling, may be released. The present research addresses the environmental release of GEMs and the possible environmental impact that they may have. It also examines attempts to construct a risk assessment scheme to predict the fate and effects of a GEM, produced from the bacterium *Erwinia carotovora*, released into terrestrial microcosms. The methodologies developed for this organism may be useful in risk prediction with other GEMs.

Several factors must be considered before GEMs are released in large quantities into the environment. Survival of an organism under laboratory conditions does not ensure survival in natural ecosystems. Conversely, ability to contain and control species in the laboratory does not preclude their establishment, survival, and adverse effects outside (Cairns and Pratt, 1986). Biotechnological products, especially the altered or engineered organisms themselves, present unique regulatory problems. Unlike toxic chemicals, organisms have the potential to increase in quantity in the environment at the site of release or following transport to some unintended and unexpected location.

GEMs may potentially affect the environment in through any one of three ways: (1) by transferring DNA from or into the GEM, thus altering the genetic composition of the GEM or indigenous bacterial populations, (2) by affecting the structural integrity of communities, both microbial and macrobial, and (3) by affecting functional processes, including geochemical cycling of nutrients.

Bacteria possess genetic mechanisms for the dispersal and alteration of their DNA; such mechanisms enhance bacterial survival in the environment (Grant and

Long, 1981). Four basic mechanisms for exchanging genetic material are known (Freifelder, 1987): (1) transformation, the uptake of naked DNA as plasmids in the case of Gram-negative bacteria or as linear DNA fragments by Gram-positive bacteria; (2) transduction, the incorporation of bacterial DNA in bacteriophage particles and their transfer to recipient bacteria; (3) conjugation, in which the DNA is transferred by direct cell-to-cell contact; and (4) fusion and recombination, a mechanism that is limited to actinomycetes.

Most, if not all, bacteria in the environment may exchange genetic information with nearby microbes (Slater, 1985; Wellington *et al.*, 1988). In considering the risk from potential transfer of the genome from a GEM to indigenous bacteria, several factors must be considered. First, molecular techniques confirm that gene transfer has indeed occurred. Such techniques may include DNA: DNA or DNA: RNA hybridization and sequencing of the DNA region in question. Second, if a transfer has indeed occurred, one must ascertain if the newly created genetic information will be sustained and expressed in the bacterium which acquired it. Third, if genetic information is maintained and expressed, the effects that such expression may have on other biota must be evaluated (Office of Technology Assessment, 1988). Several criteria, including gene transfer frequency and genetic distance among species, can be used in executing an assessment along with consideration of the construction of the gene itself and the vector on which the gene resides (Office of Technology Assessment, 1988).

While studies on potential genetic exchange mechanisms have been undertaken, few have examined these mechanisms outside the laboratory. Transfer frequencies on membranes are useful, but fail to provide information concerning selective pressures. In addition, DNA is rarely found in the naked, available state in environmental samples; rather, it is either degraded by exogenous nucleases or sorbed to soil or sediment particles and thus unavailable for transfer (Ogram *et al.*, 1988).

Such sorption is regulated by soil type, clay and organic material content, ionic strength, pH, and length of DNA (Ogram *et al.*, 1988).

Plasmid DNA transfer, via conjugation, in the environment has been studied in both terrestrial and aquatic systems. Plasmids (autonomously replicating, extrachromosomal, circular pieces of DNA) are ubiquitous in bacteria. Many environmental isolates have been shown to be recipients of plasmids. Genthner *et al.* (1988) tested 68 bacterial isolates from aquatic sources and found 26 of them able to receive plasmids from *Pseudomonas aeruginosa* donors. Schilf and Klingmuller (1983), using pRD1 carried by *E. coli*, discovered that 1.3% of soil bacteria and 17.3% of aquatic bacteria could receive the plasmid from a donor. Evidence for plasmid selection or exchange in the environment is also based upon high incidence of plasmids in polluted areas (Hada and Sizemore, 1981), coexistence of identical plasmids in different strains, and data from *in situ* transfer experiments (Grimes *et al.*, 1988). While plasmids are known to affect the growth of microorganisms, Cruz-Cruz *et al.* (1988) found that bacteria containing large (100 megadalton) plasmids may survive *in situ* equally well as those with smaller plasmids. They also suggest that genetic alteration of indigenous microorganisms is made possible by either conjugation or transformation in aquatic habitats. Bacterial conjugation has been observed in both non-sterile and sterile soils (Weinberg and Stotzky, 1972).

Transfer of plasmids also appears to be influenced by environmental influences. Stotzky and Krasovsky (1981) found that recombination was affected markedly by pH with the survival of both donors and recipients increasing up to a pH of 6.9. Moisture content of soil appears not to be influential if this is between 20-100% of the soils total holding capacity (Trevors and Oddie, 1986). In addition to transfer in soil and aquatic systems, plasmids may be transferred in plants (Lacy *et al.*, 1984).

The vast majority of DNA transfers in regard to GEMs appear to be innocuous; this is expected since antibiotic resistance from the marker plasmid is often the only parameter monitored. Nevertheless, it is conceivable that transfer of plasmids may affect important functional characteristics of the organism. Rafii and Crawford (1988) found that several conjugative plasmids were transferred in sterile soil from the donor, *Streptomyces lividans*, to several *Streptomyces* strains isolated from environmental samples. In a concurrent study, Wang and Crawford (1988) found that a similar *Streptomyces* strain caused significantly higher soil carbon mineralization rates in nonsterile soil amended with lignocellulose. They believe the effect is caused by a foreign DNA insert. The transfer of such an insert could alter the function of other organisms. In addition, Glick *et al.* (1986) found that introduction of plasmid DNA into *Azotobacter vinelandii*, via transformation, reduced its capacity for nitrogen fixation, mean cell size, and siderophore production.

Because plasmids are frequently passed from one organism to another (Slater, 1985), they may not be suitable for environmental applications. However, because of ease of manipulation and potential usefulness, they cannot simply be neglected; any risk attributed to them, however, must be predicted. Walter *et al.* (1987b) found that no single mating technique was superior in predicting the frequency of plasmid transfer; they developed a new technique, a combination of several older ones, that predicts the frequency with which plasmids move from one cell to another. Another group at the EPA's Corvallis laboratory (Knudsen *et al.*, 1988) developed a predictive computer simulation model that accurately forecasts transconjugant populations in both the rhizosphere and on leaf surfaces. This and other models not yet developed may eventually lead to accurate assessments of plasmid transfer prior to environmental release.

Transfer of DNA via transduction has also been documented in environmental samples from both soil and water. Zeph *et al.* (1988) found that transduction of *E.*

coli by bacteriophage P1 occurred in soil, both sterile and nonsterile; however, they did not observe transduction of indigenous organisms. It appears that a minimum number of bacteria are needed to ensure replication of the phage (Wiggins and Alexander, 1985). This limit minimizes viral damage in the environment because of the relatively low populations of individual bacteria (Wiggins and Alexander, 1985). Transduction in aquatic samples has also been demonstrated by Saye *et al.* (1987), using *P. aeruginosa* containing plasmid Rms149, although the presence of natural microbial communities significantly decreased transduction frequency.

Transformation, especially in *Bacillus subtilis*, has been shown in nonsterile soil (Graham and Istock, 1978, 1979). However, Greaves and Wilson (1970) found that DNA or RNA added to nonsterile soil was rapidly degraded, thus causing an increase in indigenous non-transformed populations.

Transfer of genetic information is a viable concern when designing a risk assessment scheme to predict potential environmental consequences. Recent technologies, such as suicide vectors (Bej *et al.*, 1988) may eventually prevent movement of recombinant genes. Even more important than the actual transfer of genetic information, however, is the expression of that genetic information. If such expression is to be manifested, the bacterium must survive after it is released. Alexander (1985) surmises that if a GEM is to have an effect, good or bad, it must survive. The question of survival is central to both the pro and con arguments regarding the efficacy of GEMs.

A great deal of information has been acquired regarding survival of single species of bacteria, especially *Rhizobium* (Stacey, 1985). However, scant data have been obtained concerning the release of non-root colonizing bacteria into an environment that may or may not have originally contained the bacterium being released. The data collected from introductions of *Rhizobium* (and other bacteria) are

useful in comprehending biotic and abiotic factors that affect microbial survival. These data will be reviewed here.

Abiotic factors that affect bacterial growth in the environment include substrate type, nutrient availability, temperature, desiccation, salinity, and pH (Grant and Long, 1981; Stacey, 1985). Among the most important for a newly released GEM are nutrient availability, temperature, and pH. Severe fluctuations in any of these may severely compromise GEM integrity. Even if the GEM encounters conditions favorable for its establishment, it must contend with predation by phages or protozoans. Predation has been shown to adversely affect *Rhizobium* populations (Stacey, 1985). In addition, deleterious enzymes or antibiotics produced by indigenous microbes may cause elimination of the introduced species. However, effects of many of these factors can be minimized by increasing concentrations of bacteria being applied to the area of interest. With this rationale, though, comes an increased chance of producing runoff that may carry the GEM to an area not originally intended.

It has been demonstrated that bacteria, engineered or not, do not survive for extended periods of time because of environmental pressures exerted on them. Indeed, if the bacteria being introduced have been derived from indigenous species, they will have to outcompete these species which are already firmly established in their niche. To make these introduced microbes predominate, strong selective pressure will have to be applied (Stacey, 1985). Alexander (1985) summarizes the situation bacteria encounter by saying that bacteria die when they fail to overcome any of the biotic or abiotic factors that they encounter.

Predicting survival prior to release is an important parameter in risk assessment. Walter *et al.* (1987a) developed a technique that allows determination of survival of bacteria in soil extracts. This technique appears to be useful for an industrial assay in initial data generation. The group found that engineered strains survive longer in sterile soil than in nonsterile soil. This is not surprising since a lack of competi-

tion would encourage survival. Using antibiotic-resistant strains produced by mutagenesis, Van Elsas *et al.* (1986) released *P. fluorescens* and *Bacillus subtilis* into field plots. They observed that *B. subtilis* declined rapidly while *P. fluorescens* declined at a slow, steady rate and survived for up to 120 days at detectable levels. Using rifampin-resistant spontaneous mutants of *P. fluorescens* and *P. putida*, Compeau *et al.* (1988) found that limited sites exist in soil for colonization by either bacterial strain. In addition, they determined that rifampin resistance is not always harmless and may, in fact, confer a competitive disadvantage to the bacterium. They suggest that all strains being used in a study be compared for growth rate in media to ensure that no differences are present. In all of the GEM soil survival studies to date, bacteria have always declined in concentration over time; this is not unexpected because of the enormous competition they encounter.

Little data exists for GEM release into aquatic systems. Like their terrestrial counterparts, bacteria decline when released into aquatic microcosms (Burton *et al.*, 1987). Sostarec *et al.* (1988) found that engineered *E. carotovora*, the same strain as used in this research, declined at the same rate as its wildtype parent.

While recent documentation exists for survival of GEMs in soil, few studies have been executed to measure aerial dispersion of microorganisms from plants and soil; little or no research has been undertaken to elucidate the mechanisms, such as wind speed, plant leaf condition and age, inoculum age, and moisture, that may influence dispersal of GEMs into unanticipated areas. Because of their small mass, bacteria and viral particles take extensive periods of time to settle out of the atmosphere; therefore, they may be transported over long distances (Barnthouse and Palumbo, 1986). Bacteria have been shown to travel for hundreds of kilometers on air currents, however, bacteria carried by plant tissue or airborne soil particles settle far more rapidly (Barnthouse and Palumbo, 1986).

Bacterial plant pathogens have been found to disseminate in aerosols produced by raindrop impacts, irrigation systems, and mechanical manipulation (Harrison, 1980) and are capable of inducing pathogenesis after movement. Lindemann & Upper (1985) observed that bacteria reentrain on plant surfaces and soil after being disseminated in aerosols; in addition, they discovered that plant canopies are excellent sources of these aerosols when leaves are dry on sunny days. More recently, two studies have documented that bacteria altered by natural processes do indeed aerially disperse from a site of introduction. Marshall *et al.* (1988) found that *E. coli*, containing a Tn*5* derivative of the plasmid ColE1, dispersed from the sites of introduction, a laboratory office and a barn, with maximal dispersion being observed in the first 30 minutes after release. While rapid aerosol die-off was observed, it is conceivable that many of the bacteria attained a viable, but non-culturable status (Colwell *et al.*, 1985) that was not detected. Such bacteria could increase in concentration if favorable conditions are encountered. Marshall *et al.* also learned that, in several instances, *E. coli* containing ColE1 and Tn*5* demonstrated enhanced survival, apparently related to a growth advantage from the transposon. In a more environmentally realistic study, Lindow *et al.* (1988) determined that viable *Pseudomonas syringae* cells experienced an exponential decrease in concentration as distance from aerosol application site increased. While survival rate of bacteria was 10 times higher on the intended plants than on adjacent plants, such dispersion was demonstrated even with relatively calm winds (0.73 to 1.5 m/s) that were not favorable to dissemination. Organisms on the soil surface appeared to die rapidly as expected since *P. syringae* is an epiphyte.

In a study of the first releases of a recombinant bacterium, Seidler and Hern (1988) reported that low numbers of *P. syringae* and *P. fluorescens* left test plots even in the presence of minimal wind conditions; they also found that strong vertical mixing occurred at three spray releases. Even though 99.99% of the introduced

bacteria remained on the soil and plants for which they were intended, some 1.2 x 10^6 colony forming units (CFU) of bacteria migrated onto a 15 meter buffer zone during the course of one experiment; bacteria could be found around the site two days after the test. In addition, a greater drift of bioaerosol particles occurred in an experiment conducted at Tulelake, CA as compared to experiments carried out at Brentwood, CA. Seidler and Hern believe that a larger sprayed area and a greater source strength of bacterial inoculum may have significantly affected aerosol drift during the one release. Clearly, additional investigation is warranted to determine which factors contribute to the aerial dispersion of GEMs during and after application.

A major problem in ascertaining GEM survival are techniques available to the investigator. Currently, indirect methods such as the plate count are limited in that no one medium will support the growth of all bacterial species present in a particular sample (Mills and Bell, 1986). However, in the studies described later in this work, the plate count has proven to be an inexpensive, yet reliable, method of determining concentrations of GEMs and indigenous bacteria despite the views of many microbial ecologists (Mills and Bell, 1986). This conclusion is also supported by Olsen and Bakken (1987). In addition, use of selective media allows enumeration of communities of interest. Direct counting of bacteria using simple or fluorescent (Daley, 1979) stains contributes little information since it is impossible to detect the organism of interest from the remainder of the sample. Use of fluorescent antibodies, specific for the organism in question, have not been as useful as initially hoped because of low sensitivity and presence of contaminating substances in environmental samples (Levin *et al.*, 1987).

Molecular techniques for detection of GEMs show promise but are more complicated and expensive than conventional technologies. Methods have been developed to ascertain the amount of dissolved environmental DNA (DeFlaun *et al.*, 1986)

as well as DNA from bacterial cells (Holben *et al.*, 1988; Steffan *et al.*, 1988). Use of this DNA in hybridization studies allows detection of GEMs by genotype rather than phenotype as well as allowing detection of nonculturable microbes (Barkay and Sayler, 1988). Application of these techniques have allowed detection of as few as 10 cells/g soil (Fredrickson *et al.*, 1988). However, such use of probes in the past usually resulted in low sensitivity (Barkay and Sayler, 1988; Levin *et al.*, 1987). The application of the polymerase chain reaction (PCR) (Saiki *et al.*, 1988) for use in environmental samples by Steffan and Atlas (1988) has shown promise for GEM detection. Steffan and Atlas (1988) reported the detection of 1 cell/gram sediment using *P. cepacia* containing 15 to 20 copies of a 1.3 kilobase (kb) gene. However, use of the PCR technique with GEMs containing only one gene per organism has not been especially productive because of environmental contamination and low initial DNA amounts (James Tiedje, personal communication). Hence, further methods development is indicated before the technique can be exploited to its maximum efficiency.

The problem in using GEM survival in developing a risk assessment protocol is that the GEM may indeed be present but undetectable. Colwell *et al.* (1985) coined the term 'viable, but non-culturable' for such a situation. In their study, Colwell's group found that, in both microcosms and field tests, *Vibrio cholerae* were present in higher numbers when determined by immunofluorescent microscopy than by plate counts. Thus, even when plate counts yielded negative information concerning survival, bacteria were indeed present.

Two other parameters exist where GEMs may cause an unexpected effect. One is that GEMs may cause alterations in community structure. Ecosystems have two fundamental properties: 1) they are composed of structures, and 2) these structures facilitate specific functions (Cairns & Pratt, 1986). Since structure and function are closely related, an alteration in one may soon affect the other. A variety of studies

will be required to estimate probable effects resulting from the controlled or uncontrolled release of GEMs (Chakrabarty *et al.*, 1984; Rissler, 1983; Cairns & Pratt, 1986). Complex ecological effects resulting from displacement of native bacterial populations may occur, especially when GEMs are derived from common free-living taxa (Liang *et al.*, 1982). Displacement of bacterial groups could produce an alteration of detrital processing and nutrient cycling that could have broad consequences, especially if introduced organisms have high fitness and are transferred among ecosystems (Cairns & Pratt, 1986). Many ecologists believe that important processing bacteria are the same for virtually all comparable ecosystems. The probability of such adverse effects may be small, but failure to evaluate the potential risks could be disastrous.

Functional effects would have severe consequences if a crucial parameter was affected or if the assimilative capacity of the ecosystem (Cairns, 1977) was exceeded. Bacteria decomposing detritus produce the majority of energy in most aquatic ecosystems (Wetzel, 1983); the mineralization of this material is the most important role of bacteria (Fenchel & Blackburn, 1979). Most processing occurs in sediments and is linked tightly to the processing of other vital nutrients such as nitrogen, sulfur, and phosphorus (Fenchel & Blackburn, 1979). These processes, mediated mostly by bacteria, are vital to continued ecosystem function. Surface-dwelling communities of microorganisms are exceedingly complex (Shapiro, 1985) and demonstrate the rapid exchange of materials being recycled. In addition to having similar functional attributes, most ecosystems boundaries are imprecisely defined. The exports of energy and nutrient bearing materials from one ecosystem become the imports of another. At the microbial level, most imported and exported materials carry a portion of the resident biota (Pimentel, 1985) thus providing the opportunity for introduction of GEMs into new habitats. However, no consensus exists on what parameters in the environment should be monitored even when

dealing with chemical impacts. The number of parameters that may be monitored are numerous and include nutrient and enzyme concentrations, production/respiration ratios, oxidation-reduction potentials, and pH shifts. However, no agreement exists on what is a "normal" value for a particular parameter under a particular set of circumstances (Cairns and Pratt, 1986). Indeed, far more research is indicated in this area for both chemicals and GEMs.

It has been shown that GEMs are able to produce functional effects that are not observed in the parent organism. Glick *et al.* (1986) found that the introduction of plasmid DNA into *Azotobacter vinelandii* caused a decrease in nitrogen fixation, mean cell size, and siderophore synthesis. Glick *et al.* hypothesized that the plasmid introduction imparted a metabolic load on the bacterium that impaired its ability to function. Wang and Crawford (1988) found that a recombinant strain of *Streptomyces lividans* significantly affected soil organic carbon turnover rates. They noted that the organism displayed an increased mineralization rate, particularly in soils amended with lignocellulose; these rates remained significantly higher over the 30 day duration of the test.

Because of the uncertainity of GEM releases, it is likely that small, easily contained microcosms will form the basis for preliminary assessments of ecological effects (A.D. Little, Inc., 1984). Such studies have proved useful for studying a number of ecological problems, although results must be interpreted with caution (King, 1980). The small size and contained nature of these microcosms make possible effective dose-response studies for possible dissemination of recombinant DNA. Small or large innoculum levels of donor and recipient bacteria may be used with safety since the laboratory environments may be autoclaved. Further, the small size of the chambers makes introducing various environmental stresses possible; such stresses may effect the frequency of DNA exchange. Microcosm construction, optimization, and limitations will be discussed further in the next chapter.

Before GEMs can be released into the environment, manufacturers must comply with existing Federal and local regulations. Most commercial releases are controlled by the Environmental Protection Agency whose regulation is mandated by the Toxic Substances Control Act (TSCA) (Cohrssen, 1988). At the present time, those desiring to release a GEM must first issue a premanufacturing notice (PMN) describing the organism and its characteristics such as survival and potential effects. These PMNs must comply with the Coordinated Framework for Regulation of Biotechnology (Federal Register, 1986). PMNs have been used by the chemical industry for well over a decade. However, it is important to note that risk assessment protocols have been developed and proven for chemical use whereas they have not been validated for use with self-multiplying GEMs.

Suitable risk assessment protocols have not yet been developed, in part, because an agreement of what should be monitored and when has not been reached. Prior to a release of a chemical or GEM into the environment, data must be collected so as to provide a baseline for data that is collected after the release (Cairns and Pratt, 1986). Many suitable risk assessment protocols have been developed for chemicals since these have been proven and used by industry for some time; with modifications to accommodate the unique characteristics of GEMs, these may be useful (Dean-Ross, 1986). However, molecular biologists and microbial ecologists charged with assessing the risk from GEM release fail to understand the process of risk assessment. Many molecular biologists, especially those involved with commercial applications, feel that the only risk associated with GEM release is that of gene transfer and they believe that even that risk is remote (Brill, 1988).

Chemical hazard assessment is five-fold: risk identification, risk characterization, risk source exposure assessment, hazard assessment, and estimation of risk (Cairns, 1982; Orvos and Cairns, 1987). Risk identification for GEMs may be broadly thought of in terms of gene transfer, structural effects, and functional effects. Ability

to characterize these risks is dependent upon the site under consideration, the GEM itself, and the parameters being monitored (Molak and Stara, 1988). Assessing exposure will include studies of survival, displacement, and pathogenicity. Hazard assessment, the subject of the majority of this dissertation, involves a tier approach to determine the acute or chronic effects of a particular GEM. With increasing complexity of the test system comes increased costs, decreased replicability, and more environmentally realistic data. Estimation of risk is based upon all of the data collected as well as model development and predictive value.

GEMs, especially non-pathogenic ones, may not have clear-cut effects and the traditional toxicological dose-response paradigm may not be applicable to evaluating the ecological effects of introduced organisms. This does not mean that such a paradigm may not be present nor does it imply that protocols developed for chemicals are not useful for GEMs. Careful modification of such protocols will make them useful for GEMs. However, additional technologies are needed to enumerate GEMs at low numbers and to measure any functional alterations produced by them. While progress has recently been made on enumeration, such as the polymerase chain reaction (Steffan and Atlas, 1988; Jain *et al.*, 1988) and improved DNA isolation procedures (Steffan *et al.*, 1988), a great deal of refinement will be necessary before they are fully used (J. Tiedje, personal communication).

Efforts by the EPA have centered on methods development since they must underlie any accepted protocol (Levin et al., 1987). While some of the factors for establishment of microorganisms are known, far more research is indicated to ascertain the effects of environmental factors on GEMs and their DNA.

For the research described in subsequent chapters, *Erwinia carotovora* subsp. *carotovora* has been chosen as a model since this strain (L-833) (Allen *et al.*, 1986) has been engineered specifically to include two types of genetic alterations in the bacterial chromosome: deletion of a gene fundamental to plant pathogenesis and

inclusion of an antibiotic resistance gene. With this model, the possibility that the antibiotic resistance marker may become integrated into the genomes of other microbes that come into contact with it will be examined. *E. carotovora* is a pathogenic member of the Enterobacteriaceae that produces soft rot in plant tissues and causes more than $100,000,000 in losses of stored food annually despite control measures that are primarily environmental in nature. Soft-rotting bacteria are ubiquitous and exist in low populations as epiphytes on underground surfaces of healthy plants (Stanghellini, 1982). If the host is physiologically compromised and environmental conditions are favorable, these resident bacteria multiply rapidly and initiate pathogenesis. Therefore, disarmed bacteria applied to seed at planting will probably protect plants from soft rot since similar studies using fluorescent pseudomonads and non-pathogenic agrobacteria as biological control agents have been successful (Suslow, 1982; Lindow, 1983).

Soft rot is a result of a battery of enzymes produced by the pathogen that break down components of plant cells and release substrates for bacterial growth. These include pectic enzymes, cellulases, and proteases (Chatterjee & Starr, 1980). Pectic enzymes are responsible for maceration of plant tissues; other enzymes are ineffective without pectic enzymes. Among pectic enzymes, extracellular endo-pectate lyases play major roles in plant cell death and tissue maceration (Collmer *et al.*, 1982; Stack *et al.*, 1980). Endo-pectate lyases cleave pectic acid which is a component of all plant cell walls involved in the structural integrity of plant tissues (Bateman & Basham, 1976). Cleavage of pectic materials by endo-pectate lyase causes cell separation or tissue maceration and tissues so affected lose turgidity and become soft and slimy to the touch. Therefore, the GEM used in these studies is the precursor to an engineered biological control agent designed for environmental release.

Routine ecological assessment methods must need to be developed for use by biotechnology industries to ensure the soundness of future economic development of biotechnology products and to provide necessary information to regulatory agencies. Current case-by-case criteria will soon become useless if the expansion of biotechnology industries continues. Site-specific testing will be required and such testing must be scientifically justifiable and have general transferability to several potential receiving ecosystems. Testing schemes will need to be easily interpretable by industry and regulatory analysts. If suitable protocols are not developed, the environment will undoubtfully be compromised at some point in the future.

LITERATURE CITED

A.D. Little, Inc. 1984. Risk Analysis Methods for Environmental Applications of Biotechnology. Cambridge, MA. 35 pp.

Alexander, M. 1985. Ecological consequences: reducing the uncertainties. Issues in Science and Technology. Spring: 57-68

Allen, C., V.K. Stromberg, F.D. Smith, G.H. Lacy, and M.S. Mount. 1986. Complementation of an *Erwinia carotovora* subsp. *carotovora* protease mutant with a protease-encoding cosmid. Mol. Gen. Genet. 202:276-279.

Barkay, T. and G.S. Sayler. 1988. Gene probes as a tool for the detection of specific genomes in the environment, *In* W.J. Adams, G.A. Chapman, and W.G. Landis (eds.), Aquatic Toxicology and Hazard Assessment: 10th volume, ASTM STP 971. American Society for Testing and Materials, Philadelphia, pp. 29-36.

Barnthouse, L.W. & A.V. Palumbo. 1986. Assessing the transport and fate of bioengineered microorganisms in the environment. [In] J. Fiksel & V.T. Covello (eds.), Biotechnology Risk Assessment, Pergamon Press, pp. 109-128.

Bateman, D.F., and H.G. Basham. 1976. Degradation of plant cell walls and membranes by microbial enzymes. p.316-355. [In] R. Heitefuss and P.H. Williams, (eds.), Physiological Plant Pathology, Vol. 4. Encyclopedia of Plant Physiology, Springer-Verlag, N.Y., 880pp.

Bej, A.K., M.H. Perlin, and R.M. Atlas. 1988. Model suicide vector for containment of genetically engineered microorganisms. Appl. Environ. Micro. 54:2472-2477.

Brill, W.J. 1988. Why engineered organisms are safe. Issues in Science and Technology, Spring, 1988.

Burton, G.A., Jr., D. Gunnison, and G.R. Lanza. 1987. Survival of pathogenic bacteria in various freshwater sediments. Appl. Environ. Micro. 53:633-638.

Cairns, J., Jr. 1977. Aquatic ecosystem assimilative capacity. Fisheries 2(2):5-7, 24.

Cairns, J., Jr. 1982. Environmental Effects. [In] G.F. Bennett, F.S. Feates, and I. Wilder (eds.), Hazardous Materials Spills Handbook, McGraw-Hill, New York, pp. 415-421.

Cairns, J., Jr. and J.R. Pratt. 1986. Ecological consequence assessment: effects of bioengineered organisms. [In] J. Fiksel & V.T. Covello (eds.), Biotechnology Risk Assessment, Pergamon Press, pp. 88-108.

Chakrabarty, A.M., J.S. Karns, J.J. Kilbane, and D.K. Chatterjee. 1984. Selective evolution of genes for enhanced degradation of persistent, toxic chemicals. [In] W. Arber, K. Illmensee, W.J. Peacock, and P. Starlinger (eds.), Genetic Manipulation: Impact on Man and Society, Cambridge University Press, NY. pp. 43-54.

Chatterjee, A.K., and M.P. Starr. 1980. Genetics of *Erwinia* species. Annu. Rev. Microbiol. 34:645-676.

Cohrssen, J.J. 1988. United States biotechnology policy. Amer. Biotech. Lab. 1:22-27.

Collmer, A., P. Berman, and M. Mount. 1982. Pectate lyase regulation and bacterial soft rot pathogenesis. Pp. 395-422 [In] M.S. Mount and G. H. Lacy (eds.), Phytopathogenic Prokaryotes. Vol. 1, Academic Press, Inc., N.Y. 541 pp.

Colwell, R.R., P.R. Brayton, D.J. Grimes, D.B. Roszak, S.A. Huq, and L.M. Palmer. 1985. Viable but non-culturable Vibrio cholerae and related pathogens in the environment: Implications for release of genetically engineered microorganisms. Biotechnology. 3:817-820.

Compeau, G., B.J. Al-Achi, E. Platsouka, and S.B. Levy. 1988. Survival of rifampin-resistant mutants of *Pseudomonas fluorescens* and *Pseudomonas putida* in soil systems. Appl. Environ. Micro. 54:2432-2438.

Cruz-Cruz, N.E., G.A. Toranzos, D.G. Ahearn, and T.C. Hazen. 1988. In situ survival of plasmid-bearing and plasmidless *Pseudomonas aeruginosa* in pristine tropical waters. Appl. Environ. Micro. 54:2574-2577.

Daley, R.J. 1979. Direct epifluorescence enumeration of native aquatic bacteria: uses, limitations, and comparative accuracy. [In] J.W. Costerton and R.R. Colwell (eds.), Native Aquatic Bacteria: Enumeration, Activity, and Ecology, ATSM STP 695, American Society for Testing and Materials. pp. 29-45.

Dean-Ross, D. 1986. Release of genetically engineered organisms: hazard assessment. ASM News 52:572-575.

DeFlaun, M.F., J.H. Paul, and D. Davis. 1986. Simplified method for dissolved DNA determination in aquatic environments. Appl. Environ. Micro. 52:654-659.

Federal Register. 1986. Coordinated framework for regulation of biotechnology. Fed. Reg. 51(123):23302-23393.

Fenchel, T. and T.H. Blackburn. 1979. Bacteria and mineral cycling, Academic Press, NY., 225pp.

Fredrickson, J.K., D.F. Bezdicek, F.J. Brockman, and S.W. Li. 1988. Enumeration of Tn*5* mutant bacteria in soil by using a most-probable-number DNA hybridization procedure and antibiotic resistance. Appl. Enviro. Micro. 54:446-453.

Freifelder, D.M. 1987. Microbial Genetics, Jones and Bartlett, New York, 601 pp.

Genthner, F.J., P. Chatterjee, T. Barkay, and A.W. Bourquin. 1988. Capacity of aquatic bacteria to act as recipients of plasmid DNA. Appl. Environ. Micro. 54:115-117.

Glick, B.R., H.E. Brooks, and J.J. Pasternak. 1986. Physiological effects of plasmid DNA transformation on *Azotobacter vinelandii*. Can. J. Micro. 32:145-148.

Graham, J.B. and C.A. Istock. 1978. Genetic exchange in *Bacillus subtillis* in soil. Mol. Gen. Genet. 166:287-290.

Graham, J.B. and C.A. Istock. 1979. Gene exchange and natural selection cause *Bacillus subtillis* to evolve in soil culture. Science 204:637-639.

Grant, W.D. and P.E. Long. 1981. Environmental Microbiology. Halsted Press, Glasgow, Scotland, 215 pp.

Greaves, M.P. and M.J. Wilson. 1970. The degradation of nucleic acids and montmorillonite-nucleic acid complexes by soil microorganisms. Soil Biol. Biochem. 2:257-268.

Grimes, D.J., C.C. Somerville, W. Straube, D.B. Roszak, B.A. Ortiz-Conde, M.T. MacDonell, and R.R. Colwell. 1988. [In] W.J. Adams, G.A. Chapman, and W.G. Landis (eds.), Aquatic Toxicology and Hazard Assessment: 10th volume, ASTM STP 971. American Society for Testing and Materials, Philadelphia, pp. 37-42.

Hada, H.S. and R.K. Sizemore. 1981. Incidence of plasmids in marine *Vibrio* spp. isolated from an oil field in the northwestern Gulf of Mexico. Appl. Environ. Microbiol. 41:199-202.

Harrison, M.D. 1980. Aerial dissemination of bacterial plant pathogens. Annals of N.Y. Aca. Sci. 353:94-104.

Holben, W.E., J.K. Jansson, B.K. Chelm, and J.M. Tiedje. 1988. DNA probe method for the detection of specific microorganisms in the soil bacterial community. Appl. Environ. Micro. 54:703-711.

Jain, R.K., R.S. Burlage. 1988. Methods for detecting recombinant DNA in the environment. CRC Critical Rev. Biotech. 8:33-84.

King, D.L. 1980. Some cautions in applying results from aquatic microcosms. [In] J.P. Giesy, Jr. (ed.), Microcosm in Ecological Research, National Technical Information Service, Springfield, VA. pp. 164-191.

Knudsen, G.R., M.V. Walter, L.A. Porteous, V.J. Prince, J.L. Armstrong, and R.J. Seidler. 1988. Predictive model of conjugative plasmid transfer in the rhizosphere and phyllosphere. Appl. Environ. Micro. 54:343-347.

Lacy, G.H., V.K. Stromberg, and N.P. Cannon. 1984. *Erwinia amylovora* mutants and *in planta* derived transconjugants resistant to oxytetracycline. Can. J. Plant Pathol. 6:33-39.

Levin, M.A., R. Seidler, A.W. Borquin, J.R. Fowle III, and T. Barkay. 1987. EPA developing methods to assess environmental release. Biotechnology 5:38-45.

Liang, L.N., J.L. Sinclair, L.M. Mallory and M. Alexander. 1982. Fate in model ecosystems of microbial species of potential use in genetic engineering. Applied Env. Micro. 44:708.

Lindemann, J. and C.D. Upper. 1985. Aerial dispersal of epiphytic bacteria over bean plants. Appl. Environ. Micro. 50:1229-1232.

Lindow, S.E. 1983. Methods of preventing frost injury caused by epiphytic ice-nucleation-active bacteria. Plant Dis. 67-327-333.

Lindow, S.E., G.R. Knudsen, R.J. Seidler, M.V. Walter, V.W. Lambou, P.S. Amy, D. Schmedding, V. Prince, and S. Hern. 1988. Aerial dispersal and epiphytic survival of Pseudomonas syringae during a pretest for the release of genetically engineered strains into the environment. Appl. Environ. Micro. 54:1557-1563.

Marshall, B., P. Flynn, D. Kamely, and S.B. Levy. 1988. Survival of Escherichia coli in a laboratory and a farm environment. Appl. Environ. Micro. 54:1776-1783.

Mills, A.L., and P.E. Bell. 1986. Determination of individual organisms and their activities *in situ*. [In] R.L. Tate (ed.), Microbial Autecology, Wiley, New York, pp. 27-60.

Molak, V. and J.F. Stara. 1988. Genetically engineered microorganisms in the aquatic environment: environmental safety assessment. *In* W.J. Adams, G.A. Chapman, and W.G. Landis (eds.), Aquatic Toxicology and Hazard Assessment: 10th volume, ASTM STP 971. American Society for Testing and Materials, Philadelphia, pp. 43-50.

Ogram, A., G.S. Sayler, D. Gustin, and R.J. Lewis. 1988. DNA adsorption to soil and sediments. Environ. Sci. Tech. 22:982-984.

Office of Technology Assessment. 1988. New Developments in Biotechnology - Field-Testing Engineered Organisms: Genetic and Ecological Issues. OTA-BA-350, Washington, D.C., 150 pp.

Olsen, R.A. and L.R. Bakken. 1987. Viability of soil bacteria: optimization of plate-counting technique and comparison between total counts and plate counts within different size groups. Micro. Ecology 13:59-74.

Orvos, D.R. and J. Cairns, Jr. 1987. Hazmat and Habitat. Fire Engineering 140(7):24-35.

Pimentel, D. 1985. Using genetic engineering for biological control: reducing ecological Pectic enzyme complex from risks. [In] H.O. Halvorson, D. Pramer, and M. Rogul (eds.), Engineered Organisms in the Environment: Scientific Issues, ASM, Washington. pp. 129-140.

Rafii, F. and D.L. Crawford. 1988. Transfer of conjugative plasmids and mobilization of a nonconjugative plasmid between *Streptomyces* strains on agar and in soil. Appl. Environ. Micro. 54:1334-1340.

Rissler, J.F. 1983. Research needs for biotic environmental effects of genetically-engineered microorganisms. AAAS/EPA Science and Engineering Fellows Program. 40pp.

Saiki, R.K., D.H. Gelfand, S. Stoffel, S.J. Scharf, R. Higuchi, G.T. Horn, K.B. Mullis, H.A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239:487-491.

Saye, D.J., O. Ogunseitan, G.S. Sayler, and R.V. Miller. 1987. Potential for transfer of plasmids in a natural freshwater environment: effect of plasmid donor concentration and a natural microbial community on transduction in *Pseudomonas aeruginosa* Appl. Environ. Micro. 53:987-995.

Schilf, W. and W. Klingmuller. 1983. Experiments with *E. coli* on the dispersal of plasmids in environmental samples. Recomb. DNA Tech. Bull. 6:101-102.

Seidler, R.J. and S. Hern. 1988. Special report: The release of ice minus recombinant bacteria at California test sites. USEPA, CERL, Corvallis, OR. 83pp. + app.

Shapiro, J.A. 1985. Intercellular communication and genetic change in bacterial populations. [In] H.O. Halvorson, D. Pramer, and M. Rogul (eds.), Engineered Organisms in the Environment: Scientific Issues, ASM, Washington. pp. 63-69.

Slater, J.H. 1985. Gene transfer in microbial communities. [In] H.O. Halvorson, D. Pramer, and M. Rogul (eds.), Engineered Organisms in the Environment: Scientific Issues, ASM, Washington. pp. 89-98.

Sostarec, V., D.R. Orvos, G.H. Lacy, and J. Cairns, Jr. 1988. Persistence of genetically engineered *Erwinia carotovora* in aquatic microcosms. Abstr. Ninth Ann. Mtg. Soc. Environ. Toxicol. Chem. 152:177.

Stacey, G. 1985. The *Rhizobium* experience. [In] H.O. Halvorson, D. Pramer, and M. Rogul (eds.), Engineered Organisms in the Environment: Scientific Issues, ASM, Washington. pp. 109-121.

Stack, J.P., M.S. Mount, P.M. Berman, and J.P. Hubbard. 1980. *Erwinia carotovora:* A model for degradation and assimilation of host pectic fractions. Phytopathology 70:267-272.

Stanghellini, M.E. 1982. Soft-rotting bacteria and the rhizosphere. Pp. 225-261 [In] M.S. Mount and G.H. Lacy (eds.), Phytopathogenic Prokaryotes, Vol. 1, Academic Press, Inc., N.Y. 541pp.

Steffan, R.J. and R.M. Atlas. 1988a. DNA amplification to enhance detection of genetically engineered bacteria in environmental samples. Appl. Environ. Micro. 54:2185-2191.

Steffan, R.J., J. Goksoyr, A. Bej and R.M. Atlas. 1988b. Recovery of DNA from soil and sediments. Appl. Environ. Micro. 54:2908-2915.

Stotzky, G. and V.N. Krasovsky. 1981. Ecological factors that affect the survival, establishment, growth, and genetic recombination of microbes in natural habitats. [In] S.B. Levy, R.C. Clowes, and E.L. Koenig (eds.), Molecular Biology, Pathogenicity, and Ecology of Bacterial Plasmids, Plenum, New York, pp. 31-42.

Suslow, T. V. 1982. Role of root-colonizing bacteria in plant growth. Pp. 187-223 [In] M. S. Mount and G. H. Lacy (eds.), Phytopathogenic Prokaryotes, Vol. 1, Academic Press, Inc., N.Y., 541pp.

Trevors, J.T. and K.M. Oddie. 1986. R-plasmid transfer in soil and water. Can. J. Micro. 32:610-613.

Van Elsas, J.D., A.F. Dijkstra, J.M. Govaert, amd J.A. van Veen. 1986. Survival of *Pseudomonas fluorescens* and *Bacillus subtilis* introduced into two soils of different texture in field microplots. FEMS Micro. Ecology 38:151-160. Vidaver, A.K. 1982. Biological control of Plant Pathogens with prokaryotes. Pp. 387-397 [In] M.S. Mount and G.H. Lacy (eds.), Phytopathogenic Prokaryotes, Vol. 2, Academic Press, Inc., N.Y., 506pp.

Walter, M.V., K. Barbour, M. McDowell, and R.J. Seidler. 1987a. A method to evaluate survival of genetically engineered bacteria in soil extracts. Curr. Micro. 15:193-197

Walter, M.V., A. Porteous,and R.J. Seidler. 1987b. Measuring genetic stability in bacteria of potential use in genetic engineering. Appl. Environ. Micro. 53:105-109.

Wang, Z. and D.L. Crawford. Effects of wildtype and recombinant *Streptomyces* on mineralization of organic carbon. Abstracts of First Inter. Conf. Release Genetically Engineered Microorganisms No. 12, p. 13.

Weinberg, S.R. and G. Stotzky. 1972. Conjugation and genetic recombination of *Escherichia coli* in soil. Soil Biol. Biochem. 4:171-180.

Wellington, E.M., N. Creswell, and V.A. Saunders. 1988. The fate of seeded streptomycetes and their plasmids in soil. Abstracts First Inter. Conf. Release Genetically Eng. Micro. 39:81.

Wetzel, R.G. 1983. Limnology, 2nd Ed., W.B. Saunders, Phil., PA.

Wiggins, B.A. and M.A. Alexander. 1985. Minimum bacterial density for bacteriophage replication: implications for significance of bacteriophages in natural ecosystems. Appl. Environ. Micro. 49:19-23.

Zeph, L.R., M.A. Onaga, and G. Stotzky. 1988. Transduction of *E. coli* by bacteriophage P1 in soil. Appl. Environ. Micro. 54:1731-1737.

CHAPTER ONE

MICROCOSMS IN BIOTECHNOLOGY RISK ASSESSMENT

INTRODUCTION

Microcosms have been developed to serve as environmental models for determining impacts of anthropogenic factors. Probably no term in the biological sciences is more ambiguous. Microcosms may range in size from small 10 ml containers to large 200 liter ones. Ausmus *et al.* (1980) define a microcosm as a "laboratory experimental unit including more than one component." Such components include trophic levels and functional groups (Cairns, 1986). It is believed that most risk assessments can be accomplished in microcosm systems before conducting them in more realistic and expensive settings (Cairns, 1986).

Microcosms offer the advantages of small size, replicability, lack of confounding factors, realistic interactions between physical, chemical, and biological processes, simultaneous estimation of transport, fate and effect parameters, and an estimation of ecological effects (Ausmus *et al.*, 1980; Harte *et al.*, 1980; Van Voris *et al.*, 1980; Van Voris *et al.*, 1984). Using microcosms in biotechnology (GEM) risk assessment offers many advantages including making a preliminary determination of survival, structural and functional effects, and potential DNA transfer before the actual environmental release of the microbe. If adverse characteristics or behaviors are observed, the microcosm may be easily disposed of. Conversely, if no unusual events are found, the data may serve to justify small-scale field tests. However, microcosm test results may not be comparable to each other because of the diversity of systems in present use (King, 1980; Cairns and Pratt, 1986).

No clear consensus exists as to what parameters should be monitored (Ausmus *et al.*, 1980; Cairns, 1986; Pritchard, 1988). Possibilities include structural components such as biomass and microbial and macroinvertebrate diversity, and functional parameters including respiration, productivity, decomposition, and nutrient cycling (Cairns, 1986). Although many variables can be followed, one of the criticisms of using microcosms in biotechnology risk assessment is that the data generated are not indicative of environmental realities; furthermore, no one knows what data to collect (Teidje, 1988). Rather than using microcosms as environmental surrogates, Teidje proposes using them to answer specific questions, such as gene exchange. However, several studies have demonstrated the usefulness and replicability of both aquatic and terrestrial microcosms (Harte *et al.*, 1980; Portier *et al.*, 1983; Levy *et al.*, 1985; Portier, 1985; Federle *et al.*, 1986). Parameters monitored and compared included microbial density, phosphatase and dehydrogenase activities, ATP, mineralization (Portier, 1985), community structure, and fatty acid analysis (Federle *et al.*, 1986).

It is assumed that microcosms are indicative of the systems they are derived from. However, poor validation of commonly used microcosm systems do not support that hypothesis (Cairns, 1988). Field validation of a microcosm involves comparing responses of the microcosm with those that occur in the environment. Three types of validation are proposed: 1) scientific validation, 2) site-specific validation, and 3) monitoring validation (Cairns, 1988). Although these validations were designed for use with chemicals, two of them are applicable for microcosm use in biotechnology risk assessment. Scientific validation of an experiment mandates that observations made in a microcosm be validated using environmental samples. Responses seen in the microcosm prior to inoculation with either chemicals or GEMs can be assayed in the field site. Site-specific

validation confirms results of this general validation to a particular site (Cairns, 1988). Validating microcosm response is crucial in biotechnology applications since results will be used to predict behavior in natural systems (Cairns *et al.*, 1988.).

Microcosms are classified as single component, single unit, sequential, or linked (Ausmus *et al.*, 1980). Single component designs are the simplest and contain specific organisms or substrates. They are useful in screening and mechanism identification. Single unit microcosms use self-contained experimental units that contain all parameters that are involved in the analysis. Sequential microcosms are more complex and involve more than one substrate type, such as water and soil; however, they are more difficult to validate and monitor. Linked microcosms involve more than one ecosystem through linkage of experimental. They are rarely used, expensive, and hard to control.

Design of microcosms varies with the parameters being studied. Microcosms must be carefully designed both for yielding meaningful results and for budget constraints. They greatly vary in size and composition depending upon research needs (Pritchard and Bourquin, 1984). Size depends upon the experiment and the trophic levels being incorporated, sediment/water ratio, and sampling frequency. It appears that most microbial processes can be studied in small microcosms (Pritchard and Bourquin, 1984) as long as care is taken to avoid bottle effects. Microcosm scaling and the sediment surface area to water volume ratio is also important. Amount of surface area is also important to terrestrial microcosms since substrate depth and quantity are influential in microbial processes.

Microcosm construction must be accomplished using biologically inert materials since many commonly used substances adversely affect microbes (Price *et al.*, 1986). Carefully cleaned glass containers are preferred along with

surgical-grade synthetic tubing; however, some tubing, especially neoprene, could be toxic (Price *et al.*, 1986). Mixing of the substrate, air, and water column, if any, must also be considered. Use of inert materials, such as glass and Teflon™, is crucial. Mixing of water columns is useful to increase replicability (Pritchard and Bourquin, 1984) but may be questionable since it is not indicative of environmental conditions; indeed, it may be more realistic to mix the air above the water column. Light must be provided if it is crucial to parameters being monitored and its photoperiod and intensity must be optimized.

Few studies have used microcosms in biotechnology risk assessment. Armstrong *et al.* (1987) did develop a system to use with plants and insects. The microcosm demonstrated that GEM effects could be observed using such an environmental surrogate. Therefore, the purpose of this study was to design an efficient, low cost microcosm for conducting a preliminary risk assessment of a genetically engineered microorganism (GEM). Since only microbial effects were being studied, no plants or macroinvertebrates were to be included. The design promises to be easy to use and maintain as well as minimizing confounding factors.

METHODS AND MATERIALS

Microcosms

Terrestrial microcosms consisted of 1-liter glass mason jars containing 200 g of soil. The soil was air dried to 50% of holding capacity to allow for infusion of the inoculum; this drying did not affect the concentrations of indigenous microbes (Table 1-1). Initially, the system was sealed to prevent air movement; later, humidified, filter-sterilized air was circulated at 5 ml/min through the microcosm, as shown, for the duration of the study (Fig. 1-1). This modification minimized evaporation of soil moisture while allowing for elimination of

metabolic by-products. Both intake and exhaust air was sterilized by membrane filtration using a Gelman Maxi Capsule ™ (Gelman Sciences, Inc., Ann Arbor, MI). Soil in the microcosms was gently mixed every third day to ensure a homogenous mixture. These studies utilized soil from the Virginia Polytechnic Institute and State University (Virginia Tech) dairy farm on campus and the farm at Whitethorn on the New River. Soil was a Hayter loam in both fields. Dairy farm soil was no-tillage cropped, planted in alfalfa for at least 3 years, and consisted of 29% sand, 47% silt, and 23% clay. Whitethorn soil was no-till previously planted with corn for more than 5 years, dark grayish brown in color, and consisted of 39% sand, 41% silt, and 24% clay. Other soil characteristics, determined by standard methods (Page *et al.*, 1982), are listed in Table 1-2.

Microcosm Inoculation

Erwinia carotovora were grown in 4 liters of nutrient broth to a turbidity of approximately 1.0 at 550 nm. This strain had an ampicillin-resistant gene chromosomally inserted but had no other alterations. Bacterial cells were harvested by centrifugation at 1000 *g* for 20 min to provide approximately 6 x 10^{10} cells. The supernatant was discarded and the pellet washed in sterile distilled water (SDW), resuspended in SDW, and centrifuged at 1000 *g* This pellet was suspended in SDW each microcosm (200 g soil) to provide approximately 10^8 cells/g soil. This amount was chosen after experimentation so that soil would be neither too moist or dry. Inoculum was added to soil, which had been previously air dried for 48 h and passed through a 4.75 mm sieve after collection, and throughly mixed. This inoculated soil was passed through a 2 mm sieve, remixed, and then randomly placed into microcosms. Microcosms were incubated at 20° C.

Sampling of Microcosms

Soil samples were withdrawn from microcosms on 0, 2, 4, 8, 10, 16, 32, and 64 days after inoculation. To allow for diffusion of inoculum, day 0 samples were withdrawn approximately 2 h after inoculation. After gently mixing soil in the microcosms, soil samples (1 g) were randomly removed and placed in milk dilution bottles containing 99 ml SDW. These were mixed, first (45 ml) so that 15 ml of inoculum would be delivered to manually for a brief period and then for 20 min on an orbital shaker at 150 rpm, and subsequent dilutions prepared. Dilutions were plated onto plate count agar (PCA) diluted 1:10 for total bacteria and PCA containing 40 μg/ml kanamycin monosulfate (Sigma Chemical Co., St. Louis, MO) for *E. carotovora* using the spread plate technique. Diluted plate count agar was chosen because it yielded a significantly higher number of colonies than other media in a comparison test (Fig. 1-2). Actinomycete isolation agar (AI; Difco) was used to isolate Actinomycetes; eosin-methylene blue agar (EMB; Difco) was used to enumerate gram-negative bacteria; and pseudomonas isolation agar (PIA; Difco) was used to isolate *Pseudomonas* spp. Inoculated Petri dishes were incubated at 30° C for *E. carotovora* and 25° C for other bacteria. Plates with appropriate dilutions were then counted and results expressed as colony forming units (CFU)/g dry weight. Soil for determination of water content was removed at time of sampling.

Experimental Design and Statistical Analysis

Four microcosms were inoculated with the genetically-engineered strain of *E. carotovora* and four with sterile water as a control. On each sampling day, one sample from each of the four microcosms containing bacteria was withdrawn and diluted. Three plates were prepared from each dilution and plates with approximately 30 to 300 colonies were counted. Statistical significance

over time was evaluated using a repeated measures analysis (Sokal and Rohlf, 1981) and the Statistical Analysis System (SAS Institute, Inc., 1985).

Air Sampling

To ascertain the composition of column air above the soil after a period of incubation, air from five sealed and five flow-through microcosms was sampled for oxygen and carbon dioxide concentration using a Drager air sampler (Dragerwerk Ag Lubeck, Germany) and Drager oxygen (Cat. #67 28081) and carbon dioxide (Cat. #CH 31401 or #CH 30801) tubes. Oxygen tubes were capable of detecting 5-24% oxygen with an error of ± 5% and carbon dioxide tubes could detect 0.5-10% or 0.03-0.1% carbon dioxide with no error specified. Air samples were withdrawn through the microcosm rubber stopper 21 days after inoculation. Concentrations were expressed as % by volume oxygen or carbon dioxide.

RESULTS AND DISCUSSION

E. carotovora Survival

Genetically-engineered *E. carotovora* survived for 62 days before falling below the limit of detection (Fig. 1-3). The rate of decline followed a curve similar to those reported in other studies regardless of whether the bacteria was engineered (Lindow, 1985; Van Elsas *et al.*, 1986) or not (Liang *et al.*, 1982). Since *E. carotovora* is a plant pathogen and no such material is provided as a nutrient source, it is not surprising that the organism declined; however, it was not expected that *E. carotovora* would survive for over two months in a simple microcosm. It is believed, therefore, that the design chosen will be satisfactory for evaluating GEM survival and effects without the presence of confounding factors.

Total Bacteria Survival

Survival of total bacteria, on 1:10 PCA, was followed for 34 days (Fig. 1-4) with no information obtained on day 62 because of dilution errors. Populations of total bacteria demonstrated a significant increase ($p < .05$) in the first ten days of the study and then subsided to initial concentrations. Such an increase is probably due to the input of moisture that allowed germination of spores as well as contributing additional dissolved nutrients. It is apparent that the design is suitable for structural effects analyses since these bacteria do not significantly change over time.

Moisture Content

Moisture content in flow-through microcosms ranged from 13.7 to 16.5%, a variation of about 3%, over the course of the study (Fig. 1-5). Use of the humidification apparatus allowed the soil to remain moist yet granular throughout the experiment. This is important since excessively dry conditions could impair bacterial survival and dispersal (Grant and Long, 1981; Cox, 1987). In addition, use of humidification allows the microcosms to be placed into conditions, such as high temperatures, that favor low humidity. It is evident that moisture maintenance must be considered in microcosm construction.

Gaseous By-Products

Gas concentrations within the incubator averaged 20% oxygen and 0.02% carbon dioxide by volume. Gas concentrations within the aerated microcosms were 19.5% oxygen and 0.02% carbon dioxide; these did not differ significantly from the concentrations within the incubator. However, oxygen concentrations within the sealed microcosms averaged <5% ± while carbon dioxide concen-

trations were >10%. Exact concentrations exceeded the limitations of the Draeger tube used.

Although the Draeger tubes are not extremely accurate, they did demonstrate that sealed microcosms should not be used since they promote carbon dioxide accumulation and oxygen deprivation, even in simple designs that lack higher trophic levels. These conditions could be unfavorable to aerobic bacteria and may increase concentrations of anaerobic bacteria (Grant and Long, 1981). No attempt was made to calculate the gas concentrations within the soil itself; this often varies significantly from that of the atmosphere (Grant and Long, 1981). Finally, sealed microcosms are probably not indicative of environmental conditions that bacteria may encounter in the environment in regards to atmospheric gas accumulation.

CONCLUSIONS

The design chosen for the duration of this study allows survival of engineered *Erwinia* as well as populations of bacteria on diluted agar, does not result in the accumulation or deprivation of atmospheric gases, and does not allow the dessication of the substrate. The design complexity may be increased to introduce additional environmental factors, as may be deemed necessary.

Introduced bacteria survived for over two months in these microcosms without the presence of plant material, a known substrate for *E. carotovora*. Any adverse effects upon microbial structural integrity or functional parameters should occur within this time period when the numbers of *E. carotovora* are at detectable levels. The system is also ideal for DNA exchange studies since external selective pressures may be added.

While more environmentally realistic designs may be favored by some investigators, it has been shown that this microcosm design will allow collection

of data regarding the fate and effects of a genetically engineered microorganism without the presence of confounding factors.

LITERATURE CITED

Armstrong, J.L., G.R. Knudsen, and R.J. Seidler. 1987. Microcosm method to assess survival of recombinant bacteria associated with plants and herbivorous insects. Curr. Micro. 15:229-232.

Ausmus, B.S., P. Van Voris, and D.R. Jackson. 1980. Terrestrial microcosms: What questions do they address? [In] J.P. Giesy, Jr. (ed.), Microcosms in Ecological Research. U.S. Dept. of Energy, Springfield, Va., pp. 937-953.

Cairns, J., Jr. 1988. What constitutes field validation of predictions based on laboratory evidence? [In] W.J. Adams, G.A. Chapman, and W.G. Landis (eds.), Aquatic Toxicology and Hazard Assessment: 10th volume, ASTM STP 971. American Society for Testing and Materials, Philadelphia, pp. 361-368.

Cairns, J., Jr. 1986. Multispecies toxicity testing: a new information base for hazard evaluation. Curr. Prac. Environ. Sci. Eng. 2:37-49.

Cairns, J., Jr. and J.R. Pratt. 1986. Ecological consequence assessment: effects of bioengineered organisms. [In] J. Fiksel & V.T. Covello (eds.), Biotechnology Risk Assessment, Pergamon Press, pp. 88-108.

Cairns, J., Jr., E.P. Smith, and D.R. Orvos. 1988. The problem of validating simulation of hazardous exposure in natural systems. [In] C.C. Barnett and W.M. Holmes (eds.), Proceed. 1988 Summer Computer Simulation Conference, Soc. for Computer Simulation Conf., San Diego, pp. 448-454.

Cox, C.S. 1987. The aerobiological pathway of microorganisms. Chichester, U.K.: John Wiley & Sons, 293 pp.

Federle, T.W., R.J. Livingston, L.E. Wolfe, and D.C. White. 1986. A quantitative comparison of microbial community structure of estuarine sediments from microcosms and the field. Can. J. Micro. 32:319-325.

Grant, W.D. and P.E. Long. 1981. Environmental Microbiology. Halsted Press, Glasgow, Scotland, 215 pp.

Harte, J., D. Levy, J. Rees, and E. Saegebarth. 1980. Making microcosms an effective assessment tool. [In] J.P. Giesy (ed.), Microcosms in Ecological Research, National Technical Information Service, Springfield, VA. pp. 105-137.

King, D.L. 1980. Some cautions in applying results from aquatic microcosms. [In] J.P. Giesy, Jr. (ed.), Microcosm in Ecological Research, National Technical Information Service, Springfield, VA. pp. 164-191.

Levy, D., G. Lockett, J. Oldfather, J. Rees, E. Saegebarth, R. Schneider, and J. Harte. 1985. Realism and replicability of lentic freshwater microcosms. [In] T.P. Boyle (ed.), Validation and predictability of laboratory methods for assessing the fate and effects of contaminants in aquatic ecosystems, ASTM STP 865, Amer. Soc. Testing Materials, Philadelphia, pp. 43-56.

Liang, L.N., J.L. Sinclair, L.M. Mallory and M. Alexander. 1982. Fate in model ecosystems of microbial species of potential use in genetic engineering. Applied Env. Micro. 44:708-714.

Lindow, S.E. 1985. Ecology of *Pseudomonas syringae* relevant to the field use of ice-deletion mutants constructed in vitro for plant frost control. [In] H.O. Halvorson, D. Pramer, and M. Rogul (eds.), Engineered Organisms in the Environment: Scientific Issues, ASM, Washington. pp. 23-35.

Page, A.L., R.H. Miller, and D.R. Keeney. 1982. Methods of soil analysis, chemical and microbiological properties, 2nd edition. Amer. Soc. Agronomy, Madison, WI, 1158 pp.

Portier, R.J. 1985. Comparison of environmental effect and biotransformation of toxicants on laboratory microcosm and field microbial communities. [In] T.P. Boyle (ed.), Validation and predictability of laboratory methods for assessing the

fate and effects of contaminants in aquatic ecosystems, ASTM STP 865, Amer. Soc. Testing Materials, Philadelphia, pp. 14-30.

Portier, R.J., H.M. Chen, and S.P. Meyers. 1983. Environmental effect and fate of selected phenols in aquatic ecosystems using microcosm approaches. Dev. Industrial Micro., Vol. 24. pp. 409-424.

Price, N.M., P.J. Harrison, M.R. Landry, F. Azam, and K.J.F. Hall. 1986. Toxic effects of latex and Tygon tubing on marine phytoplankton, zooplankton, and bacteria. Mar. Ecol. Prog. Ser. 34:41-49.

Pritchard, P.H. 1988. Round table 6: Use of Microcosms. [In] M. Sussman, C.H. Collins, F.A. Skinner, and D.E. Steward-Tull (eds.), The Release of Genetically-engineered Microorganisms, Academic Press, London, pp. 265-269.

Pritchard, P.H. and A.W. Bourquin. 1984. The use of microcosms for evaluation of interactions between pollutants and microorganisms. [In] K.C. Marshall (ed.), Advances in Microbial Ecology, Vol. 7. Plenum Press, New York, pp. 133-215.

SAS Institute, Inc. 1985. SAS User's Guide: Statistics, version 5. SAS Institute, Inc., Cary. N.C.

Sokal, R.R. and F.J. Rohlf. 1981. Biometry. W.H. Freeman and Co., San Francisco, CA., 776 pp.

Tiedje, J. 1988. Round table 6: Use of Microcosms. [In] M. Sussman, C.H. Collins, F.A. Skinner, and D.E. Steward-Tull (eds.), The Release of Genetically-engineered Microorganisms, Academic Press, London, pp. 268-269.

Van Elsas, J.D., A.F. Dijkstra, J.M. Govaert, amd J.A. van Veen. 1986. Survival of *Pseudomonas fluorescens* and *Bacillus subtilis* introduced into two soils of different texture in field microplots. FEMS Micro. Ecology 38:151-160.

Van Voris, P., D.A. Tolle, M.F. Arthur, J. Chesson, T.C. Zwick. 1984. Development and validation of a terrestrial microcosm testing system for assessing ecological effects of utility wastes. Electric Power Research Institute, Palo Alto, CA.

Van Voris, P., R.V. O'Neill, W.R. Emanuel, H.H. Shugart, Jr. 1980. Functional complexity and ecosystem stability. Ecology 61:1352-1360.

TABLE 1-1. Concentrations of bacteria in dried and non-dried soil

Organisms	Dried Soil Log CFU/g (± S.D.)	Non-Dried Soil Log CFU/g (± S.D.)
Total Bacteria[a]	6.56 (0.32)	6.53 (0.19)
Gram-negative[b]	5.95 (0.36)	6.20 (0.28)
Actinomycetes[c]	6.26 (0.12)	6.20 (0.14)
Pseudomonas[d]	4.00 (0.08)	4.04 (0.11)

[a]Determined with 1:10 plate count agar at 20° C.

[b]Determined with eosin-methylene blue agar at 20° C.

[c]Determined with actinomycete isolation agar at 20° C.

[d]Determined with pseudomonas isolation agar at 20° C.

TABLE 1-2. Soil characteristics of Hayter loam soils from dairy farm (DAIRY) and Whitethorn Research Farm (WHITE) used in initial soil survival study

Sample	pH	P (ppm)	K (ppm)	Ca (ppm)	Mg (ppm)	Mn (ppm)	Zn (ppm)	NO_3-N (ppm)	Organic Matter (%)
WHITE	5.6	19	138	876	128	39	2.3	13	3.7
DAIRY	5.4	17	119	852	128	22	3.4	30	3.0

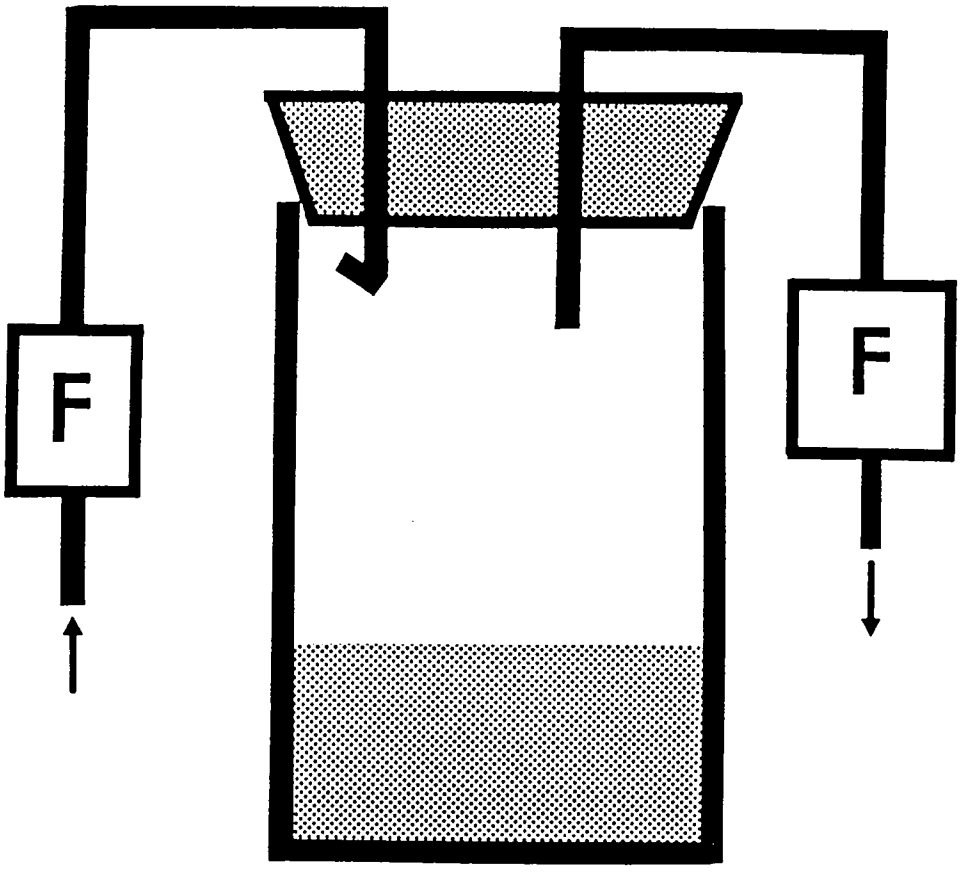

FIG. 1-1. Diagram of microcosm apparatus used in study. Soil in bottom of vessel was subjected to constant flow of humidified air that was sterlized by membrane filters (F), or was sealed for those experiments that did not utilize circulated air.

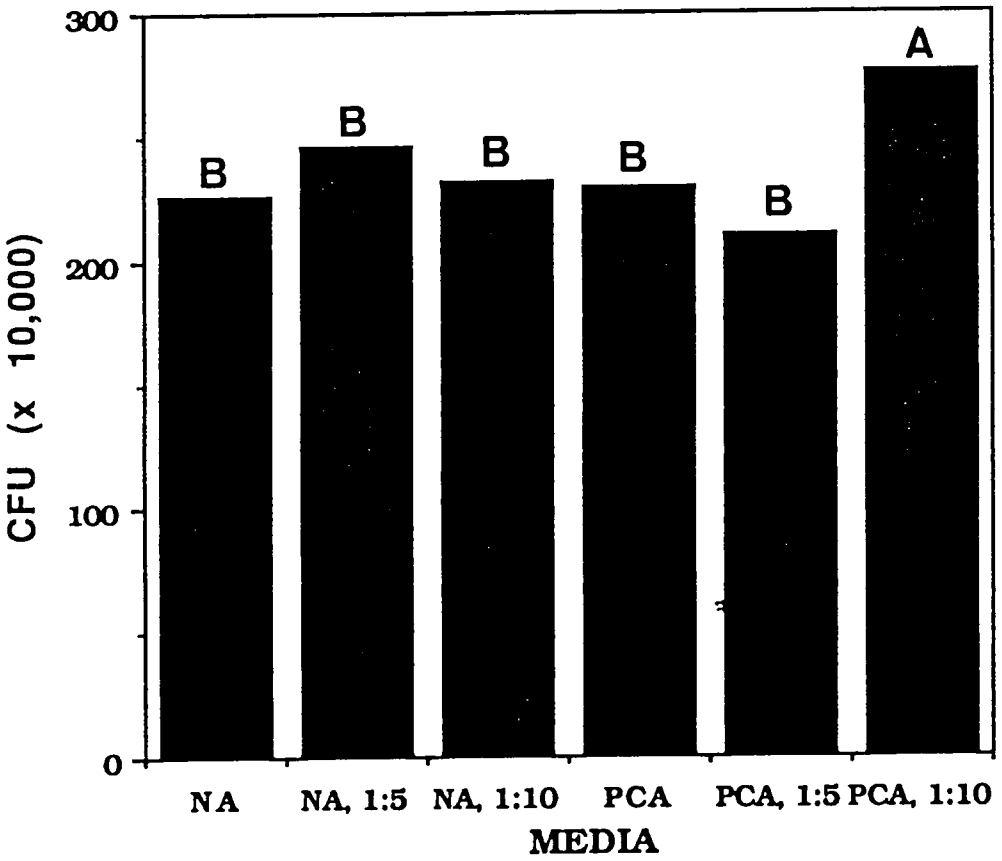

FIG. 1-2. Growth of total numbers of bacteria on different media types and dilutions. Different letters indicate groups with statistical difference based upon multiple comparison of means using the Student-Newman-Keuls test. NA- Nutrient agar, PCA- Plate count agar.

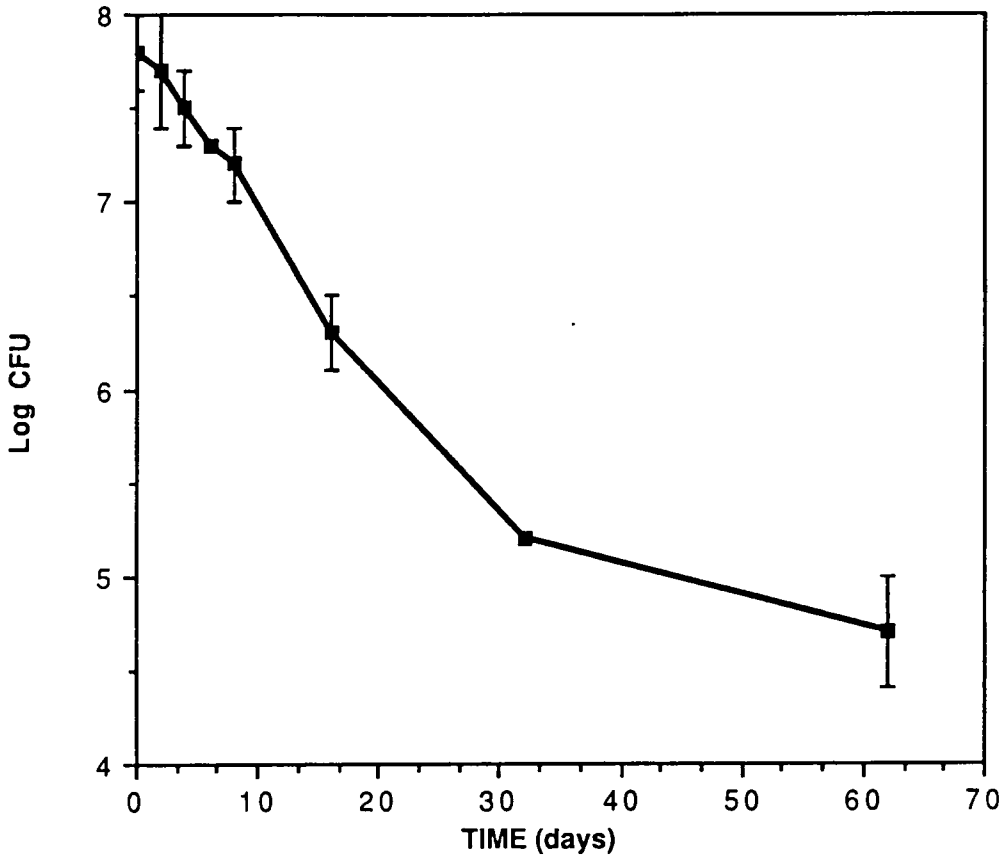

FIG. 1-3. Survival of ampicillin-resistant (L-827) *Erwinia carotovora* in soil microcosms. Error bars represent one standard deviation unit.

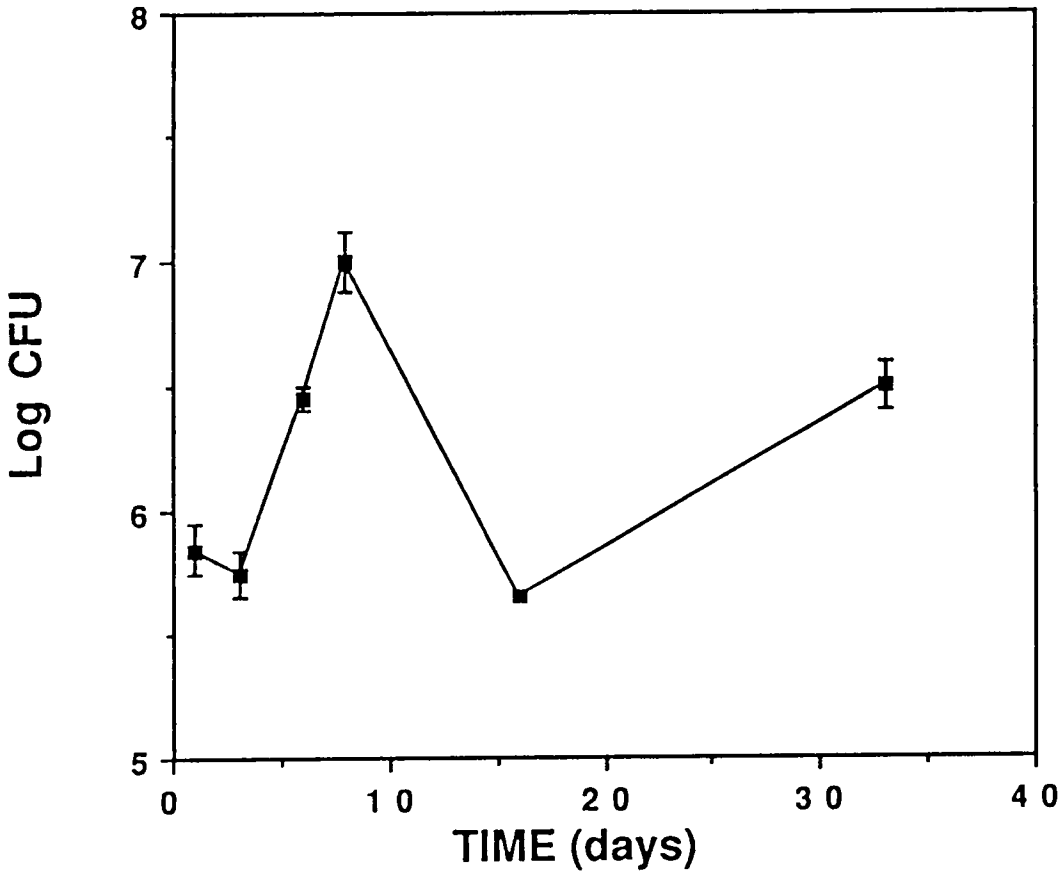

FIG. 1-4. Total bacteria survival in microcosms containing dairy farm soil. Bacteria cultured on 1:10 plate count agar and sampled over 32 d.

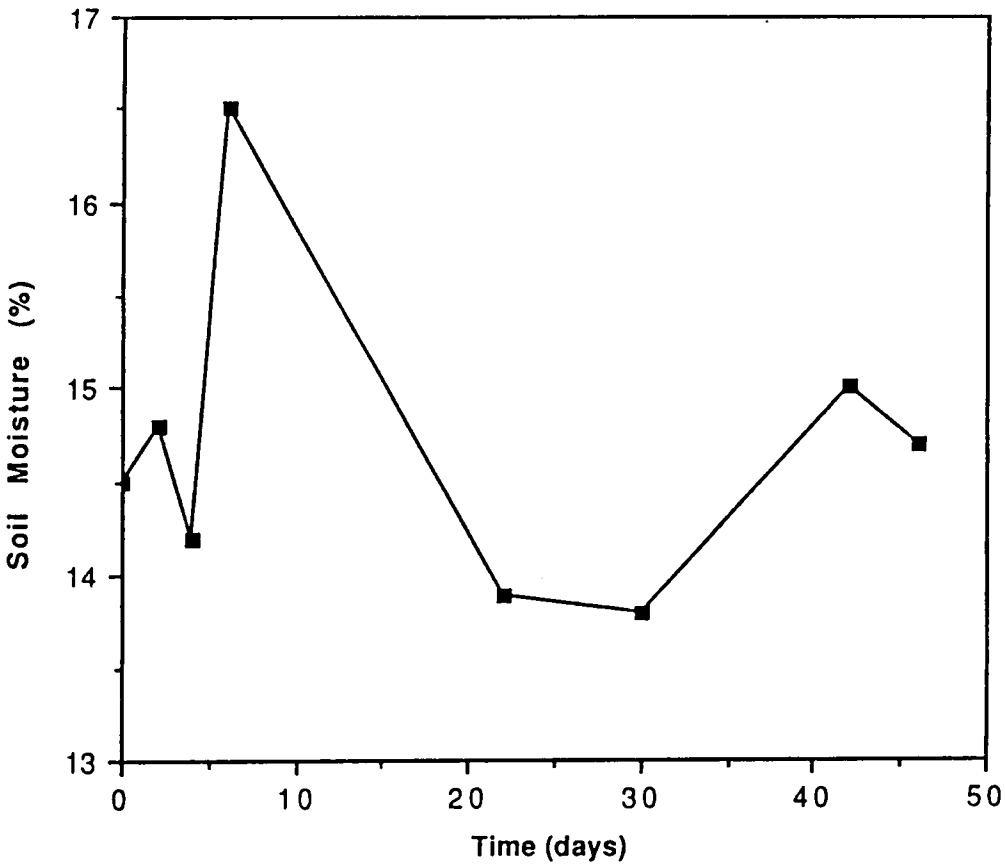

FIG. 1-5. Soil moisture in microcosms during study. Each point represents average of six samples with moisture determined by drying soil to constant weight at 105° C.

CHAPTER TWO

ACQUISITION OF ANTIBIOTIC RESISTANCE BY INDIGENOUS MICROBES

INTRODUCTION

Genetic engineering of organisms may have many benefits on society. However, use of genetically engineered microorganisms (GEMs) could have inadvertent consequences that may place the environment at risk. Environmental use of GEMs has been limited to small-scale trials because of a lack of knowledge about their impact on ecosystems and increased public awareness of and concern over recombinant organism release. GEMs may potentially affect environmental processes through any one of three ways: (1) by transferring DNA out from or into the GEM, thus altering the GEM itself or indigenous populations of bacteria, (2) by affecting structural integrity of communities, both microscopic and macroscopic, and (3) by affecting functional processes, including geochemical cycling of nutrients.

The use of genetic mechanisms for efficient dispersal and alteration of DNA is intrinsic to bacterial survival in the environment. Bacteria are known to have four basic mechanisms for exchanging genetic material: (1) transformation, the uptake of naked DNA as plasmids for Gram-negative bacteria or linear DNA fragments by Gram-positive bacteria; (2) transduction, the incorporation of bacterial DNA in bacteriophage particles and their transfer to a recipient bacterium; (3) conjugation, in which the DNA is transferred by direct cell-to-cell contact that may be plasmid mediated; and (4) fusion and recombination, knowledge of which is limited at this time to actinomycetes (Freifelder, 1987).

Most, if not all, bacteria in the environment exchange genetic information with nearby microbes on a routine basis. In assessing risk from potential genomic

transfer from a GEM to indigenous bacteria, several factors must be considered. First, using molecular hybridization techniques, it must be confirmed that gene transfer has indeed occurred. Second, if transfer has happened, it must be ascertained if novel genetic information is sustained and expressed in the bacterium which just acquired it. Third, if such new genetic information is maintained and expressed, effects that such expression may have on other biota must be evaluated (Office of Technology Assessment, 1988). Several criteria, including gene transfer frequency and genetic distance between donor and recipient species, can be used (Office of Technology Assessment, 1988).

While some studies have been accomplished on potential genetic exchange mechanisms, few have examined these mechanisms in the environment. While transfer can be evaluated in the laboratory, such studies do not take into account the various selective pressures that are present in nature. In addition, DNA is rarely found in the naked, available state in environmental samples; rather, it may be degraded by exogenous nucleases or absorbed to soil or sediment particles that may make it unavailable for transfer (Ogram *et al.*, 1988). Such sorption is regulated by the soil type and ionic strength, pH, and length of DNA (Ogram *et al.*, 1988).

Plasmid DNA transfer, via conjugation, has been studied in both terrestrial and aquatic systems. Plasmids – extrachromosomal, circular pieces of DNA – are ubiquitous in natural bacteria. Many environmental isolates have been shown to be recipients for plasmid exchange. Genthner *et al.* (1988) tested 68 bacterial isolates from aquatic sources and found 26 of them able to receive plasmids from *Pseudomonas aeruginosa* donors. Evidence for plasmid selection or exchange in the environment is based upon the high incidence of plasmids in polluted areas (Hada and Sizemore, 1981), presence of naked DNA in the environment, coexistence of identical plasmids in different strains, and data from

in situ transfer experiments (Grimes *et al.*, 1988). Transfer of plasmids also appears to be affected by environmental influences. Stotzky and Krasovsky (1981) found that recombination was markedly affected by pH with survival of both donors and recipients increasing as pH was greater. However, moisture content of soil appears not to be influential in soils adjusted to 20-100% of soil water holding capacity (Trevors and Oddie, 1986).

The vast majority of such transfers appear to be innocuous; this is not to be unexpected since antibiotic resistance from the marker plasmid is often the only parameter monitored. However, it is conceivable that transfer of plasmids may affect functional characteristics of the organism. Rafii and Crawford (1988) found that several conjugative plasmids were transferred in sterile soil from the donor, *Streptomyces lividans*, and several *Streptomyces* strains isolated from environmental samples. In a concurrent study, Wang and Crawford (1988) found that similar *Streptomyces* strains produced significantly higher soil carbon mineralization rates in nonsterile soil amended with lignocellulose. They believe this effect is due to expression of the foreign DNA insert. It, therefore, is plausible that transfer of such an insert could convey such alteration in function to other organisms. In addition, Glick *et al.* (1986) found that introduction of plasmid DNA into *Azotobacter vinelandii*, via transformation, was not without consequence since capacity for nitrogen fixation, mean cell size, and siderophore production were decreased.

In the present study, evidence for acquisition of a kanamycin-resistance gene by indigenous bacteria is presented. In addition, survival of engineered and wildtype bacteria over a 15 day period is evaluated.

METHODS AND MATERIALS

Erwinia Strains

The bacterium used in this study was Erwinia carotovora subsp. carotovora strain EC14 (Allen *et al.*, 1986), a phytopathogenic enterobacterium that causes soft rot in plants and is found in plant, soil, and aquatic habitats. Pectate lyase genes from EC14 were cloned into plasmid pBR322 on a 1.3 kilobase pair (kbp) fragment and expressed in *Escherichia coli* strain HB101 (Roberts *et al.*, 1986b). A 5.5 kbp fragment of this inserted EC14 DNA was deleted by digestion with *Bgl*II and the remaining plasmid DNA was ligated using T4 ligase. The resulting plasmid, pDR41, no longer mediated pectate lyase activity as determined by isoelectric focusing and activity stain overlays (Roberts *et al.*, 1986a). Plasmid pDR41 was further modified by inserting the *Pst*I kbp fragment of Tn*903* (kanamycin resistance) isolated from pUC4K (Vieira and Messing, 1982) into the *Pst*I site of pDR41. The new plasmid, pVS20, mediating kanamycin resistance, was transferred into EC14 by conjugal triparental mating with the helper plasmid pRK2013 (Ditta *et al.*, 1980). Substitutional mutagenesis was attempted by curing pVS20 from EC14 in low phosphate medium (Torriani, 1960). Kanamycin-resistant strain L-833 was recovered that contained no detectable plasmids, was reduced 30% in the ability to rot tuber tissue, but continued to produce pectate lyase. The Tn*903*-derived fragment therefore is probably inserted in a *cis*-orientation to the wildtype genes in the EC14 chromosome. Strain L-863 was a wildtype mutant of EC14 resistant by spontaneous mutation to 150 μg/ml rifampicin; strain L-864 is a spontaneous rifampicin-resistant (150 μg/ml) mutant of L-833. Both L-863 and L-864 strains displayed growth on laboratory media and biochemical characteristics identical to other (Fig. 2-1; Table 2-1). These characteristics were also the same for wildtype *Erwinia*.

Media and chemicals

Plate count agar (PCA; Difco Laboratories, Detroit,MI), diluted 1:10, was used to enumerate total bacteria. This dilution was chosen based upon preliminary experiments for maximum colonies recovered (Fig. 2-2). PCA with 40 μg/ml kanamycin monosulfate and 150 μg/ml rifampicin (Sigma Chemical Co., St. Louis, MO) were used to detect the genetically-engineered and wildtype *E. carotovora*, respectively.

Microcosms

Microcosms consisted of sealed 1-liter mason jars (Fig. 2-3) containing 200 g of soil from the Virginia Polytechnic Institute and State University (Virginia Tech) dairy farm complex at Blacksburg. Soil was a Hayter loam type, no-till previously planted with grass, dark brown in color, and consisted of 29% sand, 47% silt, and 24% clay with a pH of 6.1.

Microcosm inoculation

Erwinia carotovora were grown in 4 liters of nutrient broth to a turbidity of approximately 1.0 at 550 nm. Bacterial cells were harvested by centrifugation at 1000 *g* for 20 min to provide approximately 6 x 10^{10} cells. The supernatant was discarded, the pellet washed in sterile distilled water (SDW), resuspended in SDW, and centrifuged at 1000 *g*. This pellet was suspended in SDW (45 ml) so that 15 ml of inoculum would be delivered to each microcosm (200 g soil) to provide approximately 10^8 cells/g soil. Inoculum was added to soil, which had been previously air dried for 48 h and passed through a 4.75 mm sieve after collection, and throughly mixed. This inoculated soil was passed through a 2

mm sieve, remixed, and then randomly placed into microcosms. Microcosms were incubated at 20° C.

Sampling of microcosms

Samples were withdrawn from microcosms on 0, 1, 3, 6, 15 and 24 days after inoculation. To allow for diffusion of inoculum, day 0 samples were withdrawn approximately 2 h after inoculation. After gently mixing the soil in the microcosms, soil samples (1 g) were randomly removed and placed in milk dilution bottles containing 99 ml SDW. These were mixed, first manually for a brief period and then for 20 min on an orbital shaker at 150 rpm, and subsequent dilutions prepared. Dilutions were plated onto appropriate media using the spread plate technique. Inoculated Petri dishes were incubated at 25° C for 48-96 h and then plates with appropriate dilutions were counted. Results were expressed as CFU/g dry weight. Soil for determination of water content was removed at time of sampling.

Isolation and Identification of Unknown Bacteria

Bacteria displaying resistance to kanamycin in media were isolated and cultured on plate count agar containing 40 μg/ml kanamycin. Standard biochemical and morphological tests were conducted including substrate utilization, enzyme production, gas production, and staining (Gerhardt *et al.*, 1981). In addition, fatty acids were analyzed by gas chromatography using the Hewlett-Packard 5898A Microbial Identification System (Microbial ID, Inc., Newark, DE).

Isolation of Genomic DNA

Chromosomal DNA from each of the unknown bacteria as well as *Erwinia* strains used was isolated using modifications of standard techniques (Maniatis *et al.*,

1982) as follows. *Erwinia* were grown in 300 ml of nutrient broth overnight and the cells harvested by centrifugation in a Sorvall GSA rotor at 5,000 rpm for 10 min. After pouring the supernate off, the pellet was washed in 50 ml of saline-EDTA (0.15 M NaCl; 0.1 M EDTA; 0.05 M Tris HCl, pH 8.0) and centrifuged as before. The supernate was discarded, 1 g of pellet placed into a new centrifuge bottle, and 40 ml of saline-EDTA added. The pellet was dissolved in this buffer and 100 mg of lysozyme (Sigma Chemical Co., St. Louis, MO; Cat. L-6876) and 20,000 units of T_1 ribonuclease (EC 3.1.27.3; Boehringer Mannheim, Indianapolis, IN; Cat. 109-207) added. The mixture was swirled and incubated at 37° C for 20 min. After incubation, 8 ml of 5 M sodium perchlorate, 2 mg proteinase K, and 4.5 ml 10% sodium dodecyl sulfate (SDS) were added, mixed, and then the mixture incubated at 45° C for 40 min. The bottles were gently mixed at about 10 min intervals during this process. Upon completion, 40 ml of 1:1 phenol-chloroform were added, the solution gently mixed by rolling, and then incubated for 30 min followed by centrifugation for 10 min at 2,500 rpm. The phenol was removed and the extraction process repeated. After this final phenol extraction was complete, a final extraction was performed using 40 ml of 1:24 isoamyl-chloroform added for 30 min. After completion, the aqueous layer was dialyzed against 0.2x TEN Buffer (1x TEN = 0.05M NaCl; 0.005M EDTA; 0.05M Tris HCl, pH 8.0) for 24 h with changes of buffer at 4, 8, and 12 h. DNA was concentrated by ethanol precipitation and stored at 4° C. DNAs were checked for concentration and relative purity by electrophoresis.

Construction of DNA Probe

A nucleic acid probe for DNA hybridization was prepared by digesting pUC4K (Pharmacia LKB Biotechnology, Piscataway, NJ; Cat. 27-4958-01) with 30 units of *Pst*I (Bethesda Research Laboratories, Gaithersburg, MD) for 3 h at 37° C. The

pUC4K plasmid contains a kanamycin resistance marker from Tn*903* which encodes an aminoglycoside 3′-phosphotransferase gene. This is the same antibiotic marker used in the engineered strain of *E. carotovora* and it was felt that this gene, or parts of it, may have moved. The digested mixture was then electrophoresed in low melting point agar (Pharmacia LKB Biotechnology) at 17 v overnight. The band containing a 1.3 kb fragment mediating resistance to kanamycin was visualized by UV light, removed by cutting, melted at 70° C, and analyzed for purity by electrophoresis. This DNA was then nick-translated with biotin-11-dUTP using the manufacturers instructions (Bethesda Research Laboratories). The labeled DNA was purified by either ethanol precipitation or by size-exclusion chromatography with Sephadex G-50 (Pharmacia LKB Biotechnology). DNA was then checked for probe purity by developing a 2 μl aliquot using the BluGENE DNA detection system according to the manufacturers directions (Bethesda Research Laboratories).

Hybridization with Biotinylated Probe

Biotin-labeled DNA was used to probe for DNA homology in both *E. carotovora* and unknown bacteria. Genomic DNA was digested using 30 units of one of the following restriction enzymes, *Eco*RI, *Hin*cII, or *Pst*I. After electrophoresis at 17 volts, Southern transfer (Maniatis *et al.*, 1982) of the DNA onto Nitroplus 2000 (Micron Separations, Inc.) nitrocellulose was conducted using a Vacublot apparatus at 25 pKa vacuum. The filter was then baked at 80° C for one hour and stored in a plastic bag.

Hybridization was performed by first wetting the membrane in 2x SSC (1x SSC = 0.15 M NaCl; 0.15 M sodium citrate). A prehybridization solution consisting of 6x SSC, 0.5% sodium dodecyl sulfate (SDS), 5x Denhardt's solution (Maniatis *et al.*, 1982), 45% formamide, and 100 μg/ml denatured salmon sperm DNA was

added into a plastic bag containing the membrane at a volume of 200 $\mu l/cm^2$ of nitrocellulose filter. After removing air and sealing the bag, the mixture was incubated at 42° C for 3-4 hours in a waterbath. The bag was then opened, allowed to drain, and a hybridization solution added at 100 $\mu l/cm^2$ of nitrocellulose membrane. The hybridization solution consisted of 6x SSC, 0.01 M EDTA, 5x Denhardt's solution, 0.5% SDS, 100 $\mu g/ml$ salmon sperm, 45% formamide, and the biotin-labeled probe at a concentration was 0.4 $\mu g/ml$. This mixture was incubated for 12-16 hours at 42° C at which time the bag was opened and the hybridization fluid collected for later use. The filter was washed as follows:

1) 250 ml of 2x SSC/0.1% SDS at room temperature for 3 min. Repeat once.
2) 250 ml of 0.2x SSC/0.1% SDS, room temperature, 3 min. Repeat once.
3) 250 ml of 0.2x SSC/0.1% SDS, 60° C, 15 min. Repeat once.
4) Rinse in 2x SSC at room temperature for 2-3 min.

The membrane was developed using the BluGENE DNA detection system according to the manufacturers instructions (Bethesda Research Laboratories).

Experimental design and statistical analysis

Three microcosms were inoculated with the genetically-engineered strain and three with the wildtype. On each sampling day, one sample from each of the six microcosms was withdrawn and diluted. Three plates were prepared from each dilution and plates with approximately 30 to 300 colonies were counted. Statistical significance between groups at each sampling day was evaluated using Student's *t* test and between sampling intervals using a repeated measures analysis (Sokal and Rohlf, 1981). Statistical analyses were accomplished using SAS (SAS Institute, 1985).

RESULTS AND DISCUSSION

Erwinia Survival

Erwinia decreased in concentration 2 orders of magnitude during the 15 days they were accurately assessed (Fig. 2-4). Observations made after 15 days were questionable because of the proliferation of indigenous bacteria displaying kanamycin resistance. No significant difference ($p > 0.05$) in rate of decline was observed between the engineered and wildtype strain. Both strains declined about 0.5 order of magnitude during the first 6 days but then declined about 1.5 orders of magnitude over the subsequent 9 days. The amount of decline over the duration of the experiment was significant ($p < 0.05$).

It is evident that added *Erwinia*, regardless of its nature, were unable to colonize the substrate and utilize the soil nutrients present. The organic matter of the dairy farm soil is rather low (2.7%) and the microcosms were sealed, allowing metabolic by-products to accumulate. In addition, no plant material, except possible root debris, was available to *Erwinia*, a plant pathogen. All of these factors may be responsible for the increased rate of decline observed during the second half of the experiment.

These results support those of earlier studies, in which added bacteria do not survive for extended periods of time regardless of their construction or morphology (Liang *et al.*, 1982; Lindow, 1985; Van Elsas *et al.*, 1986). A wide range of factors influence bacterial survival including pH, temperature, nutrient availability, moisture, and predators (Grant and Long, 1981). While some factors were controlled, such as moisture and temperature, others were not. The correlation between these observations and those of other investigators seem to support the use of microcosms for initial data acquisition since container effect (Pritchard and Bourquin, 1984) does not appear to a problem. The concen-

trations of *Erwinia* used appear high (10^8 CFU/ml), but reflect those concentrations that have been used in earlier field releases (Seidler and Hern, 1988) as well as concentrations that will be commercially applied (M. Walter, personal communication). However, use of such high inoculum in a closed microcosm system may contribute to the ultimate demise of the organisms since nutrient depletion and formation of metabolic wastes is accelerated.

Identification of Unknown Bacteria

Indigenous bacteria, resistant to kanamycin, were observed at day 15 of the study at approximately 10^3 CFU/g soil. While the time factor appears excessive for appearance of genetic transfer, experiments with *Streptomyces* have also demonstrated similar intervals (E. Wellington, personal communication; Wellington *et al.*, 1988). The acquisition of kanamycin resistance in this study was replicated during a second trial using the same dairy farm soil but was not observed when other soil types (Whitethorn farm) were used. The frequency observed is greater than commonly reported (Slater, 1985); the reasons for this remain unclear. Although transfer of plasmids in the environment has been well documented, no plasmids are reported to be present in the engineered *Erwinia* (G. Lacy, personal communication). All of the unknown bacteria were gram-negative; this is important since gram-negative bacteria more easily exchange DNA among donors and recipients.

All unknown bacteria were assigned codes based on colony color; codes were W (white), Y (yellow), or R (red). Indigenous bacteria were identified by biochemical (Table 2-2) and fatty acid analysis as follows: *Agrobacterium tumefaciens* (W-1), *Flavobacterium indologenes* (W-2), *Cytophaga johnsonae* (Y-2) and *Flavobacterium* spp. (Y-1). Coding numbers are in parentheses. Fatty acid analysis also revealed that Y-1 may be *Cytophaga*, but several retention peaks

do not correspond, indicating differences between Y-1 and Y-2 (Fig. 2-5). No identification was possible on the red (R-1) species although it was not found to be *Serratia*. Probabilities for these matches, a measure of confidence, were obtained from fatty acid analysis as compared to a known data bank of fatty acid profiles. Probabilities were 0.198 for *Agrobacterium tumefaciens* (W-1), 0.425 for *Flavobacterium indologenes* (W-2), 0.386 for *Cytophaga johnsonae* (Y-2), and only about 0.01 for *Flavobacterium* spp. (Y-1). By comparison, values obtained for known cultures of *Erwinia carotovora* and *Pseudomonas fluorescens* were 0.826 and 0.820, respectively.

It is apparent that identification of bacteria from environmental samples is not satisfactory since most testing protocols, including the ones used, were designed for bacteria of medical importance.

Construction of Probe

A suitable probe for hybridization was constructed and labeled with biotin. The probe was pure, with no contaminating DNA or RNA bands observed by agarose electrophoresis. DNA extraction from the unknown indigenous microbes was also satisfactory and DNA appeared to be of a quality suitable for restriction (Fig. 2-6).

Hybridization

Southern hybridization of the restricted DNAs (Fig. 2-6) appeared to be successful (Fig. 2-7). However, stringency conditions were low (0.2x SSC) enough to indicate that the degree of homology is not high. In addition, bands appear fuzzy and are not tight; this could be due to excessive vacuum during the blotting process.

Regardless, homology is demonstrated in the engineered *Erwinia* (Lane A; Fig. 2-7), but not in the wildtype (Lane G). All four indigenous bacteria used (R-1 DNA would not satisfactorily restrict) had homologous regions at approximately 1.4 kbp (Lanes D, F) and 4 kbp (Lanes B, E). These results suggest transfer of the pUC4K gene present in the engineered strain to indigenous bacteria. However, before transfer can be assumed, additional studies, including sequencing and restriction analysis, will have to be executed. Use of radioactive probes may also be beneficial (Barkay and Sayler, 1988).

CONCLUSIONS

The acquisition of antibiotic resistance by indigenous bacteria was unexpected. Although transfer of the chromosomally-inserted kanamycin resistance gene is possible, it is unlikely. Evidence from the DNA homology experiments performed in this chapter do indicate that such transfer has indeed occurred, but the stringency of washing conditions was low. This indicates that the degree of homology between the target and probe DNA was low. Use of DNA amplification may help in further proving the transfer of DNA. In addition, mating experiments between indigenous organisms not displaying kanamycin resistance and engineered *E. carotovora*, recommended by EPA personnel, are planned.

LITERATURE CITED

Allen, C., V.K. Stromberg, F.D. Smith, G.H. Lacy, and M.S. Mount. 1986. Complementation of an *Erwinia carotovora* subsp. *carotovora* protease mutant with a protease-encoding cosmid. Mol. Gen. Genet. 202:276-279.

Barkay, T. and G.S. Sayler. 1988. Gene probes as a tool for the detection of specific genomes in the environment, *In* W.J. Adams, G.A. Chapman, and W.G. Landis (eds.), Aquatic Toxicology and Hazard Assessment: 10th volume, ASTM STP 971. American Society for Testing and Materials, Philadelphia, pp. 29-36.

Ditta, G., S. Stanfield, D. Corbin, and D.R. Helinski. 1980. Broad host range DNA cloning system for Gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. Proc. Nat. Acad. Sci. USA 77:7347-7351.

Freifelder, D.M. 1987. Microbial Genetics, Jones and Bartlett, New York, 601 pp.

Genthner, F.J., P. Chatterjee, T. Barkay, and A.W. Bourquin. 1988. Capacity of aquatic bacteria to act as recipients of plasmid DNA. Appl. Environ. Micro. 54:115-117.

Gerhardt, P., R.G.E. Murray, R.N. Costilow, E.W. Nester, W.A. Wood, N.R. Krieg, and G.B. Phillips (eds.). 1981. Manual of Methods for General Bacteriology, American Society for Microbiology, Washington, D.C., 524 pp.

Glick, B.R., H.E. Brooks, and J.J. Pasternak. 1986. Physiological effects of plasmid DNA transformation on *Azotobacter vinelandii*. Can. J. Micro. 32:145-148.

Grant, W.D. and P.E. Long. 1981. Environmental Microbiology. Halsted Press, Glasgow, Scotland, 215 pp.

Grimes, D.J., C.C. Somerville, W. Straube, D.B. Roszak, B.A. Ortiz-Conde, M.T. MacDonell, and R.R. Colwell. 1988. [In] W.J. Adams, G.A. Chapman, and W.G. Landis (eds.), Aquatic Toxicology and Hazard Assessment: 10th volume, ASTM STP 971. American Society for Testing and Materials, Philadelphia, pp. 37-42.

Hada, H.S. and R.K. Sizemore. 1981. Incidence of plasmids in marine *Vibrio* spp. isolated from an oil field in the northwestern Gulf of Mexico. Appl. Environ. Micro. 41:199-202.

Liang, L.N., J.L. Sinclair, L.M. Mallory and M. Alexander. 1982. Fate in model ecosystems of microbial species of potential use in genetic engineering. Appl. Environ. Micro. 44:708-714.

Lindow, S.E. 1985. Ecology of *Pseudomonas syringae* relevant to the field use of ice-deletion mutants constructed in vitro for plant frost control. [In] H.O. Halvorson, D. Pramer, and M. Rogul (eds)., Engineered Organisms in the Environment: Scientific Issues, ASM, Washington. pp. 23-25.

Maniatis, T., E.F. Fritsch, J. Sambrook. 1982. Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 545 pp.

Ogram, A., G.S. Sayler, D. Gustin, and R.J. Lewis. 1988. DNA adsorption to soil and sediments. Environ. Sci. Tech. 22:982-984.

Office of Technology Assessment. 1988. New Developments in Biotechnology - Field-Testing Engineered Organisms: Genetic and Ecological Issues. OTA-BA-350, Washington, D.C., 150 pp.

Pritchard, P.H. and A.W. Bourquin. 1984. The use of microcosms for evaluation of interactions between pollutants and microorganisms, [In] K.C. Marshall (ed.), Advances in Microbial Ecology, Vol. 7. Plenum Press, New York, pp. 133-215.

Rafii, F. and D.L. Crawford. 1988. Transfer of conjugative plasmids and mobilization of a nonconjugative plasmid between *Streptomyces* strains on agar and in soil. Appl. Environ. Micro. 54:1334-1340.

Roberts, D.P., P.M. Berman, C. Allen, V.K. Stromberg, G.H. Lacy, and M.S. Mount. 1986a. *Erwinia carotovora:* Molecular cloning of a 3.4 kilobase DNA fragment mediating production of pectate lyase. Can. J. Plant Pathol. 8:17-27.

Roberts, D.P., P.M. Berman, C. Allen, V.K. Stromberg, G.H. Lacy, and M.S. Mount. 1986b. Requirement for two or more *Erwinia carotovora* subsp. *carotovora* pectolytic gene products for maceration of potato tuber tissue by Escherichia coli. J. Bacteriol. 167:279-284.

SAS Institute, Inc. 1985. SAS User's Guide: Statistics, version 5. SAS Institute, Inc., Cary. N.C.

Seidler, R.J. and S. Hern. 1988. Special report: The release of ice minus recombinant bacteria at California test sites. USEPA, CERL, Corvallis, OR. 83 pp.+ app.

Slater, J.H. 1985. Gene transfer in microbial communities. [In] H.O. Halvorson, D. Pramer, and M. Rogul (eds.), Engineered Organisms in the Environment: Scientific Issues, ASM, Washington. pp. 89-98.

Sokal, R.R. and F.J. Rohlf. 1981. Biometry. W.H. Freeman and Co., San Francisco, CA. 776 pp.

Stotzky, G. and V.N. Krasovsky. 1981. Ecological factors that affect the survival, establishment, growth, and genetic recombination of microbes in natural habitats. [In] S.B. Levy, R.C. Clowes, and E.L. Koenig (eds.), Molecular Biology, Pathogenicity, and Ecology of Bacterial Plasmids, Plenum, New York, pp. 31-42.

Torriani, A. 1960. Influence of inorganic phosphate on the fermentation of phosphatases by *E. coli*. Biochem. Biophys. Acta 38:460-469.

Trevors, J.T. and K.M. Oddie. 1986. R-plasmid transfer in soil and water. Can. J. Micro. 32:610-613.

Van Elsas, J.D., A.F. Dijkstra, J.M. Govaert, amd J.A. van Veen. 1986. Survival of *Pseudomonas fluorescens* and *Bacillus subtilis* introduced into two soils of different texture in field microplots. FEMS Micro. Ecology 38:151-160.

Vieira, J., and J. Messing. 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19:259-268.

Wang, Z. and D.L. Crawford. 1988. Effects of wildtype and recombinant *Streptomyces* on mineralization of organic carbon. Abstracts of First Inter. Conf. Release Genetically Engineered Microorganisms No. 12, p. 13.

Wellington, E.M., N. Cresswell, and V.A. Saunders. 1988. The fate of seeded streptomycetes and their plasmids in soil. Abstracts First Inter. Conf. Release Genetically Eng. Micro. 39:81.

Table 2-1. Biochemical characteristics of engineered (L-863) and wildtype (L-864) *Erwinia carotovora*.

Parameter	L-863	L-864
Gram stain	G-	G-
Cell Morphology	Bacillus	Bacillus
Colony Morphology	White, circular	White, circular
Catalase	weak	weak
Glucose	+	+
Gas	-	-
Lysine decarboxylase	-	-
Lactose	+	+
Arabinose	+	+
Sorbitol	-	-
V-P	-	-
Urease	-	-
Citrate	+	+
Anaerobic Dextrose	+	+
OF Xylose	+	+
Indole	-	-
Resistance to:		
Tetracycline	-	-
Rifampicin	+	+
Kanamycin	-	+

Table 2-2. Biochemical characteristics of unknown bacteria. Unknowns are described by colony color- Y = Yellow, W = White, R = Red.

Parameter	Y-1	Y-2	W-1	W-2	R-1
Gram stain	G-	G-	G-	G-	G-
Cell Morphology	Bacillus	Bacillus	Bacillus	Bacillus	Bacillus
Cell Length	long, diplobacilli	very long	short	moderate	moderate
Colony Morphology	Yellow, irregular	Yellow, smooth	White, mucoid	Yellowish-white	Reddish-white mucoid
Oxidase	-	-	+	+	-
Catalase	+	+	weak	strong	-
Glucose	-	-	-	-	-
Gas	-	-	-	-	-
Lysine decarboxylase	-	-	-	-	-
Ornithine decarboxylase	-	-	-	-	-
Lactose	-	-	-	-	+
Arabinose	-	-	-	-	-
Sorbitol	-	-	-	-	-
V-P	-	-	-	-	-
Dulcitol	-	-	-	-	-
Urease	-	+	+	-	-
Citrate	-	-	-	-	-
Anaerobic Dextrose	-	-	-	-	-
Aerobic OF Dextrose	+	-	-	-	-
Arginine dihydrolase	-	-	-	-	-
Hydrogen sulfide	-	-	-	-	-
OF Xylose	-	-	-	-	-
Indole	-	-	-	-	-
Resistance to:					
Tetracycline	-	-	-	-	-
Rifampicin	-	-	-	-	-
Kanamycin	+	+	+	+	+

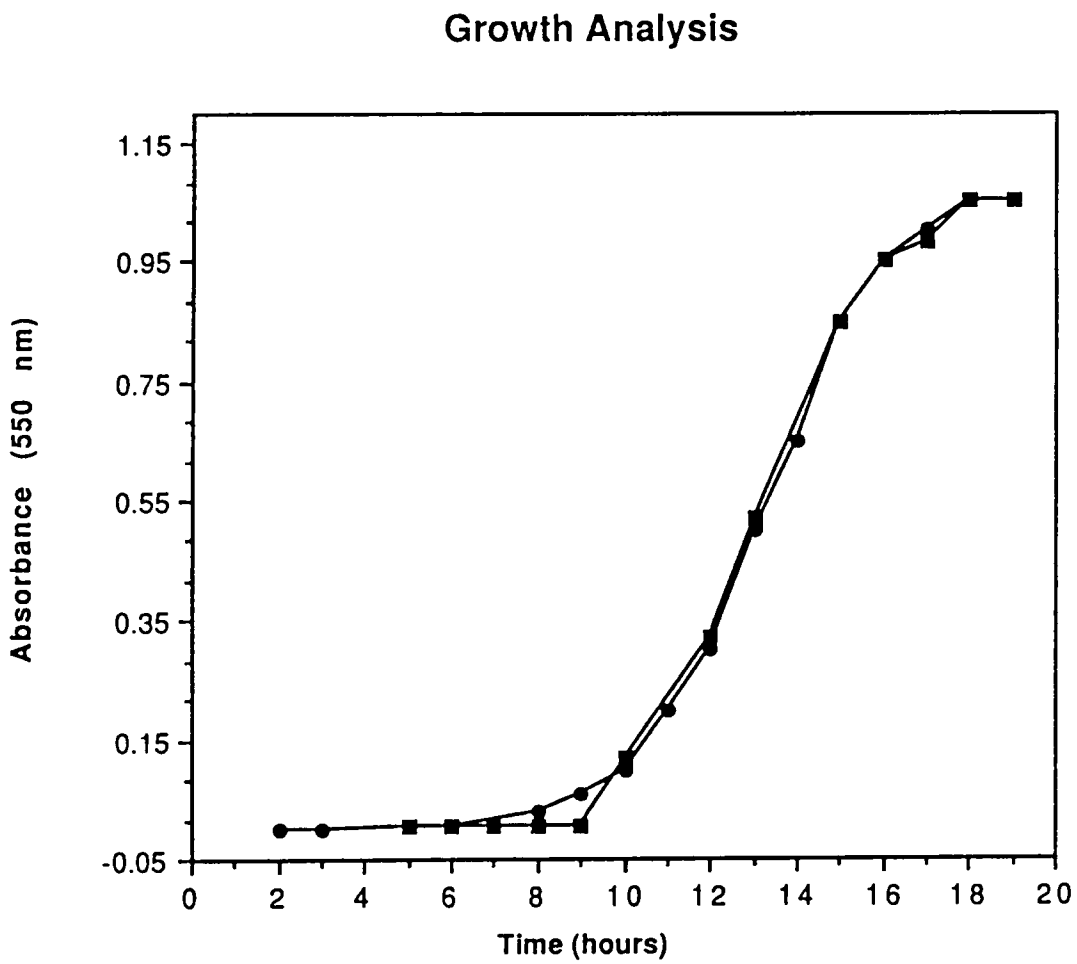

FIG. 2-1. Analysis of growth characteristics of genetically-engineered *Erwinia carotovora* (•) and wildtype *E. carotovora* (▪). A 200 μl aliquot of identical turbidity of either strain was inoculated into nutrient broth and absorbance subsequently measured.

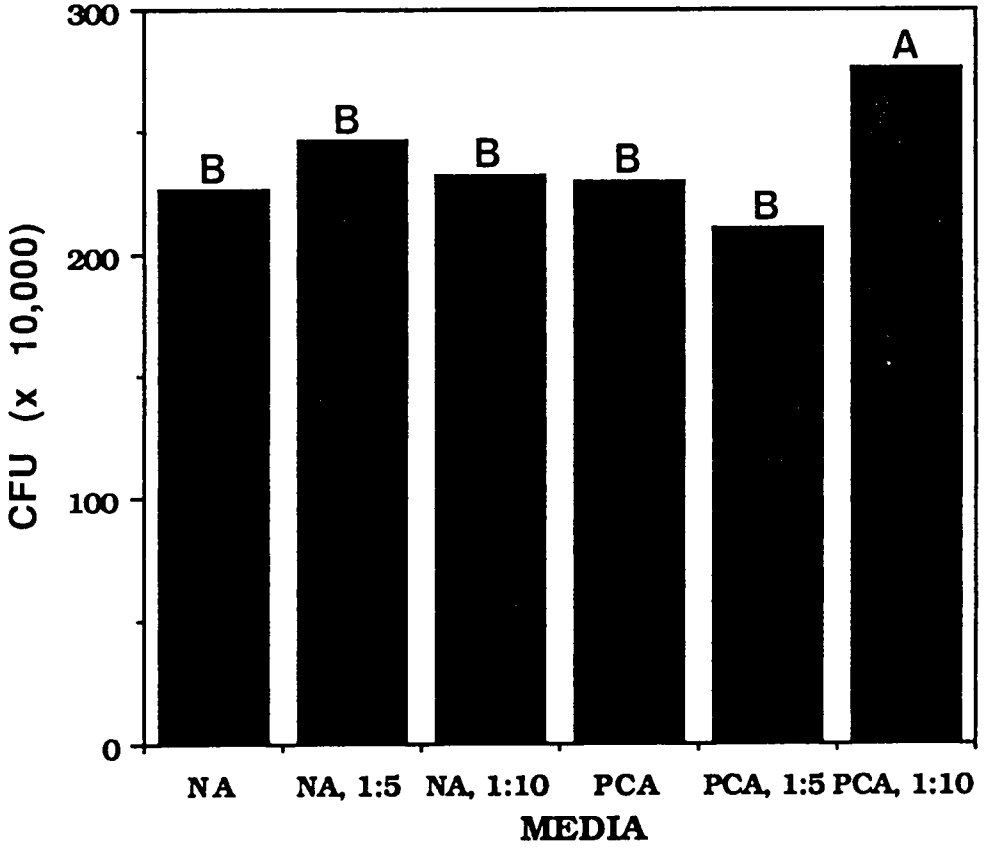

FIG. 2-2. Effect of different media types and dilutions on growth of total numbers of bacteria. Different letters indicate groups with statistical difference based upon multiple comparison of means using the Student-Newman-Keuls test. NA-Nutrient agar, analysis by Student-Newman-Kuels test. NA- Nutrient agar, PCA-Plate count agar.

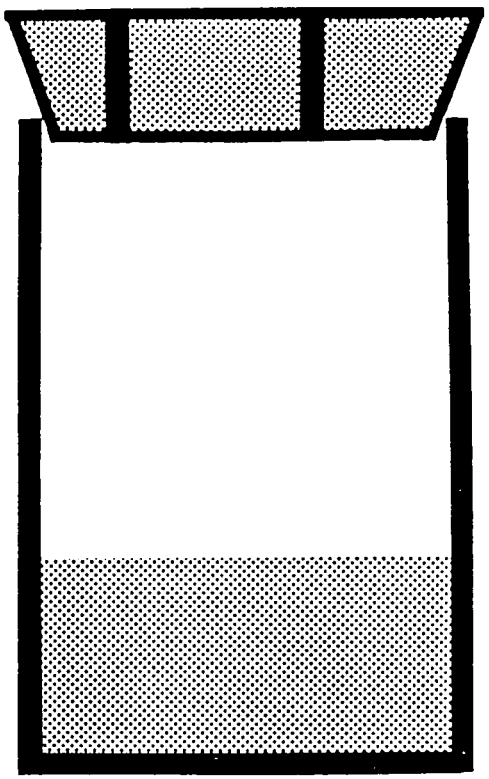

FIG. 2-3. Diagram of microcosm apparatus used in study. Soil was placed in bottom of vessel and jar sealed.

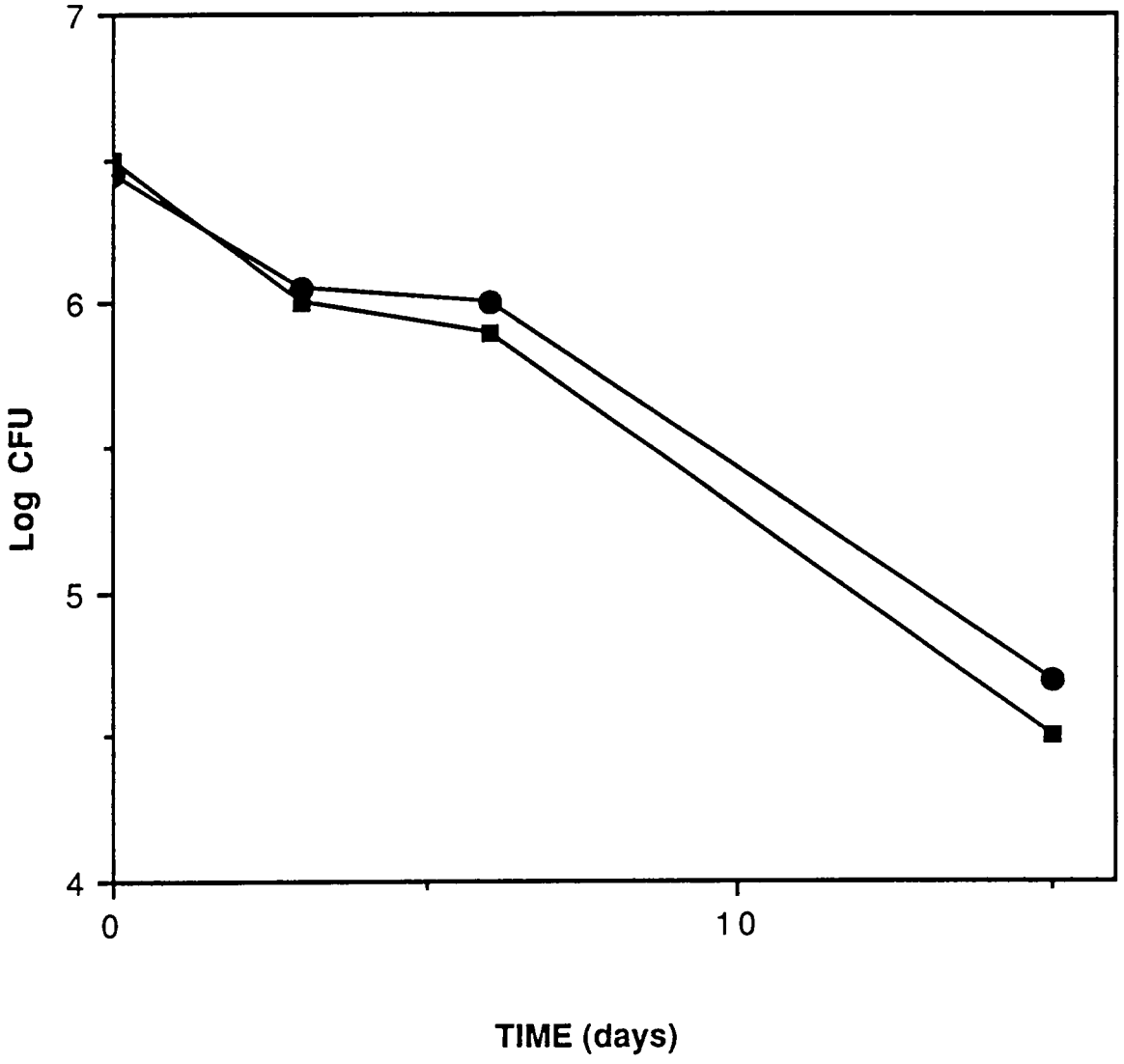

FIG. 2-4. Survival of genetically-engineered (L-833) (•) and wildtype (▪) *Erwinia carotovora* in terrestrial microcosms. Bacteria were inoculated into 200 g of soil and mixture placed into microcosms.

FIG. 2-5. Gas chromatograms of fatty acids from Y-1 (*Flavobacterium* spp.) (top) and Y-2 (*Cytophaga johnsonae*) (bottom). Although some peaks have identical retention times, several do not indicating that the organisms are not identical, even though colony morphology is alike.

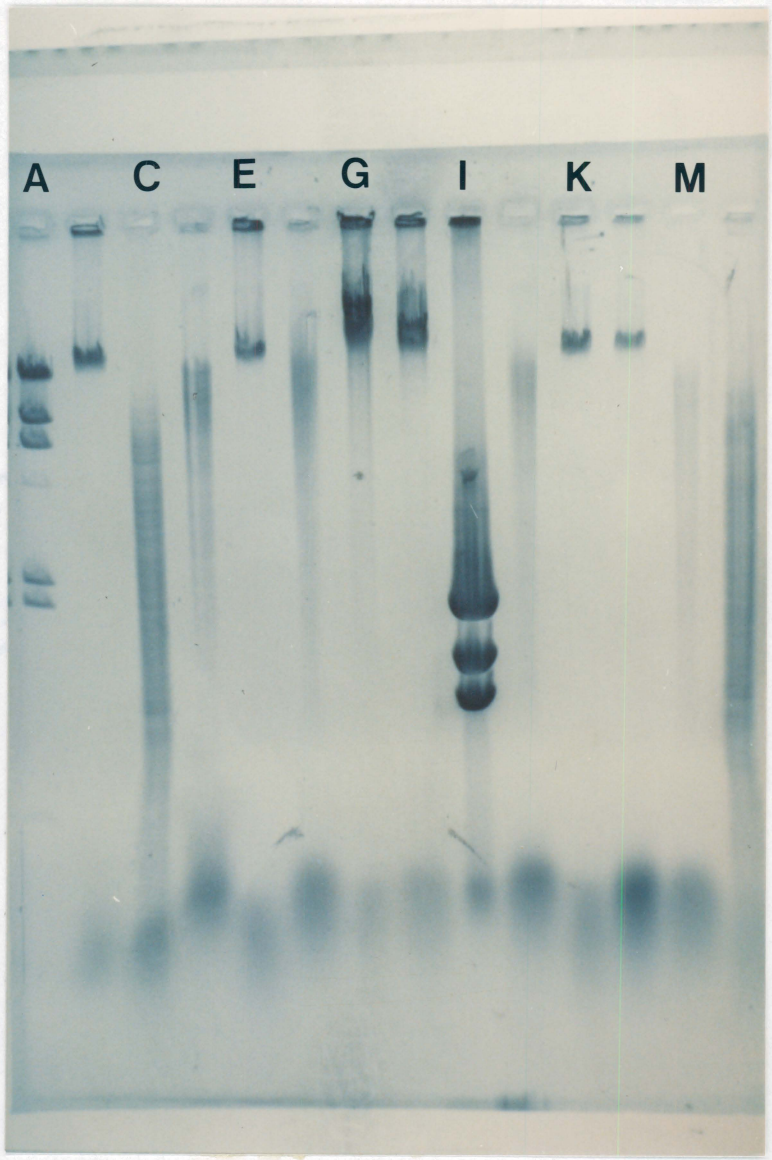

FIG. 2-6. Agarose gel with DNAs from *Erwinia* and unknown bacteria. Some samples restricted with enzymes as described. A - λ/*Hin*dIII marker; B - *Erwinia carotovora* (L-864); C - *Erwinia carotovora* (L-864)/*Hin*cII; D - *Erwinia carotovora (L-864)/Eco*RI; E, F - *Flavobacterium* spp. restricted with *Hin*cII (E) or *Eco*RI (F). G, H - *Cytophaga* restricted with *Hin*cII (G) or *Eco*RI (H). I - pUC4K plasmid restricted with *Hin*cII used as a positive control. J, K - *Agrobacterium tumefaciens* restricted with *Hin*cII (J) or *Eco*RI (K). L, M - *Flavobacterium indologenes* restricted with *Hin*cII (L) or *Eco*RI (M). N - Lane containing rifampin-resistant *Erwinia/Hin*cII.

FIG. 2-7. Southern hybridization of blot from agarose gel on Nitroplus 2000 membrane probed for DNA homology in indigenous microcosm bacteria. Probe is kanamycin resistance marker from pUC4K. A - λ/*Hind*III marker; B - *Erwinia carotovora*/*Eco* RI; C,D,F,G - Indigenous bacteria/*Eco*RI; E - pUC4K/*Hin*cII; H - Lane containing rifampin-resistant *Erwinia*/*Hin*cII (No homology).

CHAPTER THREE

SURVIVAL OF GENETICALLY-ENGINEERED *ERWINIA CAROTOVORA* AND EFFECTS UPON SELECTED MICROBIAL GENERA

INTRODUCTION

Genetic engineering is being used to develop microorganisms capable of performing commercial tasks such as producing pesticides and pharmaceuticals, fixing nitrogen, leaching ores, recovering oil, degrading hazardous wastes, and constructing biological control agents for pests or diseases. Use of genetically-engineered microorganisms (GEMs) has been limited to small-scale trials due to lack of knowledge about their survival, impact on ecosystems, and public concern over release. A need exists for development of risk assessment methodologies to predict environmental risk, if any, prior to release.

A number of factors need to be considered before GEMs are released into the environment in large quantities. Success of an organism under laboratory conditions does not ensure success in natural ecosystems. Conversely, the ability to contain and control species in the laboratory does not preclude their establishment, survival, and adverse effects outside the laboratory. In addition, GEMs present unique regulatory problems since, unlike toxic chemicals, organisms have the potential to increase in numbers in the environment at the site of release or following transport to some unintended and unexpected location (2). GEMs may compete with and displace native populations, thereby affecting the normal processing of materials in soils and sediments (2, 16). Also, the use of genetic mechanisms for efficient dispersal of DNA is intrinsic to survival of the organism in the environment; thus DNA from the GEM may be transferred to native flora (16). Finally, strains possessing enzymes for the assimilation and utilization of growth substrates may survive for extended periods when sub-

jected to nutrient poor environments and thus pose additional problems when developing risk assessment protocols (2).

Some confusion exists concerning the nature of GEMs for ecological studies or the environmental fate of the recombinant DNA they contain. Many researchers have proposed models using plasmids containing recombinant DNA; however, molecular biologists constructing GEMs for environmental release prefer insertion of the recombinant DNA directly into the bacterial chromosome since such constructions are more stable (9, 10). In this study, a GEM was used that was constructed specifically to include two types of genetic alterations in the bacterial chromosome: deletion of a gene fundamental to plant pathogenesis and inclusion of an antibiotic resistance gene. In this study, the survival of the genetically-engineered strain, as well as its parent strain, was examined and the effect of adding both strains upon populations of indigenous bacteria in microcosms was determined.

MATERIALS AND METHODS

Bacterial strains and plasmids

The bacterium used in this study was *Erwinia carotovora* subsp. *carotovora* (ECC) strain EC14 (1), a phytopathogenic enterobacterium that causes soft rot in tissues of many plants and is ubiquitous in nature, occurring in plant, soil, and aquatic habitats (4, 6, 14, 17). This bacterium may be transmitted by insects (7, 8). Pectate lyase genes from EC14 were cloned into plasmid pBR322 on an 8.7 kilobase pair (kbp) fragment and expressed in *Escherichia coli* strain HB101 (19). A 5.5 kbp fragment of the EC14 DNA was deleted by digestion with *Bgl*II and the remaining plasmid DNA was ligated using T4 ligase. The resulting plasmid, pDR41, no longer mediated pectate lyase activity as determined by isoelectric focusing and activity stain overlays (18). Plasmid pDR41 was further modified

by inserting the *Pst*I 1.4 kbp fragment of Tn*903* (kanamycin resistance) isolated from pUC4K (25) into the *Pst*I site of pDR41. The new plasmid, pVS20, mediating kanamycin resistance, was transferred into EC14 by conjugal triparental mating with the helper plasmid pRK2013 (5). Substitutional mutagenesis was attempted by curing pVS20 from EC14 in low phosphate medium (24). Kanamycin-resistant strain L-833 was recovered that contained no detectable plasmids and was reduced up to 30% in the ability to rot tuber tissue but was not disarmed for the production of the target pectate lyase. The Tn*903* derived fragment, therefore, is probably inserted in a *cis*-orientation to the wildtype pectate lyase genes in the EC14 chromosome. Strain L-863 was a spontaneous mutant of wildtype EC14 resistant to 150 μg/ml rifampicin; strain L-864 is a spontaneous rifampin-resistant (150 μg/ml) mutant of L-833. Both L-863 and L-864 strains displayed growth and biochemical characteristics identical to the wildtype strain.

Media and Chemicals

Plate count agar (PCA; Difco Laboratories, Detroit, MI), diluted 1:10, was used to enumerate total bacteria. This dilution was chosen based upon preliminary experiments for maximum colonies recovered (data not shown). PCA amended with 40 μg/ml kanamycin monosulfate and 150 μg/ml rifampicin (Sigma Chemical Co., St. Louis, MO) were used to detect the genetically-engineered and wildtype *E. carotovora*, respectively. Actinomycete isolation agar (AI; Difco) was used to isolate Actinomycetes; mannitol salt agar (MSA; Difco) was used to enumerate *Staphylococcus* spp.; and pseudomonas isolation agar (PIA; Difco) was used to isolate *Pseudomonas* spp.

Microcosms

Terrestrial microcosms consisted of 1-liter glass mason jars containing 200 g of soil (Fig. 3-1). Humidified, sterile air was circulated at 5 ml/min through the microcosm, as shown, for the duration of the study. This minimized evaporation of soil moisture while allowing for elimination of metabolic by-products. Both intake and exhaust air was sterilized by membrane filtration using a Gelman Maxi Capsule ™ (Gelman Sciences, Inc., Ann Arbor, MI). Soil in microcosms was gently mixed every other day to ensure a homogenous distribution of microorganisms. This study utilized soil from the Virginia Polytechnic Institute and State University (Virginia Tech) farm at Whitethorn on the New River. Soil was a Hayter loam type, no-till previously planted with corn, dark grayish brown in color, and consisted of 39% sand, 41% silt, and 20% clay. Other soil characteristics, determined by the Virginia Tech soil testing laboratory, are listed in Table 3-1.

Microcosm Inoculation

Erwinia carotovora were grown in 4 liters of nutrient broth to an absorbance of approximately 1.0 at 550 nm. Bacterial cells were harvested by centrifugation at 10,000 *g* for 20 min to provide approximately 6 x 10^{10} cells. The supernatant was discarded and the pellet washed in sterile distilled water (SDW), resuspended in SDW, and centrifuged at 10,000 *g*. This pellet was suspended in SDW (45 ml) so that 15 ml of inoculum would be delivered to each microcosm (200 g soil) to provide approximately 10^8 cells/g soil. Inoculum was added to soil, which had been previously air dried for 48 h and passed through a 4.75-mm sieve after collection, and throughly mixed. This inoculated soil was passed through a 2-mm sieve, remixed, and then randomly placed into microcosms. Microcosms were incubated at 20°C.

Sampling of Microcosms

Samples were withdrawn from microcosms on 0, 1, 3, 6, 12, 24, 42, and 46 days after inoculation. To allow for diffusion of inoculum, day 0 samples were withdrawn approximately 2 h after inoculation. After gently mixing the soil in the microcosms, soil samples (1 g) were randomly removed and placed in milk dilution bottles containing 99 ml SDW. These were mixed, first manually for a brief period and then for 20 min on an orbital shaker at 150 rpm, and subsequent dilutions prepared. Dilutions were plated onto appropriate media using the spread plate technique. Inoculated Petri dishes were incubated at 25°C (PCA, AI) or 30° C (PCA with antibiotics; PIA; MSA) for 48-96 h, and then plates with appropriate dilutions were counted. Results were expressed as CFU/g dry weight. Soil for determination of water content was removed at time of sampling.

Experimental Design and Statistical Analysis

Three microcosms were inoculated with the genetically-engineered strain and three with the wildtype. On each sampling day, one sample from each of the six microcosms was withdrawn and diluted. Three plates were prepared from each dilution and plates with approximately 30 to 300 colonies were counted. Statistical significance between groups at each sampling day was evaluated using Student's *t* test and between sampling intervals using a repeated measures analysis (21). Statistical analysis was accomplished using SAS (20).

RESULTS

Soil characteristics after the 46-day study are shown in Table 1. While pH and concentrations of phosphorus, potassium, calcium, and magnesium remained essentially unchanged, nitrogen levels increased by a factor of 2.5 and organic

carbon decreased by approximately 20% in all microcosms. Using humidified air, the moisture content of microcosm soil fluctuated only about 3% during the course of the study.

Populations of genetically-engineered and wildtype *E. carotovora* declined at significantly ($p \leq 0.05$) different rates over the duration of the experiment; at days 1, 3, 6, 12, and 46, the genetically-engineered *E. carotovora* (L-864) was present at a significantly lower concentration ($p \leq 0.05$) than the L-863 (wildtype) (Fig. 3-2). Initial concentrations of engineered and wildtype *E. carotovora* although not exactly the same, were not statistically different. Over the course of the experiment, the engineered strain declined significantly faster ($p \leq 0.05$) than the wildtype as determined by the repeated measures analysis. The threshold of detection for both engineered and wildtype was approximately 10^3 CFU/g dry weight.

Populations of total bacteria remained relatively stable in both treatment groups and declined only one order of magnitude during the study (Fig. 3-3A). There were no significant differences in initial populations, but significant decreases in populations were observed between 1 and 3 days as well as 6 and 12 days. No difference was observed due to group effect ($p > 0.05$).

No significant differences were also noted between group effects upon populations of *Pseudomonas* spp. (Fig. 3-3B), *Staphylococcus* spp. (Fig. 3-4B), and Actinomycetes (Fig. 3-4A). While *Pseudomonas* spp. and Actinomycetes experienced significant declines of 0.5 and 1 order of magnitude, respectively, *Staphylococcus* spp. of both test groups actually increased in number with significant increase being observed between days 24-46. Initial and final populations of *Staphylococcus* spp. (Fig. 3-4B) and *Pseudomonas* spp. (Fig. 3-3B) were not significantly different ($p > 0.05$); however, beginning concentrations of

Actinomycetes were significantly ($p \leq 0.05$) different while ending populations were not ($p > 0.05$) (Fig. 3-4).

DISCUSSION

Both the genetically-engineered and wildtype strains of *E. carotovora* significantly declined at different rates over the duration of the experiment. Although the decline in numbers has been previously observed (D.R. Orvos, G.H. Lacy, and J. Cairns, Jr., Abstr. Annu. Meet. Soc. Environ. Toxicol. Chem. 1987, p. 212), the more rapid decline of the engineered strain was unexpected. Although statistical significance between the genetically-engineered and the wildtype bacteria was observed at day 1, 3, 6, 12 and 46, the reason for this significance is not clear and may, in fact, simply be a reflection of inherent variability from the sampling technique rather than any actual difference in rate of decline since no difference was seen at day 24. It is possible, however, that insertion of the kanamycin sequences in the GEM conferred a competitive disadvantage. Regardless, it is important to note that the genetically-engineered strain did not survive any better compared to the wildtype strain; this was expected based upon the phenotypes and genotypes of both introduced strains. Overall, it appears that, even at high initial concentrations, the added bacteria, regardless of origin, were not able to compete with indigenous oligotrophic bacteria. This has been observed with wildtype *E. carotovora* (3) and other species of genetically-engineered bacteria after release into soil systems (11, 12, 13) as well as for wildtype *E. carotovora* (3). The high initial concentration of both strains reflected concentrations of *E. carotovora* found near rotting tissue as well as the minimum number of other rhizobacteria required for colonization (23). Lower detection limits for both genetically-engineered and wildtype strains was approximately

10^3 CFU/g; this correlates well with other studies (D.R Orvos, G.H. Lacy, and J. Cairns, Jr., Abstr. Annu. Meet. Soc. Environ. Toxicol. Chem. 1987, p. 212; 22).

Proliferation of indigenous microbes on antibiotic-fortified media was noted when high inoculum levels were used; detection limits may indeed be even lower if these problems are overcome. Indigenous microbes did not grow on plates containing rifampin at dilutions less than 10^3; however, kanamycin, by itself, was not found to be a satisfactory marker antibiotic since some indigenous soil bacteria, particularly *Streptomyces,* are already resistant to it. No acquisition of the marker antibiotic (Kan) was observed in indigenous bacteria as was suggested but not confirmed in our previous studies (D.R. Orvos, G.H. Lacy, and J. Cairns, Jr., Abstr. First Int. Conf. Release Genetically Engineered Micro. 1988, No. 56). It is important to note that a different soil type was used for this study as was a new microcosm design allowing air flow through the containers. It is not known if these factors may have prevented the potential exchange of the marker antibiotic resistance genome. Regardless, the need for sensitive detection methodologies remains clear.

Total populations of bacteria experienced a significant increase between day 0 and 1 in both microcosm groups (Fig. 3-3). This has been reported previously (D.R. Orvos, G.H. Lacy, and J. Cairns, Jr., Abstr. Annu. Meet. Soc. Environ. Toxicol. Chem. 1987, p. 212) and is probably due to added water and organic matter, in the form of bacteria, from the inoculum. The small decrease could be reflective of cropping by nematodes and protozoa though no conclusive evidence was obtained (4). Although no one medium will allow growth of all indigenous bacteria (15), it is apparent that use of a 1:10 dilution of plate count agar allowed accurate sampling of a subset of total bacteria. The number sampled remained consistent throughout the experiment and therefore allowed one to determine the effect of GEM release on this subset.

Actinomycetes in both microcosm groups declined significantly ($p \leq 0.05$) over time, although the decline was less in microcosms containing the engineered *E. carotovora*. The initial populations in both groups differed significantly by an order of magnitude, and this may partially explain this finding (Fig. 3-4). Actinomycetes may have increased to high levels while soil was air dried, then declined to equilibrium in moist soil.

Pseudomonas spp. declined by only 0.5 orders of magnitude during the study and, although no significant treatment effects were observed, some group-time interactions were noted (Fig. 3-3). Increased variability was also noted among pseudomonads as compared to the other bacteria sampled, possibly due to fluctuations in moisture or nutrients in the microcosms. Pseudomonads in microcosms containing the engineered strain demonstrated a significant decrease between day 3 and 6, while those in microcosms containing the wildtype did not change. While an interaction between pseudomonads and engineered *E. carotovora* is possible, it is probably unlikely. The ramifications of this observation are unclear.

Staphylococcus spp. were found to have stable populations over time but experienced a significant increase in both treatment groups between day 24 and 46 (Fig. 4-4). No similar increase was seen in any of the other selected genera or in total populations. It is hypothesized that this may be due to increased moisture content of the soil, an increase in nutrients due to dead *E. carotovora* or some unknown factor.

The slow decline of total bacteria, *Pseudomonas* spp., and Actinomycetes, as well as the small increase of *Staphylococcus* spp., were reflective of a microcosm system that permitted initial populations of bacteria to remain viable during the course of the experiment. Lack of other confounding factors, such as plant growth and other environmental influences, permitted us to determine

directly if the genetically-engineered bacterium affected these important groups of soil microorganisms in a manner different than that of the wildtype. Each of these groups, as well as the total populations, are important within their own ecological niches, are easy to monitor, and may be indicative of organisms that may be affected by addition of an exogenous bacterium.

Low standard deviations between and within (data not shown) replicates were observed. This documents that the spread plate technique method is an inexpensive, yet useful, method for gathering preliminary and confirmatory data when determining the environmental fate of GEMs. Although the viable plate count method is acknowledged to have many potential sources of error (3, 15), these sources can be minimized by personnel training and development of standard operating procedures for a particular study; we have found that these measures can nearly eliminate operator error regardless of source and increase the usefulness of the technique.

The increase in nitrogen by-products, especially ammonia, may have contributed to the large reduction of exogenous bacteria as well as the slow decline of indigenous species. We speculate that although the relatively small reduction in total organic carbon did not affect indigenous populations, it may have contributed to the decrease of *E. carotovora*

Even with the small reduction in organic matter and possible accumulation of nitrogen metabolites, this microcosm design appeared to be satisfactory for preliminary studies regarding the risk assessment of genetically-engineered microorganisms released into the environment. By removing extraneous confounding factors, direct determination of GEM effects upon microbial communities is permissible. In addition, GEMs and their wildtype survived for over 40 days after inoculation at high concentrations. Perturbation of structural or func-

tional parameters, as well as potential genetic exchange, would be expected to occur within this time period.

Although a simple microcosm design was employed, this design yielded preliminary data concerning the survival of a GEM and structural effects upon selected bacterial populations without the presence of confounding factors. Future research will examine the effect of such confounding factors as vegetation and temperature fluctuations, upon survival and structural effects as well as potential functional effects of different GEMs released into the environment.

LITERATURE CITED

1. Allen, C., V.K. Stromberg, F.D. Smith, G.H. Lacy, and M.S. Mount. 1986. Complementation of an *Erwinia carotovora* subsp. *carotovora* protease mutant with a protease-encoding cosmid. Mol. Gen. Genet. **202**: 276-279.

2. Cairns, J., Jr., and J.R. Pratt. 1986. Ecological consequence assessment: effects of bioengineered organisms, p. 88-108. *In* J. Fiksel and V.T. Covello (ed.), Biotechnology risk assessment: issues and methods for environmental introductions. Pergamon Press, New York.

3. DeBoer, S.H. 1982. Survival of phytopathogenic bacteria in soil, p. 285-305. *In* M.S. Mount and G.H. Lacy (ed.), Phytopathogenic prokaryotes, vol. 1. Academic Press Inc., New York.

4. DeBoer, S.H. 1983. Frequency and distribution of *Erwinia carotovora* serogroups associated with potato in the Pemberton Valley of British Columbia. Can. J. Plant Pathol. **5**: 279-284.

5. Ditta, G., S. Stanfield, D. Corbin, and D.R. Helinski. 1980. Broad host range DNA cloning system for Gram-negative bacteria: Construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77**: 7347-7351.

6. Harrison, M.D., G.D. Franc, D.A. Maddox, J.E. Michard, and N.J. McCarter-Zorner. 1987. Presence of *Erwinia carotovora* in surface water in North America. J. Appl. Bacter. **62**: 565-570.

7. Kloepper, J.W., M.D. Harrison, and J.W. Brewer. 1979. The association of *Erwinia carotovora* var. *atroseptica* and *Erwinia carotovora* var. *carotovora* with insects in Colorado. Am. Potato J. **56:** 351-361.

8. Kloepper, J.W., M.D. Harrison, and J.W. Brewer. 1981. Effect of temperature on differential persistence and insect transmission of *Erwinia carotovora* var. *atroseptica* and *Erwinia carotovora* var. *carotovora*. Am. Potato J. **58:** 585-592.

9. Lacy, G.H., and J.V. Leary. 1975. Transfer of antibiotic resistance plasmid RP-1 into *Pseudomonas glycinea* and *Pseudomonas phaseolicola in vitro* and *in planta*. J. Gen. Microbiol. **88:** 49-57.

10. Lacy, G.H., and J.V. Leary. 1976. Plasmid-mediated transmission of chromosomal genes in *Pseudomonas glycinea*. Genet. Res. (Camb.) **27:** 363-368.

11. Liang, L.N., J.L. Sinclair, L. Mallory, and M. Alexander. 1982. Fate in model ecosystems of microbial species of potential use in genetic engineering. Appl. Environ. Microbiol. **44:** 708-714.

12. Lindow, S.E. 1985. Ecology of *Pseudomonas syringae* relevant to the field use of ice- deletion mutants constructed in vitro for plant frost control, p. 23-35. *In* H.O. Halvorson, D. Pramer, and M. Rogul (ed.), Engineered organisms in the environment: scientific issues. American Society of Microbiology, Washington, D.C.

13. Marshall, B., P. Flynn, D. Kamely, and S.B. Levy. 1988. Survival of *Escherichia coli* with and without ColE1: : Tn5 after aerosol dispersal in a laboratory and a farm environment. Appl. Environ. Microbiol. **54**: 1776-1783.

14. McCarter-Zorner, N.J., G.D. Franc, M.D. Harrison, J.E. Michaud, C.E. Quinn, I.A. Sells, and D.C. Graham. 1984. Soft rot *Erwinia* bacteria in surface and underground waters in southern Scotland and in Colorado, United States. J. Appl. Bacter. **57**: 95-105.

15. Mills, A.L., and P.E. Bell. 1986. Determination of individual organisms and their activities *in situ*, p. 27-60. *In* R.L. Tate (ed.), Microbial autecology. Wiley, New York.

16. Office of Technology Assessment. 1988. Field-testing engineered organisms: genetic and ecological issues. OTA-BA-350, U.S. Government Printing Office, Washington, D.C., 150 pp.

17. Perombelon, M.C.M., and A. Kelman. 1980. Ecology of the soft rot erwinias. Annu. Rev. Phytopathol. **18**: 361-387.

18. Reid, J.L., and A. Collmer. 1985. Activity stain for rapid characterization of pectic enzymes in isoelectrically focusing and sodium dodecyl sulfate-polyacrylamide gels. Appl. Environ. Microbiol. **50**: 615-622.

19. Roberts, D.P., P.M. Berman, C. Allen, V.K. Stromberg, G.H. Lacy, and M.S. Mount. 1986. Requirement for two or more *Erwinia carotovora* subsp.

carotovora pectolytic gene products for maceration of potato tuber tissue by Escherichia coli. J. Bacteriol. **167**: 279-284.

20. SAS Institute, Inc. 1985. SAS user's guide: statistics, version 5. SAS Institute, Inc., Cary, N.C.

21. Sokal, R.R. and F.J. Rohlf. 1981. Biometry. W.H. Freeman, New York.

22. Stanghellini, M.E. 1982. Soft-rotting bacteria in the rhizosphere, p. 249-261. *In* M.S. Mount and G.H. Lacy (ed.), Phytopathogenic prokaryotes, vol. 1. Academic Press Inc., New York.

23. Suslow, T.V. 1982. Role of root-colonizing bacteria in plant growth, p. 187-223. *In* M.S. Mount and G.H. Lacy (ed.), Phytopathogenic prokaryotes, vol. 1. Academic Press Inc., New York.

24. Torriani, A. 1960. Influence of inorganic phosphate on the fermentation of phosphatases by *E. coli*. Biochem. Biophys. Acta **38**: 460-469.

25. Vieira, J., and J. Messing. 1982. The pUC plasmids, and M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**: 259-268.

TABLE 3-1. Soil characteristics of Hayter loam soil before inoculation and 46 days after inoculation at experiment termination

Sample	pH	P (ppm)	K (ppm)	Ca (ppm)	Mg (ppm)	Mn (ppm)	Zn (ppm)	NO_3-N (ppm)	Organic Matter (%)
PRE[a]	5.6	19	138	876	128	39	2.3	13	3.7
GEM[b]	5.4	17	119	852	128	22	3.4	30	3.0
MUT[c]	5.4	18	124	864	129	22	2.4	33	3.1

[a]Pretest soil sample.

[b]Post-experiment soil from microcosm inoculated with genetically-engineered *Erwinia carotovora*.

[c]Post-experiment soil from microcosm inoculated with wildtype bacteria.

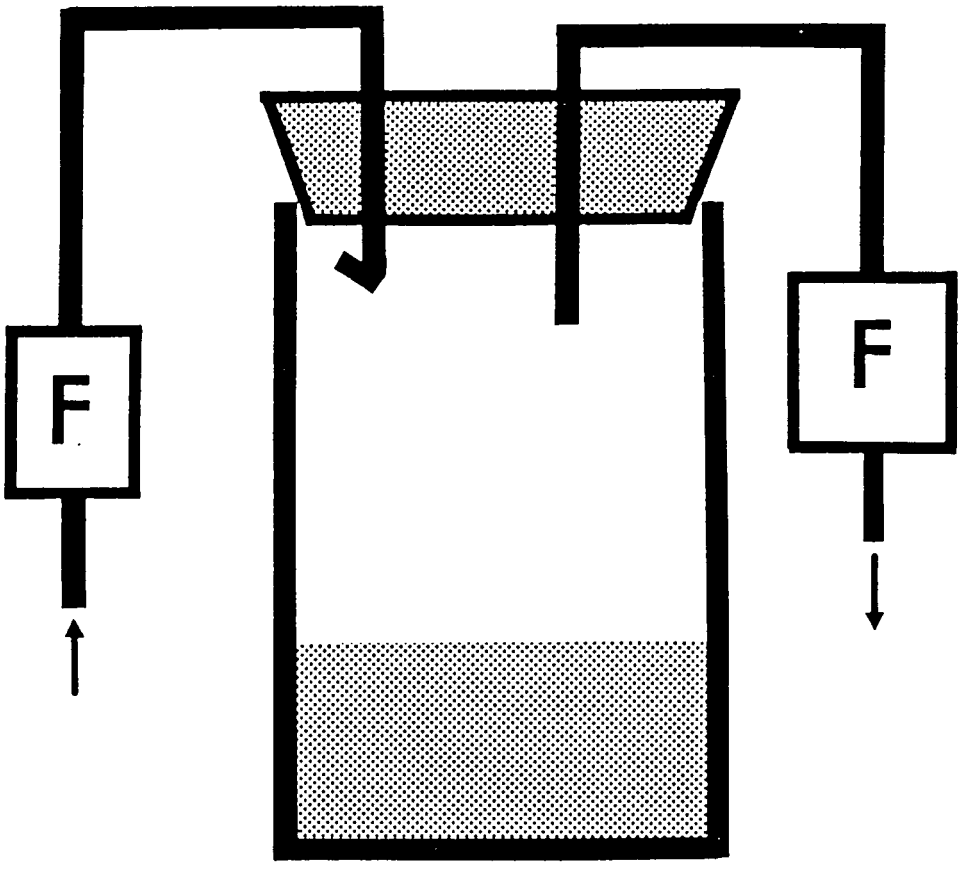

FIG. 3-1. Diagram of microcosm apparatus used in study. Soil in bottom of vessel was subjected to constant flow of humidified air that was sterlized by membrane filters (F).

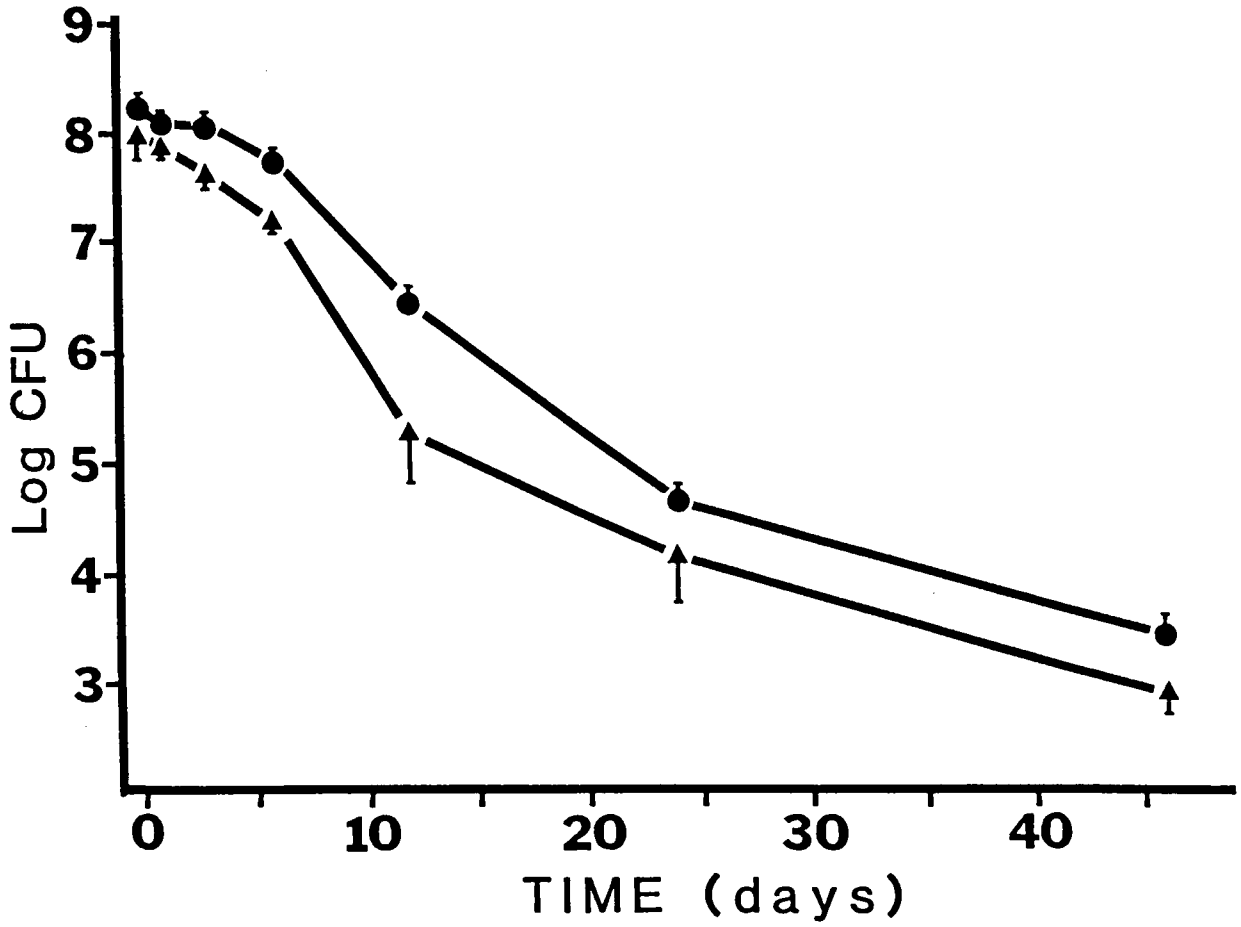

FIG. 3-2. Survival of wildtype *E. carotovora* (L-863) (•) and genetically-engineered *E. carotovora* (L-864) (▲) in soil microcosms. Error bars represent one standard deviation unit and are included in the symbol when not shown.

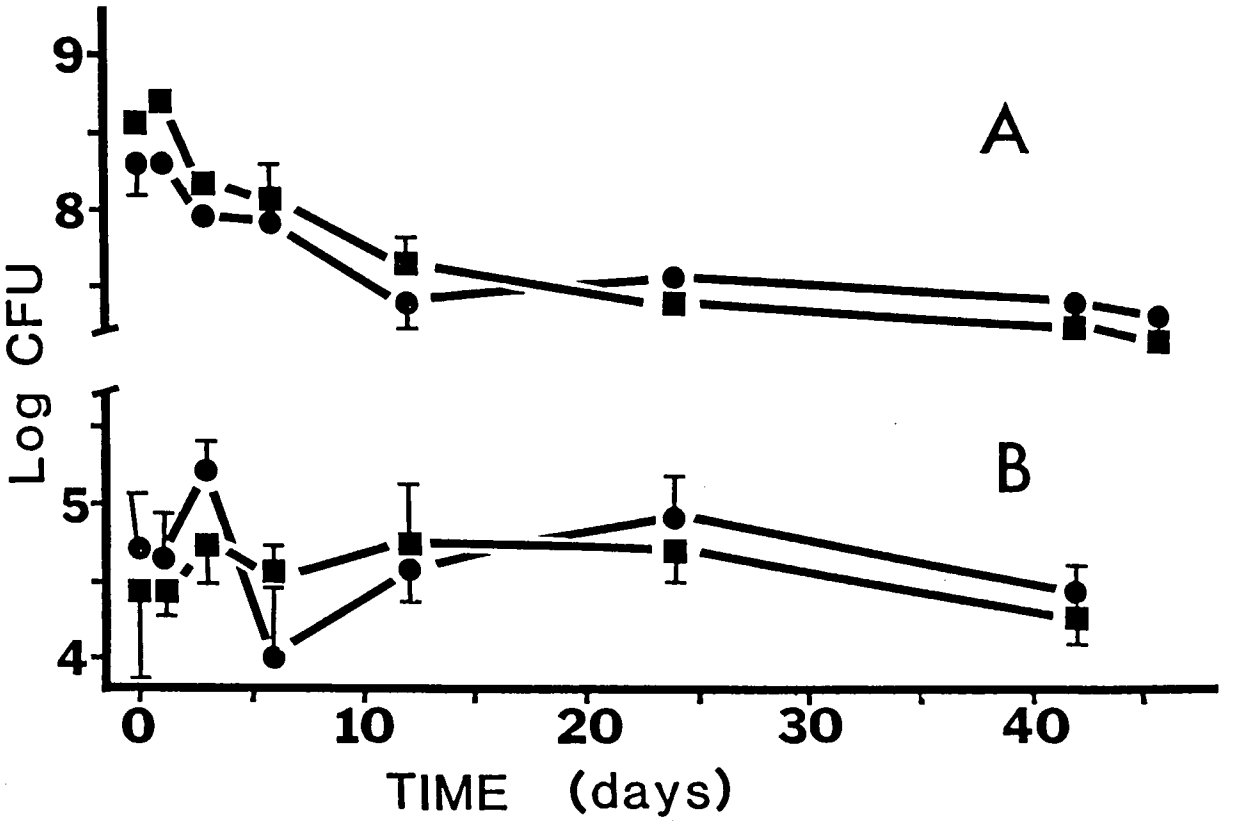

FIG. 3-3. (A) Populations of total bacteria and (B) populations of pseudomonads in microcosms inoculated with wildtype *E. carotovora* (L-863) (■) and genetically-engineered *E. carotovora* (L-864) (●) in soil microcosms. Error bars represent one standard deviation unit and are included in the symbol when not shown.

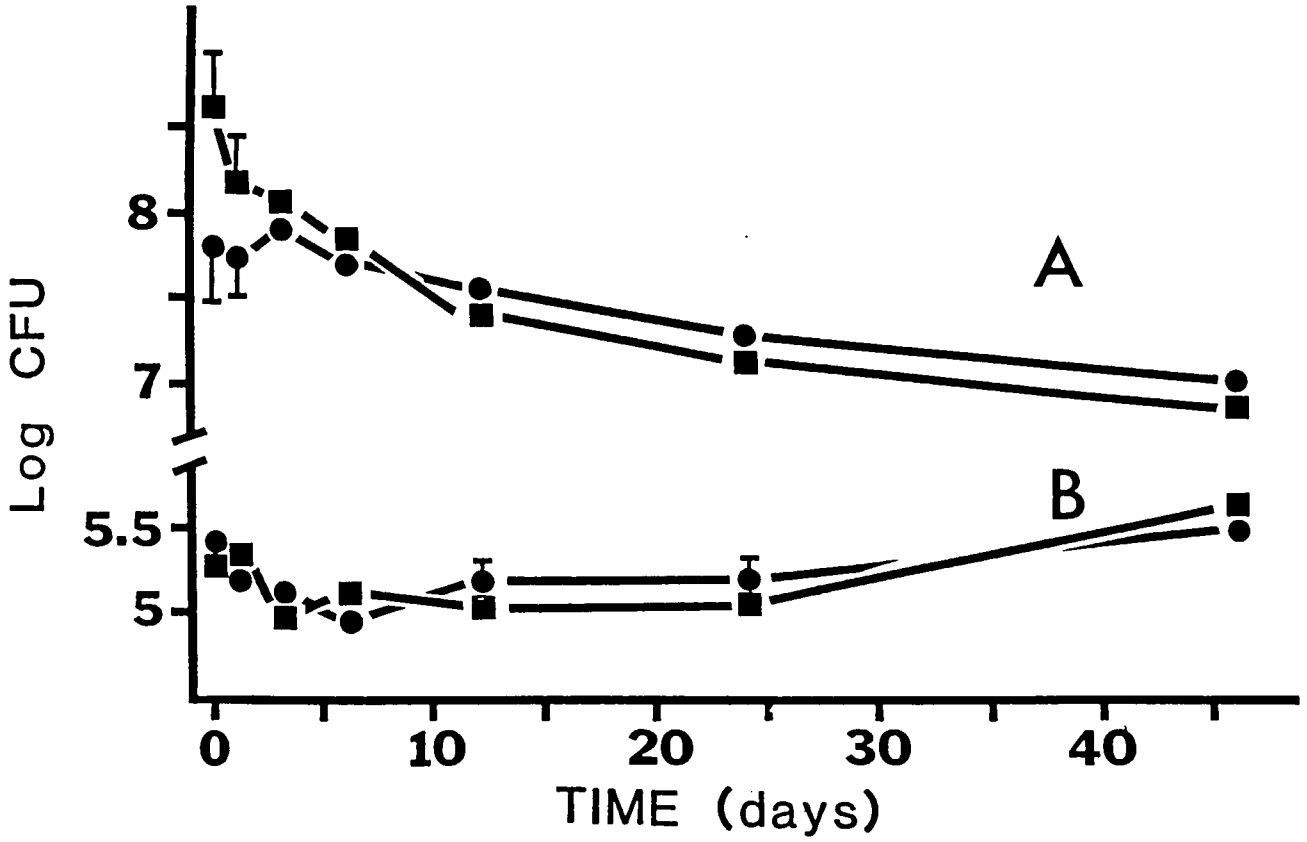

FIG. 3-4. (A) Populations of actinomycetes and (B) populations of *Staphylococci* in microcosms inoculated with wildtype *E. carotovora* (L-863) (■) and genetically-engineered *E. carotovora* (L-864) (•) in soil microcosms. Error bars represent one standard deviation unit and are included in the symbol when not shown.

CHAPTER FOUR

INTRASPECIFIC COMPETITION

INTRODUCTION

Environmental release of genetically-engineered microorganisms (GEMs) has raised concerns over potential ecological effects. Assessment of survival will be useful in preliminary testing for risk assessment when predicting potential adverse structural, functional, and genetic effects. Survival is dependent upon many environmental parameters including substrate, nutrients, temperature, pH, moisture, and predators (Stacey, 1985; Grant and Long, 1981). Survival and competition are interrelated and often used interchangably in microbial ecology. However, while survival is an important parameter, competition of the introduced bacteria with its parental strain is another way to ascertain if the GEM will possess an ability for enhanced survival. In addition, removing competitors by substrate sterlization will also provide data on survival in perturbed systems. Such perturbations may enhance GEM survival (Office of Technology Assessment, 1988).

Competition is defined by Ricklefs (1979) as the use of a resource by an organism in such a way that it reduces resource availability to other organisms. Competition may be intraspecific or interspecific; both types are poorly understood in microbial ecosystems. Interspecific competition in nonsterile soil has been addressed in earlier chapters.

This study used a genetically-engineered strain of *Erwinia carotovora* that was constructed to include two types of genetic alterations in the bacterial chromosome: insertion of a disarmed gene fundamental to plant pathogenesis in a *cis*-orientation to the wildtype and inclusion of an antibiotic resistance gene. Either alteration may affect intraspecific competitiveness of the engineered

strain. The competitiveness of the GEM strain with varied concentrations of its parental strain as well as its survival in sterilized substrate are the topics of this report.

MATERIALS AND METHODS

Bacterial strains and plasmids

The bacterium used in this study was *Erwinia carotovora* subsp. *carotovora* (Allen *et al.*, 1986), a phytopathogenic enterobacterium that produces soft rot in plant tissue. Pectate lyase genes from *E. carotovora* were cloned into plasmid pBR322 on a 1.3 kilobase pair (kbp) fragment and expressed in *Escherichia coli* strain HB101. A 5.5 kbp fragment of the inserted *E. carotovora* DNA was deleted by digestion with *Bgl*II and the remaining plasmid DNA ligated using T4 ligase. The resulting plasmid, pDR41, no longer mediates pectate lyase activity as determined by isoelectric focusing and activity stain overlays. Plasmid pDR41 was further modified by inserting the *Pst* fragment of Tn*903* (kanamycin resistance) isolated from pUC4K into the *Pst*I site of pDR41. This new plasmid, pVS20, mediating kanamycin resistance, was transferred into *E. carotovora* by conjugal triparental mating with the helper plasmid pRK2013. Substitutional mutagenesis was attempted by curing pVS20 from EC14 in low phosphate medium. Kanamycin-resistant strain L-833 was recovered that contained no detectable plasmids, was reduced up to 30% in ability to rot tuber tissue, but was not affected in the production of the target pectate lyase. The Tn*903* derived fragment, therefore, is probably inserted in a *cis*-orientation to the wildtype genes in the EC14 chromosome. Strain L-863 was a wildtype mutant of *E. carotovora* resistant by spontaneous mutation to 150 μg/ml rifampicin; strain L-864 is a spontaneous rifampin-resistant mutant of L-833. Both L-863 and L-864 strains displayed growth and biochemical characteristics identical to the wildtype strain.

Media and chemicals

Plate count agar (PCA; Difco Laboratories, Detroit,MI), amended with 40 μg/ml kanamycin monosulfate and 150 μg/ml rifampicin (Sigma Chemical Co., St. Louis, MO) was used to detect genetically-engineered *E. carotovora* and PCA with 150 μg/ml rifampicin was used to enumerate wildtype *E. carotovora*. Cycloheximide (100 μg/ml; Sigma Chemical Co.) was added to both media to inhibit fungal growth.

Microcosms

Terrestrial microcosms consisted of 1-liter glass mason jars containing 200 g of soil. Humidified, sterile air was circulated at 5 ml/min through the microcosm, as shown, for the duration of the study to minimize evaporation of soil moisture and allow for elimination of metabolic by-products. Both intake and exhaust air was sterilized by membrane filtration usinga Gelman Maxi Capsule ™ (Gelman Sciences, Inc., Ann Arbor, MI). Soil in microcosms was gently mixed by shaking every third day to ensure a homogenous mixture. This study utilized soil from the Virginia Polytechnic Institute and State University farm at Whitethorn on the New River. Soil was a Hayter loam type, no-till previously planted with corn, dark grayish brown in color, and consisted of 39% sand, 41% silt, and 24% clay. Soil moisture was 11%.

Microcosm inoculation

Erwinia carotovora were grown in 4 liters of nutrient broth to a turbidity of approximately 1.0 at 550 nm. Bacterial cells were harvested by centrifugation at 1000 *g* for 20 min to provide approximately 6 x 10^{10} cells. The supernatant was discarded, the pellet washed in sterile distilled water (SDW), resuspended

in SDW, and centrifuged at 1000 *g*. This pellet was suspended in SDW (45 ml) so that 15 ml of inoculum would be delivered to each microcosm (200 g soil) to provide approximately 10^8 cells/g soil. Amount of inoculum added was calculated depending upon the concentration desired in soil, as described below. Inoculum was added to soil, which had been previously air dried for 48 h and passed through a 4.75 mm sieve after collection, and throughly mixed. Inoculated soil was passed through a 2 mm sieve, remixed, and then randomly placed into microcosms. Microcosms were incubated at 20° C.

Experimental design

Four experiments were conducted: 1) soil containing equal concentrations (1:1) of engineered and wildtype bacteria, 2) soil with 10 times as many engineered as wildtype bacteria, 3) soil with 10 times as many wildtype as engineered bacteria, and 4) soil that had been radiation-sterilized and inoculated with either engineered or wildtype strains of *E. carotovora*.

For each competition study, six microcosms were inoculated with bacteria. Experiments with sterile soil used three microcosms with genetically-engineered strain and three with wildtype. On each sampling day, one sample was randomly withdrawn from each microcosm and diluted. Three plates were prepared from each dilution and plates with approximately 30 to 300 colonies were counted.

Sampling of microcosms

Samples were withdrawn from microcosms at varied intervals: 0, 2, 4, 6, 10, 15, 20, and 25 days for 1:1 competition experiments; 0, 2, 5, 10, and 15 days for other competition studies; and 0, 2, 5, 10, 16, 20, 40, and 55 days for sterile soils experiments. To allow for diffusion of inoculum, day 0 samples were withdrawn

approximately 2 h after inoculation. After gently mixing the soil in the microcosms, soil samples (1 g) were randomly removed and placed in milk dilution bottles containing 99 ml SDW. These were mixed, first manually for a brief period and then for 20 min on an orbital shaker at 150 rpm, and subsequent dilutions prepared. Dilutions were spread onto appropriate media. Inoculated petri dishes were incubated at 30°C for 48 h and then plates with appropriate dilutions were counted. Results were expressed as CFU/g dry weight. Soil for determination of water content was removed at time of sampling.

Soil Sterilization

Soil was sterilized by exposing it to 1.5 Mrad of gamma radiation from a ^{60}Co source over a 15 hour period at the University of Virginia Reactor Facility, Charlottesville, VA. Soil was checked for sterility by plating onto nonselective media; no growth was observed.

Statistical analysis

Statistical significance between groups at each sampling day was evaluated using Student's *t* test and between sampling intervals using a repeated measures analysis (Sokal and Rohlf, 1981).

RESULTS AND DISCUSSION

Erwinia Survival in Sterile Soil

Both strains of *E. carotovora* survived longer in sterile soil (Fig. 4-1) than they did in nonsterile soil, as described in earlier chapters. Survival in sterile soil demonstrated that both *Erwinia* strains persisted for a longer period of time in the absence of competitors declining only two orders of magnitude over 55 days.

Longer survival in sterile soil is not unexpected since less competition for resources probably occurs.

Intraspecific Competition

Both engineered and wildtype *E. carotovora* declined at similar rates when added in equal proportions to the same microcosms (Fig. 4-2). No significance difference was observed on sampling days ($p > .05$) but overall decline over time was significant ($p < .05$). Rate of decline differed from earlier studies (Chapter 3) in that the characteristic large initial decrease followed by a smaller rate of decrease was not seen. Instead, decline was linear with density diminishing about 2 orders of magnitude every 10 days.

Inoculating microcosms with concurrent varied doses of engineered and wildtype *E. carotovora* did not affect survival. Both strains declined in concentration at similar rates regardless of whether the engineered strain concentration was ten times higher (Fig. 4-3) or lower (Fig. 4-4) than the wildtype. However, rate of decline was higher in microcosms receiving mixed concentrations of organisms than in microcosms receiving the same concentration of both strains. This could be due to the higher number of bacteria present in these microcosms as opposed to the 1:1 competition study microcosms. This higher number would utilize nutrients at a faster rate.

Microbial competition is difficult to understand in nature due to its complexity. However, intraspecific competition is easily determined in the laboratory and field-test situation. It is an important parameter since it may indicate if the engineered strain will survive longer than the parental wildtype. However, field validation of results (Cairns, 1988) must be executed since laboratory observations may not correlate with field data. Meade *et al.* (1985) found that indigenous

strains of *Rhizobium leguminosarum* out-competed inoculant strains in the field but demonstrated no difference in competitiveness in the laboratory.

The ability of an engineered strain, often bred in nutrient-rich media, to compete successfully with oligotrophic microbes in an oligotrophic environment is difficult. However, introduced microbes must displace indigenous organisms already occupying a niche (Adelberg, 1985) since the engineered strain must survive long enough to perform its desired task; otherwise, it is of no commercial value. This displacement may be transient. Sparse data exists regarding introduction of microbes. Introduction of *Rhizobium* has provided some data for interspecific competition as described in earlier chapters (Stacey, 1985). In addition, Lindow (1985) studied intraspecific competition between engineered (ice-minus) and wildtype (ice-plus) strains of *Pseudomonas syringae*. Lindow found that leaf surfaces inoculated with ice-minus strains will effectively inhibit ice-plus strains by competitive exclusion (Lindow, 1987).

However, it appears that the vast majority of mutations will impart a competitive disadvantage rather than an advantage (Office of Technology Assessment, 1988), especially with transposon insertion (F.J. Brockman, M.S. thesis, Washington State University, Pullman, WA., 1988). It appears that gene insertion did not affect the ability of *Erwinia* to compete.

Factors that may make a system more susceptible to GEM invasion include environments lacking potential predators, release of ecological generalists, organisms that can exploit a variety of resources, and perturbed systems. Perturbed ecosystems may be more susceptible to invasion than first thought (Adelberg, 1985; Cairns and Pratt, 1986; Office of Technology Assessment, 1988) Cairns and Pratt (1986) determined, using protozoan models, that ability to resist invasion appears to be related to the species richness and the particular

successional state that the ecosystem is in. Additional studies are warranted to determine if this also applies to GEMs.

LITERATURE CITED

Adelberg, E.A. 1985. Summary of Proceedings. [In] H.O. Halvorson, D. Pramer, and M. Rogul (eds.), Engineered Organisms in the Environment: Scientific Issues, ASM, Washington. pp. 233-235.

Allen, C., V.K. Stromberg, F.D. Smith, G.H. Lacy, and M.S. Mount. 1986. Complementation of an *Erwinia carotovora* subsp. *carotovora* protease mutant with a protease-encoding cosmid. Mol. Gen. Genet. 202:276-279.

Cairns, J., Jr. 1988. What constitutes field validation of predictions based on laboratory evidence? [In] W.J. Adams, G.A. Chapman, and W.G. Landis, (eds.), Aquatic Toxicology and Hazard Assessment, ASTM STP 971, Phil., pp. 361-368.

Cairns, J., Jr. and J.R. Pratt. 1986. Ecological consequence assessment: effects of bioengineered organisms. [In] J. Fiksel & V.T. Covello (eds.), Biotechnology Risk Assessment, Pergamon Press, pp. 88-108.

Grant, W.D. and P.E. Long. 1981. Environmental Microbiology. Halsted Press, Glasgow, Scotland, 215 pp.

Lindow, S.E. 1985. Ecology of *Pseudomonas syringae* relevant to the field use of ice-deletion mutants constructed in vitro for plant frost control. [In] H.O. Halvorson, D. Pramer, and M. Rogul (eds.), Engineered Organisms in the Environment: Scientific Issues, ASM, Washington. pp. 23-35.

Lindow, S.E. 1987. Competitive exclusion of epiphytic bacteria by ice- *Pseudomonas syringae*. Appl. Environ. Micro. 53:2520-2527.

Meade, J., P. Higgins, and F. O'Gara. 1985. Studies on the inoculation and competitiveness of *Rhizobium leguminosarum* in soils containing indigenous rhizobia. Appl. Environ. Micro. 49:899-903.

Office of Technology Assessment. 1988. New Developments in Biotechnology - Field-Testing Engineered Organisms: Genetic and Ecological Issues. OTA-BA-350, Washington, D.C., 150 pp.

Ricklefs, R.E. 1979. Ecology. Chiron Press, New York, 966 pp.

Sokal, R.R. and F.J. Rohlf. 1981. Biometry. W.H. Freeman, New York.

Stacey, G. 1985. The *Rhizobium* experience. [In] H.O. Halvorson, D. Pramer, and M. Rogul (eds.), Engineered Organisms in the Environment: Scientific Issues, ASM, Washington. pp. 109-121.

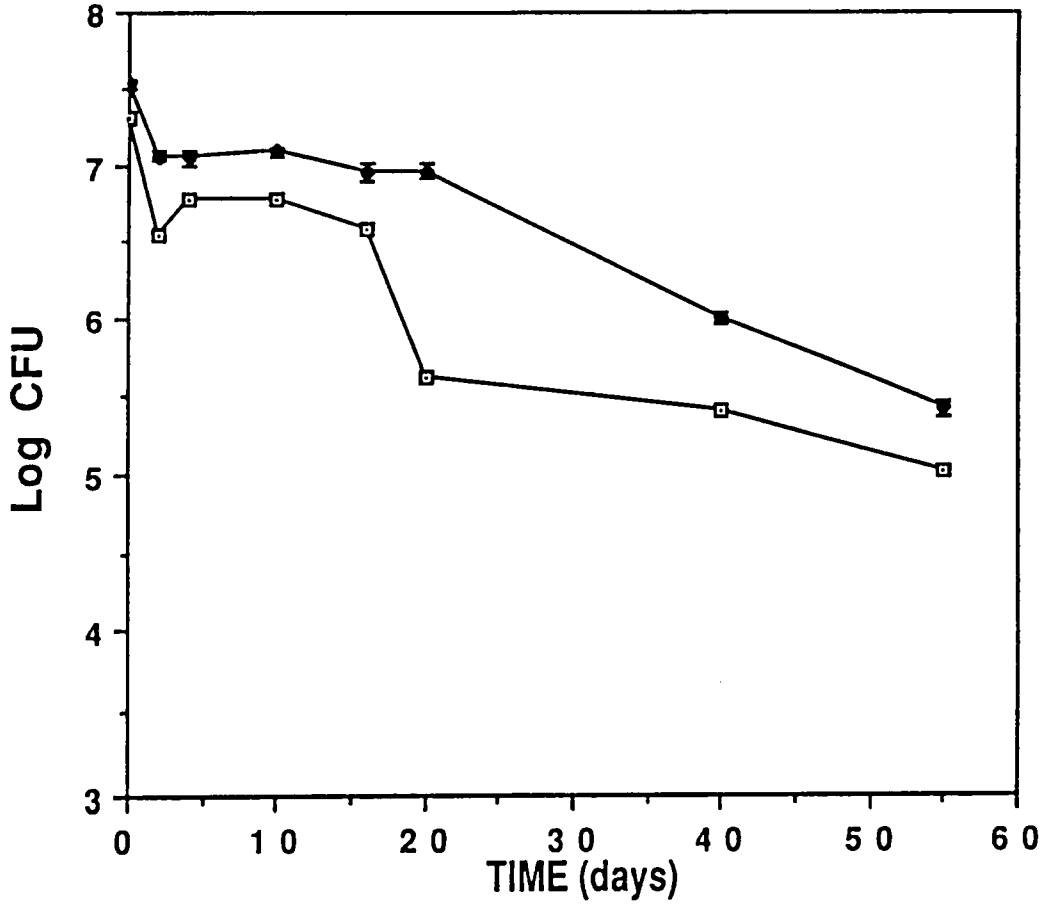

FIG. 4-1. Survival of genetically-engineered (L-864) (■) and wildtype (L-863) (•) *Erwinia carotovora* in sterile soil. Error bars represent one standard deviation unit and are contained in the symbol when not shown.

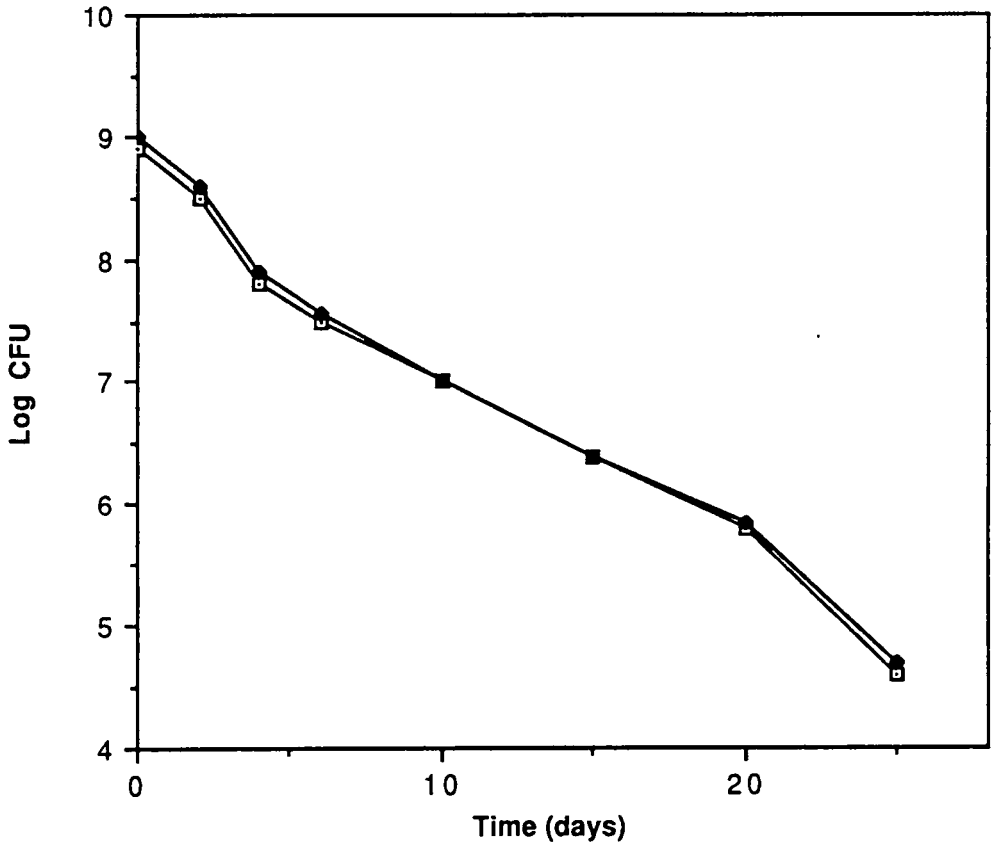

FIG. 4-2. Intraspecific competition of genetically-engineered (solid symbol) and wildtype (open symbol) *Erwinia carotovora* in non-sterile soil. Bacteria were added in equal (1:1) amounts to soil prior to placing soil into microcosms.

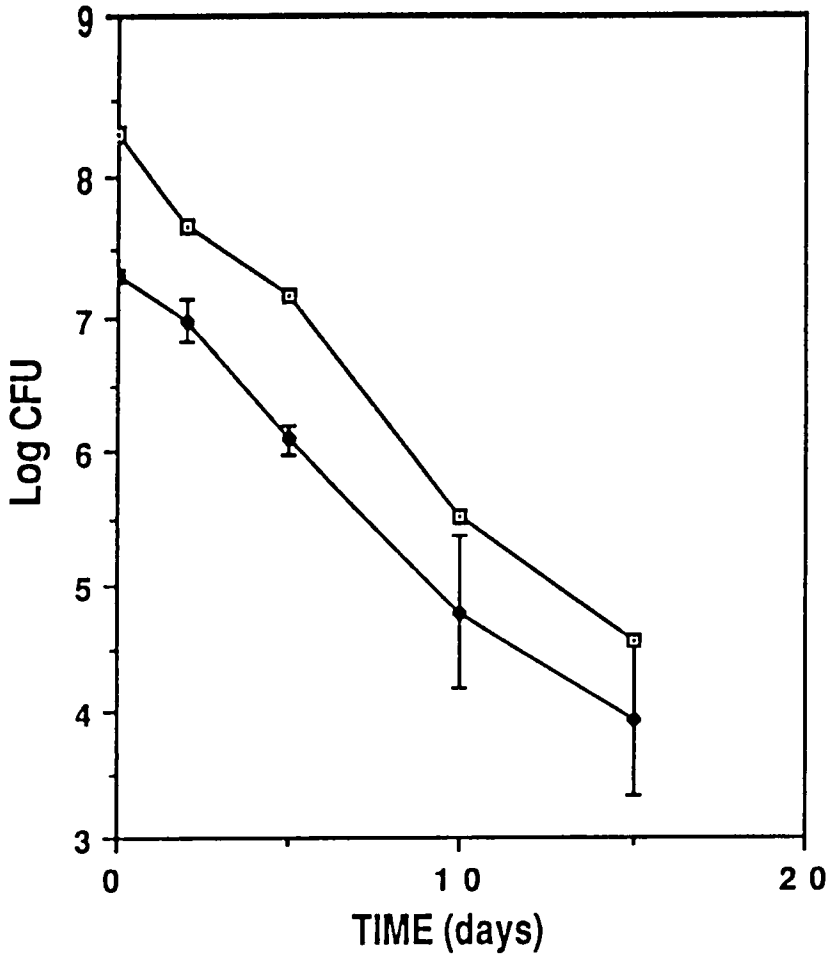

FIG. 4-3. Intraspecific competition of genetically-engineered (open symbol) and wildtype (solid symbol) *Erwinia carotovora* in non-sterile soil. Engineered bacteria were added in 10 times the amount of wildtype bacteria. Error bars represent one standard deviation unit.

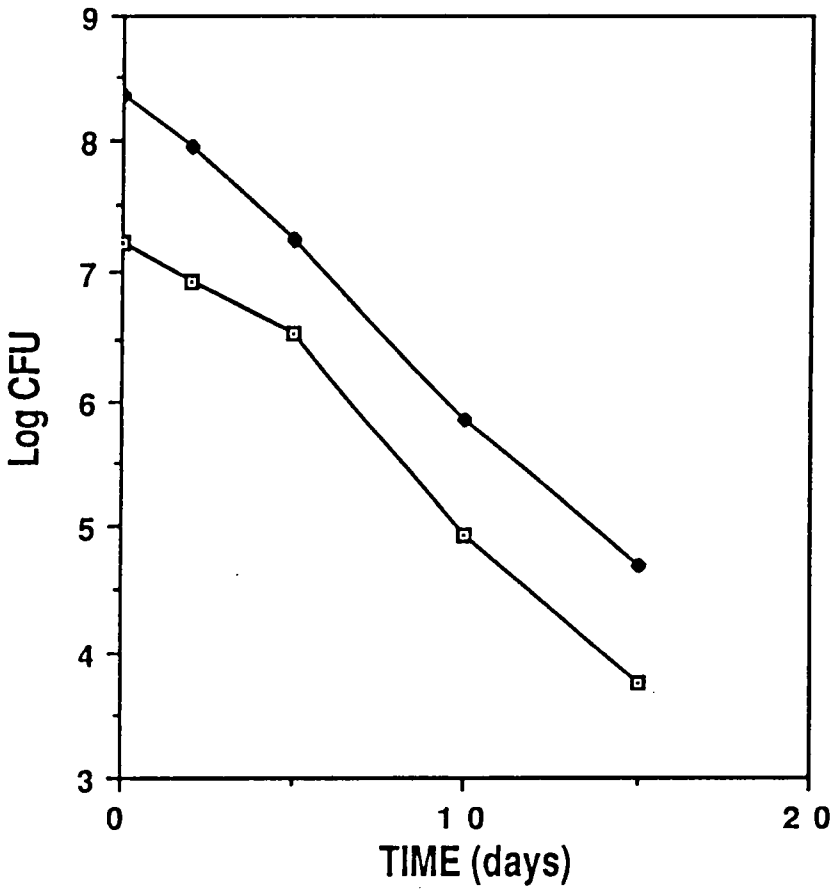

FIG. 4-4. Intraspecific competition of genetically-engineered (open symbol) and wildtype (solid symbol) *Erwinia carotovora* in non-sterile soil. Wildtype bacteria were added in 10 times the amount of engineered bacteria. Error bars represent one standard deviation unit.

CHAPTER FIVE

GEM EFFECTS UPON SOIL CHEMICAL PARAMETERS AND ENZYME ACTIVITIES

INTRODUCTION

Survival of genetically-engineered microorganisms (GEMs) in the environment has raised concerns over potential ecological effects. While assessment of survival, competition, and gene exchange will be useful in preliminary testing for risk assessment, the ultimate manifestation of any adverse effect will be alteration of ecosystem function. While such changes may have profound effects (Alexander, 1985), others point out that the probability of such adverse effects is small, especially since few exogenous organisms can even compete with indigenous microbes (Hardy and Glass, 1985).

Many functional processes could be affected by the environmental release of GEMs (Cairns and Pratt, 1986). While some processes may be intentionally altered for commercial purposes, it is plausible that processes may be inadvertently changed. Wang and Crawford (1988) found that an engineered *Streptomyces* strain caused significantly higher soil carbon mineralization rates in nonsterile soil amended with lignocellulose. They believe the effect is due to the expression of the foreign DNA insert. It is plausible that transfer of such an insert could convey such alteration in function to other organisms.

While many parameters can be monitored, soil enzyme activities, nutrient concentrations, and other chemical variables such as pH and organic matter, are among the most important since their stability is critical to biogeochemical processes (Fenchel and Blackburn, 1979). Assaying soil enzyme activities provides some indication of soil capacity to carry out biochemical processes (Ross, 1971). Dehydrogenase enzymes are important in the initial stages of soil organic

matter oxidation by transferring hydrogen or electrons from substrates to acceptors. In actuality, several enzymes and enzyme systems are involved in total dehydrogenase activity. Phosphatases are capable of cleaving orthophosphate groups from organic phosphate compounds. Free phosphatase results from the natural loss of the enzyme either from the periplasmic space or because of an environmental stress, such as osmotic shock (Sayler, 1979). Presence of phosphatases in nature directly relates to phosphorous cycling.

This study used a genetically engineered strain of *Erwinia carotovora* that was constructed to include two types of genetic alterations in the bacterial chromosome: deletion of a gene fundamental to plant pathogenesis and inclusion of an antibiotic resistance gene. Potential functional effects of adding these strains to microcosms was determined by monitoring concentrations of alkaline phosphatase, dehydrogenase, nutrients, and other selected soil attributes such as organic matter and pH.

MATERIALS AND METHODS

Bacterial strains and plasmids

Erwinia carotovora subsp. *carotovora* (Allen *et al.*, 1986), a phytopathogenic enterobacterium that produces soft rot in plant tissue and is ubiquitous in nature, was used in this study. Pectate lyase genes from *E. carotovora* were cloned into plasmid pBR322 on a 1.3 kilobase pair (kbp) fragment and expressed in *Escherichia coli* strain HB101 (Roberts *et al.*, 1986). A 5.5 kbp fragment of the *E. carotovora* DNA was deleted by digestion with *Bgl*II and the remaining plasmid DNA ligated using T4 ligase. The resulting plasmid, pDR41, no longer mediated pectate lyase activity as determined by isoelectric focusing and activity stain overlays. Plasmid pDR41 was further modified by inserting the *Pst* fragment of Tn*903* (kanamycin resistance) isolated from pUC4K into the *Pst*I site

of pDR41. This new plasmid, pVS20, mediating kanamycin resistance, was transferred into *E. carotovora* by conjugal triparental mating with the helper plasmid pRK2013. Kanamycin-resistant strain L-833 was recovered that contained no detectable plasmids, was reduced up to 30% in the ability to rot tuber tissue, but not reduced in the ability to produce the target pectate lyase. The Tn*903* derived fragment is probably inserted in a *cis*-orientation to the wildtype genes in the EC14 chromosome. Strain L-863 was a wildtype mutant of *E. carotovora* resistant by spontaneous mutation to 150 μ/ml rifampicin; strain L-864 is a spontaneous rifampin-resistant μg/ml) mutant of L-833. Both L-863 and L-864 strains displayed growth on laboratory media and biochemical characteristics identical to the wildtype strain.

Media and chemicals

Plate count agar (PCA; Difco Laboratories, Detroit, MI), with 40 μg/ml kanamycin monosulfate and 150 μg/ml rifampicin (Sigma Chemical Co., St. Louis, MO) was used to enumerate the genetically-engineered *E. carotovora* strain and PCA with 150 μg/ml rifampicin was used to enumerate wildtype *E. carotovora*. Cycloheximide (100 μg/ml; Sigma Chemical Co.) was added to both media to inhibit fungal growth.

Microcosms

Terrestrial microcosms consisted of 1-liter glass mason jars containing 200 g of soil. Humidified, sterile air was circulated at 5 ml/min through the microcosm for the duration of the study to minimize soil moisture evaporation and eliminate metabolic by- products. Both intake and exhaust air was filter sterilized (Gelman Maxi Capsule™; Gelman Sciences, Inc., Ann Arbor, MI). Soil in microcosms was gently mixed every third day to ensure a homogenous dis-

tribution of microorganisms. This study utilized soil from the Virginia Polytechnic Institute and State University farm at Whitethorn on the New River. Soil was a Hayter loam type, dark grayish brown in color, and consisted of 39% sand, 41% silt, and 24% clay.

Microcosm inoculation

Erwinia carotovora were grown in 4 liters of nutrient broth to a turbidity of approximately 1.0 at 550 nm. Bacterial cells were harvested by centrifugation at 1000 *g* for 20 min to provide approximately 6 x 10^{10} cells. The supernatant was discarded and the pellet washed in sterile distilled water (SDW), resuspended in SDW, and centrifuged at 1000 *g*. This pellet was suspended in SDW (45 ml) so that 15 ml of inoculum would be delivered to each microcosm (200 g soil) to provide approximately 10^8 cells/g soil. Amount of inoculum added was calculated depending upon the concentration desired in soil, as described below. Inoculum was added to soil, which had been previously air dried for 48 h and passed through a 4.75 mm sieve after collection, and throughly mixed. Inoculated soil was passed through a 2 mm sieve, remixed, and then randomly placed into microcosms. Microcosms were incubated at 20° C.

Experimental design

Two experimental series were conducted to (1) determine if soil nutrients or other soil chemical parameters were affected by the addition of *Erwinia* as compared to controls which were inoculated with water, and (2) ascertain if exogenous bacteria affected soil enzyme activities of dehydrogenase and alkaline phosphatase.

For each experiment, three microcosms were inoculated with 10^8 cells of either the engineered strain, the wildtype strain, or sterile water as a control. On

each sampling day, one sample was randomly withdrawn from each microcosm in the amount required for the tests described below as well as for dilution so that the density of bacteria could be monitored.

Dehydrogenase activity

Dehydrogenase activities were determined as follows (Ross, 1971): Two grams of wet soil/sediment mixture was added to 4 ml 0.5 M Tris HCl (pH 7.5) plus 2 ml 1% triphenyl-tetrazolium (TTC). One ml of 1% glucose was added and the solution mixed and incubated at 25-30 C in dark for 96 h. The mixture was swirled daily. After incubation, the reaction was stopped by adding 20 ml methanol, shaking for 30 sec, and incubating an additional 3 h in the dark. The same amount of methanol was added to each sample. A 1.0 ml aliquot of the mixture was then centrifuged briefly at 11,000 rpm in a microcentrifuge to remove particulate matter and 200 μl of this solution was carefully aspirated and placed into a microtiter plate. Absorbance was read at 490 nm using a Dynatech microplate reader and activity calculated as mg formazan/g dry sediment or soil. Standards were prepared using triphenyl formazan at concentrations of 0.004 mg per 20 ml methanol to 0.4 mg per 20 ml methanol. Blanks contained no substrate.

Alkaline phosphatase activity

To determine alkaline phosphatase (Sayler *et al.*, 1979), one gram of soil was added to 4 ml 1 M Tris (pH 8.5) and sonicated for 30 seconds at 10 watts. One ml of p-nitrophenylphosphate (p-NPP, 1 mg/ml) was added, the mixture vortexed and incubated in the dark at 30° C for 2-3 h. After incubation, the reaction was stopped with 1 ml of 1 N NaOH. A 200 μl aliquot was then placed into a microtiter

plate and read at 410 nm. Activity was calculated as μg p-NP/g dry wt. Standards were run using p-nitrophenol (p-NP) and blanks contained no substrate.

Soil analysis

Soil was assayed for concentrations of phosphorus, potassium, calcium, magnesium, zinc, manganese, nitrate, organic matter, soluble salts, and pH by the Virginia Tech Soils Testing Laboratory using standard methods (Page *et al.*, 1982). To ascertain the accuracy of tests, pH and organic matter were assayed according to standard protocols (Page *et al.*, 1982) and not found to be different.

Statistical analysis

Statistical significance between groups for each variable at each sampling time was evaluated using an analysis of variance rationale and a Student-Newman-Keuls means analysis (Sokal and Rohlf, 1981). Significance between sampling intervals was evaluated using a repeated measures analysis (Sokal and Rohlf, 1981). All analyses were accomplished using the Statistical Analysis System (SAS Institute, 1985).

RESULTS AND DISCUSSION

Enzyme activities

No treatment effects were observed for either alkaline phosphatase (Fig. 5-1) or dehydrogenase (Fig. 5-2). Both groups with added bacteria did not differ statistically ($p > 0.05$) from the control group and no time effects were observed among either treatment group or the control group ($p > 0.05$).

Although both phosphatase and dehydrogenase are important natural enzymes, there is some indication that these proteins are markedly affected only

when the stress placed on the system is severe, as in the case of chemicals. Lack of impairment from the engineered and wildtype strains was not unexpected since the genetic alterations should not have affected such enzyme production. However, it was expected that enzyme activities in soil with added bacteria would be greater than soil with only water added, since microbial activity would be higher. This was not observed. Apparently, the added bacteria did not contribute significant amounts of either dehydrogenase or phosphatase. Daily fluctuations of enzyme activity have been reported (Huber and Kidby, 1984) and must be incorporated into experimental designs. Recent studies using genetically-engineered pseudomonads have produced differences in dehydrogenase and phosphatase based upon analyses after peak production periods (J. Doyle, personal communication). These peak periods were detected by measuring carbon dioxide evolution. Elucidation of this mechanism could improve the reliabilty of using enzyme activities as indicators of environmental stress.

Soil nutrients and other chemical parameters

None of the parameters monitored demonstrated a treatment effect between engineered and wildtype *E. carotovora* strains ($p > 0.05$) (Table 5-1). However, several time effects were observed.

Decrease in pH from 5.9 on day 0 to 5.63 on day 30 was significant ($p = .003$) and indicated that metabolically-active bacteria probably affected soil pH by excretion of waste products. However, other factors, such as salt and carbon dioxide content may also affect soil pH (McLean, 1982). Carbon dioxide soil concentrations were not measured but, based upon air concentrations from earlier studies (Chapter 1), should not have been very high.

Other variables also demonstrated changes over time. Soluble salts increased from about 115 to 221 ppm over the 30 day course of the study in both treatment groups; the reasons for this remain unclear but soluble salts from decaying bacteria may be responsible since data from day 0 samples indicated that salt concentrations increased from 90 to 120 ppm. Nitrate-nitrogen also increased significantly ($p = 0.003$) by 120% from day 0 to day 30 in both groups, probably because of the metabolic products excreted by microbes or released from dying bacteria. Analysis of day 0 soil samples indicated only a 15% increase in nitrate compared to pretest samples. Analysis of urease in future studies may be beneficial in elucidating ammonia, urea, and nitrate fluctuations.

CONCLUSIONS

The parameters monitored in this research did not demonstrate any appreciable change from the engineered or wildtype strains of *E. carotovora*. This could be due to one of two reasons: (1) the variables used were not sensitive enough for use in assessing risk, or (2) the engineered and wildtype strains did not significantly differ in phenotype as to produce a difference, therefore the risk posed by the engineered strain is minimal.

Using of enzyme activities to detect functional effects of GEMs has proven useful at the EPA Research Laboratory at Corvallis, Oregon (J. Doyle, personal communication). However, such differences in activity were transient and occurred with flushes of carbon dioxide from sealed microcosms. The enzymes monitored in this study are important in biogeochemical cycles and have been used with some success in ascertaining chemical effects upon microbial activity (Sayler *et al.*, 1979; Killham *et al.*, 1983; Arthur and Frea, 1988). However, the inherent error involved makes results interpretation difficult.

Clearly, functional effects will be the ultimate manifestation of any harmful GEM release (Cairns and Pratt, 1986). Determining what functional parameters to monitor and when to monitor them will require additional research.

LITERATURE CITED

Alexander, M. 1985. Ecological consequences: reducing the uncertainties. Issues in Science and Technology. Spring: 57-68

Allen, C., V.K. Stromberg, F.D. Smith, G.H. Lacy, and M.S. Mount. 1986. Complementation of an *Erwinia carotovora* subsp. *carotovora* protease mutant with a protease-encoding cosmid. Mol. Gen. Genet. 202:276-279.

Arthur, M.F. and J.I. Frea. 1988. Microbial activity in soils contaminated with 2,3,7,8-TCDD. Environ. Toxicol. Chem. 7:5-13.

Cairns, J., Jr. and J.R. Pratt. 1986. Ecological consequence assessment: effects of bioengineered organisms. [In] J. Fiksel & V.T. Covello (eds.), Biotechnology Risk Assessment, Pergamon Press, pp. 88-108.

Fenchel, T. and T.H. Blackburn. 1979. Bacterial and mineral cycling, Academic Press, NY. 225 pp.

Hardy, R.W.F. and D.J. Glass. 1985. Our investment: what is at stake? Issues Sci. Tech. Spring, 69-82.

Huber, A.L., and D.K. Kidby. 1984. An examination of the factors involved in determining phosphatase activities in estuarine waters. 2. Sampling procedures. Hydrobiologia 111:13-19.

McLean, E.O. 1982. Soil pH and lime requirement. [In] A.L. Page, R.H. Miller, and D.R. Keeney (eds.), Methods of Soil Analysis, Part 2. Chemical and Microbiological Properties, Amer. Soc. Agronomy, Madison, WI, pp. 199-224.

Page, A.L., R.H. Miller, and D.R. Keeney. 1982. Methods of soil analysis, chemical and microbiological properties, 2nd edition. Amer. Soc. Agronomy, Madison, WI, 1158 pp.

Roberts, D.P., P.M. Berman, C. Allen, V.K. Stromberg, G.H. Lacy, and M.S. Mount. 1986. Requirement for two or more *Erwinia carotovora* subsp. *carotovora*

pectolytic gene products for maceration of potato tuber tissue by Escherichia coli. J. Bacteriol. 167:279-284.

Ross, D.J. 1971. Some factors influencing the estimation of dehydrogenase activities of some soils under pasture. Soil Biol. Biochem. 3:97-110.

SAS Institute, Inc. 1985. SAS User's Guide: Statistics, version 5. SAS Institute, Inc., Cary. N.C.

Sayler, G.S., M. Puziss, and M. Silver. 1979. Alkaline phosphatase assay for freshwater sediments: application to perturbed sediment systems. Appl. Environ. Micro. 38:922-927.

Sokal, R.R. and F.J. Rohlf. 1981. Biometry. W.H. Freeman and Co., San Francisco, CA. 776 pp.

Wang, Z. and D.L. Crawford. Effects of wildtype and recombinant *Streptomyces* on mineralization of organic carbon. Abstracts of First Inter. Conf. Release Genetically Engineered Microorganisms No. 12, p. 13.

Table 5-1. Concentrations of nutrients, organic matter, and soluble solids in soil innoculated with genetically-engineered (GEM) and wildtype (WILD) *Erwinia carotovora*. Sampling days are indicated by 0, 5, 10, and 30; a pre-inoculation soil analysis is also shown (PRE). Each value represents the average of two separate samples. Elements are represented by conventional symbols; OM = organic matter; SS = soluble salts; NO = nitrate-nitrogen.

Sample	pH	P (ppm)	K (ppm)	Ca (ppm)	Mg (ppm)	OM (%)	SS (ppm)	NO (ppm)	Zn (ppm)	Mn (ppm)
PRE	5.8	13	75	828	93	2.5	90	23	0.9	19.4
GEM 0	5.9	14	78	900	103	2.7	102	25	1.2	20.8
GEM 5	5.7	12.5	76	864	95	2.55	172.5	41.5	1.05	19.8
GEM 10	5.65	13.5	78.5	900	99	2.8	230.5	53	1.05	20.5
GEM 30	5.6	14.5	76.5	924	100.5	2.6	224	54	1.1	20.4
WILD 0	5.9	13	74	852	95	2.5	128	28	1.1	20.1
WILD 5	5.75	14.5	73.5	900	99	2.75	185.5	47.5	1.1	22.4
WILD 10	5.7	12.5	69.5	870	94.5	2.65	211.5	49	0.95	19.5
WILD 30	5.65	13.5	77	912	101	2.7	211.5	54	1.05	20.4

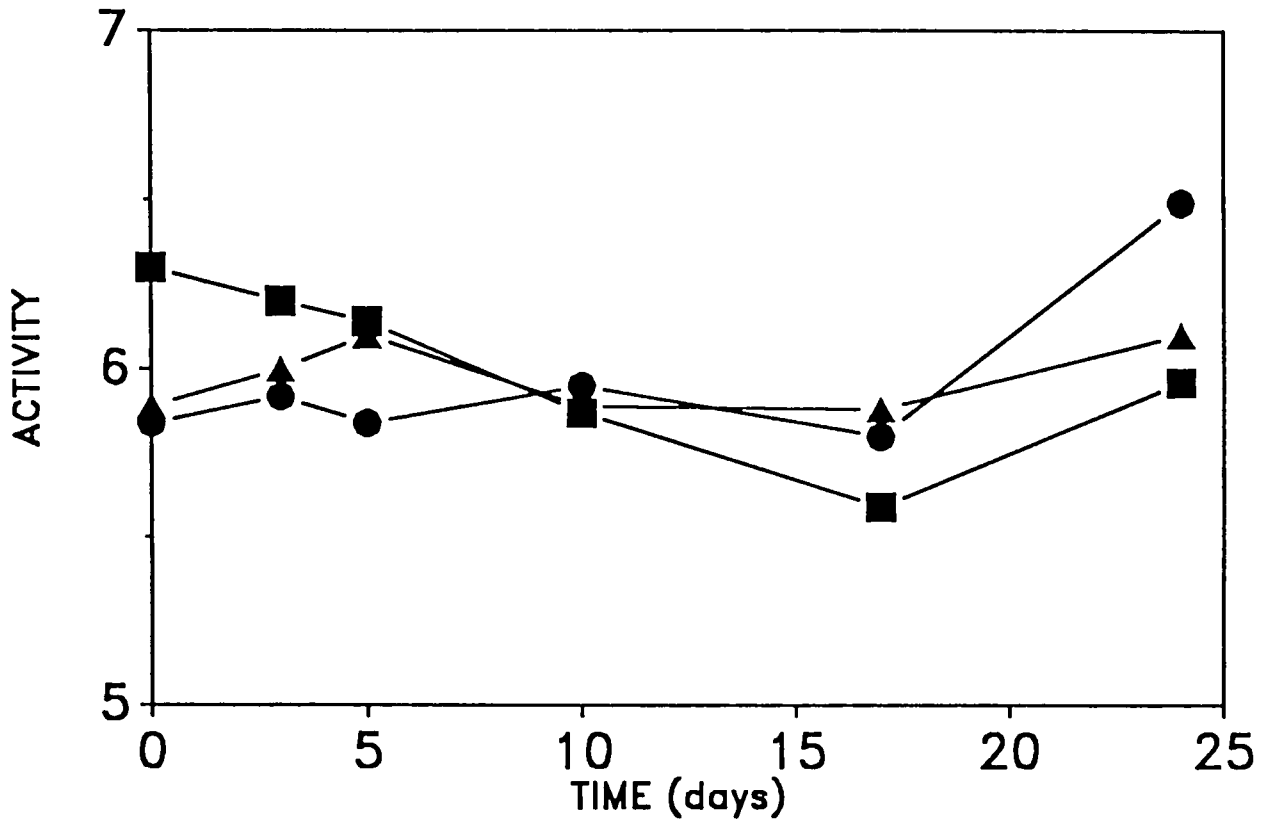

FIG. 5-1. Soil alkaline phosphatase activity in microcosms inoculated with engineered *Erwinia carotovora* (•), wildtype *E. carotovora* (▲), or sterile water (■) as a control. Error bars are deleted for clarity, but no significance differences were observed.

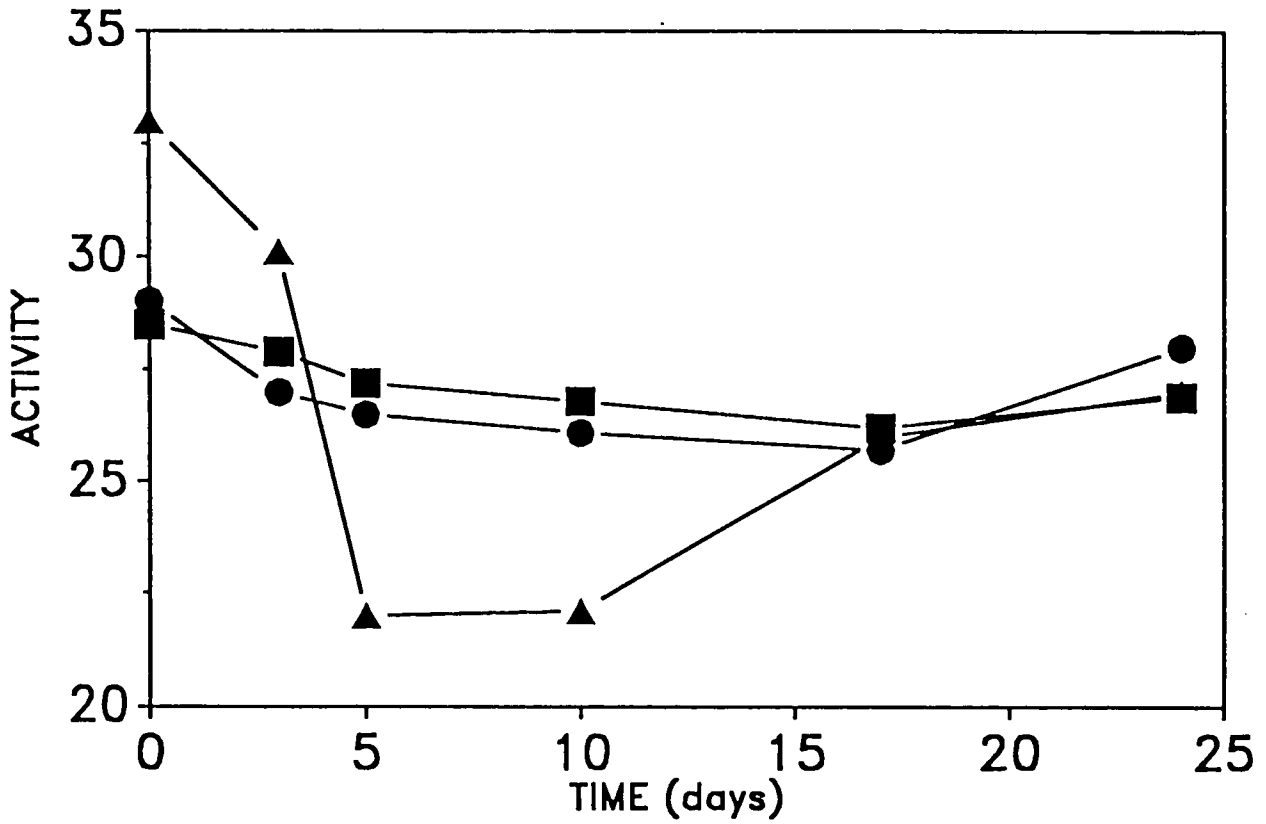

FIG. 5-2. Soil dehydrogenase activity in microcosms inoculated with engineered *Erwinia carotovora* (■), wildtype *E. carotovora* (●), or sterile water (▲) as a control. Error bars are deleted for clarity, but no significance differences were observed.

EPILOGUE

Risk assessment of genetically-engineered microorganisms is still in its infancy and the lack of literature, including methodology, became more apparent as this project proceeded. No consensus exists as to which parameters are important to monitor and which are not, which organisms are "safe" to release and which are not, and how the scheme of biotechnology risk assessment should proceed.

Risk is defined as the "potential for adverse consequences of an event or activity" (Fiksel and Covello, 1986) while risk assessment is defined as "use of scientific data to estimate the effects of exposure to hazardous materials or conditions" (Office of Technology Assessment, 1988). The risk assessment process for genetically-engineered microorganism (GEM) introduction, according to the Office of Technology Assessment (OTA)(1988) is: (1) risk identification, (2) risk-source characterization, (3) exposure assessment, (4) dose-response assessment, and (5) risk estimation. This scheme is identical to those used for chemicals. While it is very effective for predicting risk from chemicals, it fails when applied to GEMs because, as documented in the OTA report, protocols do not currently exist for predicting kinds of risk or the magnitude of potential risk. While primary differences exist between GEMs and chemicals, as described in earlier chapters, other fundamental differences are: (1) sensitive detection methodologies, often parts per trillion, exist for many toxic substances, (2) toxic chemicals, normally, are not present in high amounts prior to the episode being studied, and (3) the most fundamental principle of environmental toxicology, the dose-response curve, is easily applied to toxics to allow formulation of such values as maximum acceptable toxicant concentration, lethal concentration, and no-observable-effects-concentration (Rand and Petrocelli, 1985). GEMs are not

easily detectable, do not lend themselves well to dose-response curves, and are often already in the environment in the form of parental strains.

Since no clear agreement exists regarding what parameters to monitor, one of the purposes of this research was to ascertain which variables may be useful in predicting environmental risk. Survival is probably the most single important factor to observe. If the GEM cannot survive, the probability of risk decreases dramatically (Alexander, 1985; Office of Technology Assessment, 1988). *Erwinia* survived for sufficient periods of time (> 42 days) to allow possible adverse effects to occur. Although the microcosm did not include plant material, the primary substrate for *Erwinia*, survival occurred for a longer period than initially anticipated. Future studies in this laboratory will investigate survival with plant material present as well as bacterial survival using starved cells cultured in low-nutrient media. Survival must be assessed in more than just the laboratory environment. Both laboratory tests with microcosms and small-scale field tests must be executed in a variety of receiving environments including terrestrial and aquatic. The environments should include those similar to the anticipated one as well as environments not analogous to the expected receiving habitat. Validation of laboratory results must occur if such data are to be useful (Cairns, 1988).

Structural effects from GEM introduction appear to be remote because of the diversity and fitness of indigenous organisms. While such effects are possible, they may be difficult to detect on the microbial level since enumeration of microbes is an inexact science at best. This research demonstrated no adverse effects upon any of the bacterial genera monitored (Chapter 3). Such results were expected since all genera detected were in large quantities (> 10^5 CFU/g) and were already adapted to an oligotrophic environment. However, alteration of any one of several microbial groups performing critical roles in

biogeochemical cycling, such as *Nitrobacter* or *Desulfovibrio*, may be disastrous (Grant and Long, 1981). No attempt was made to examine effects upon higher taxa. Macroinvertebrates and fish are often used in chemical toxicity tests but are not particularly useful at this time for GEMs because of the paucity of data for even lower taxa. However, it is conceivable that GEMs may affect algae, protozoans, nematodes, and other organisms that feed upon them.

Probability of adverse functional effects may increase as GEM introductions become more common, if adequate environmental impact assessments are not performed. Disruption of fundamental ecosystem processes would be the worst possible ecological impact from a planned introduction (Office of Technology Assessment, 1988). However, OTA believes that the probability of such an event occurring in the next few years will be remote since introductions will involve GEMs with genome deletions (such as ice-minus), rather than genomic insertions that may intentionally or unintentionally be constructed to alter functional processes. Parameters that may be useful to monitor include nutrient retention and cycling, detritus processing, respiration, productivity, and enzyme activity (Cairns and Pratt, 1986). No consensus exists as to which parameters are important and this research did little to eliminate that confusion. This work did demonstrate that monitoring of enzyme activities and essential soil nutrients is feasible. Studies at EPA's Corvallis Environmental Research Laboratory have shown that genetically-engineered *Pseudomonas* strains affect phosphatase and dehydrogenase activities differently than do wildtype strains (J. Doyle, personal communication). However, these changes were transient and occurred in sealed microcosms using a microbe carrying a plasmid-bearing pesticide-degrading gene.

The use of intraspecific competition data in risk assessment protocols should allow detection of those engineered strains that will survive longer than their

parental strains. The data from this research indicated that no difference exists in intraspecific competition and this was expected based upon the organisms used and the substrate into which they were inoculated. Competition data will be particularly useful when the engineered strain is capable of altering natural functional processes for commercial gain.

Of all the possible adverse GEM effects, none promotes controversy more than potential transfer of recombinant DNA from the GEM to indigenous organisms. As discussed in the prologue of this document, DNA exchange in the environment is natural and allows for enhanced survival of bacteria when detrimental conditions are encountered. Therefore, expression of recombinant DNA may occur after the GEM itself has died out. But, as OTA emphasizes, most engineered strains will differ from the parent in only one or a few genes; such an alteration should be insignificant since changes in a few genes only rarely affects survival or function (Office of Technology Assessment, 1988). This research indicates that transfer of recombinant DNA may have occurred, but, as described in Chapter 2, conclusive evidence will require use of advanced methodologies, including gene amplification and sequencing. Such studies were beyond the scope of this dissertation but are planned.

Present guidelines for environmental release require that a premanufacturing notice (PMN) be filed with the Environmental Protection Agency (EPA). EPA receives its regulatory mandate under the Toxic Substances Control Act (TSCA), the statute that is applied to the release of new chemicals and puts the burden of product safety on the manufacturer. TSCA requires that "adequate data be developed with respect to the effect of chemical substances ... on health and the environment" (West Publishing Co., 1985). But TSCA also requires that such regulations be executed in a manner "as not to impede unduly or create unnecessary economic barriers to technological innovation" (West Publishing Co,

1985). In regards to engineered organism release, these goals may be at opposite ends of the spectrum since biotechnology development has surpassed the ability of ecotoxicology to devise suitable risk assessment protocols specific to biotechnology. Biotechnology has immense commercial value and any restriction on its development may allow foreign competitors to have unfair advantages (Hardy and Glass, 1985). However, unregulated development may lead to environmental impacts, similar to those occurring in the chemical and nuclear industries. The net result of any such cataclysm would likely impair future biotechnological advances for some time.

Another facet of the problem is the influence of the legal system upon environmental impact matters. To predict something beyond reasonable doubt is difficult, since one can question what reasonable doubt is (Anderson *et al.*, 1984). With the paucity of relevant data available, the mechanisms by which GEMs could even inflict harm are vague. Such "ignorance of mechanism" (Anderson *et al.*, 1984) can be used to support release of an unsafe product because of the uncertainity involved in product regulation. The legal complexities are numerous when one applies TSCA to GEM releases because TSCA was written for chemicals in the mid-1970s and many ambiguities are present when interpretation of the law is conducted.

To overcome these scientific and legal complications, the Office of Science and Technology Policy issued a "Coordinated Framework for Regulation of Biotechnology" in 1986 (Federal Register, 1986). The document was an interagency attempt to eliminate jurisdictional duplication in approving safety of biotechnology products and research. According to this framework, EPA would be the primary lead agency for approving environmental releases of GEMs, except those used in agriculture. EPA would then conduct an assessment in conjunction with the Animal and Plant Health Inspection Service (APHIS). A

Biotechnology Science Coordinating Committee (BSCC) would investigate the merit of planned releases. While this framework did address some problem areas, it did not propose assessment protocols, did not recommend extensive testing of organisms derived from opportunistic pathogens or those containing non-coding insertions, and exempted "small manufacturers, importers, and processors" as well as noncommercial research and development firms from TSCA regulation (Federal Register, 1986). Some of these inconsistencies have been corrected after public scrutiny.

It remains clear that risk assessment and regulation of GEMs is complex, inaccurate, and political. Use of risk assessment to predict risk will only be satisfactory if suitable parameters are determined and monitored. By using a simplistic hazard assessment scheme (Fig. 6-1) that allows flexibility, diverse genera and engineering constructs can be evaluated. Factors such as survival, intraspecific competition, and potential for DNA dissemination must be evaluated using techniques that are commonly available. Evaluating selected groups of bacteria, such as pseudomonads and nitrifiers, may indicate if vulnerable groups are at risk to be affected, but, more likely will not serve any useful purpose. More vague is which functional attributes to monitor and for how long but far more research is indicated before an answer can be produced.

The purpose of this research was to 1) develop methods to predict risk from a GEM environmental release, and 2) use these methods to ascertain risk from engineered *Erwinia carotovora*. Based upon the acquired data, the engineered strain is no more likely to cause harm than releasing the parental strain. The methods developed and refined should be useful in future studies with this and other organisms.

LITERATURE CITED

Alexander, M. 1985. Ecological consequences: reducing the uncertainties. Issues in Science and Technology. Spring: 57-68.

Anderson, F.R., D.R. Mandelker, and A.D. Tarlock. 1984. Environmental law. Little, Brown and Co., Boston, 978 pp.

Cairns, J., Jr. 1988. Validating biological monitoring, [In] D. Gruber and J.M. Diamond (eds.), Automated Biomonitoring, Horwood, Ltd., Chichester, pp. 40-48.

Cairns, J., Jr. and J.R. Pratt. 1986. Ecological consequence assessment: effects of bioengineered organisms. Water Resources Bull. 22:171-182.

Federal Register. 1986. Coordinated framework for regulation of biotechnology. 51:23301-23350.

Fiksel, J.R. and V.T. Covello. 1986. The suitability and applicability of risk assessment methods for environmental apllications of biotechnology. [In] J.R. Fiksel and V.T. Covello (eds.), Biotechnology Risk Assessment: Issues and Methods for Environmental Introductions, Pergamon Press, New York, pp. 1-34.

Grant, W.D. and P.E. Long. 1981. Environmental Microbiology. Halsted Press, Glasgow, Scotland, 215 pp.

Hardy, R.W.F. and D.J. Glass. 1985. Our investment: What is at stake? Issues in Science and Tech. Spring, 69-82.

Office of Technology Assessment. 1988. New Developments in Biotechnology-Field Testing Engineered Organisms: Genetic and Ecological Issues. OTA-BA-350, Washington, D.C., 150 pp.

Rand, G.M. and S.R. Petrocelli. 1985. Fundamentals of Aquatic Toxicology. Hemisphere Publishing, Cambridge, 666 pp.

West Publishing Co. 1985. Selected environmental law statutes. West Publishing Co., St. Paul, Minn., 839 pp.

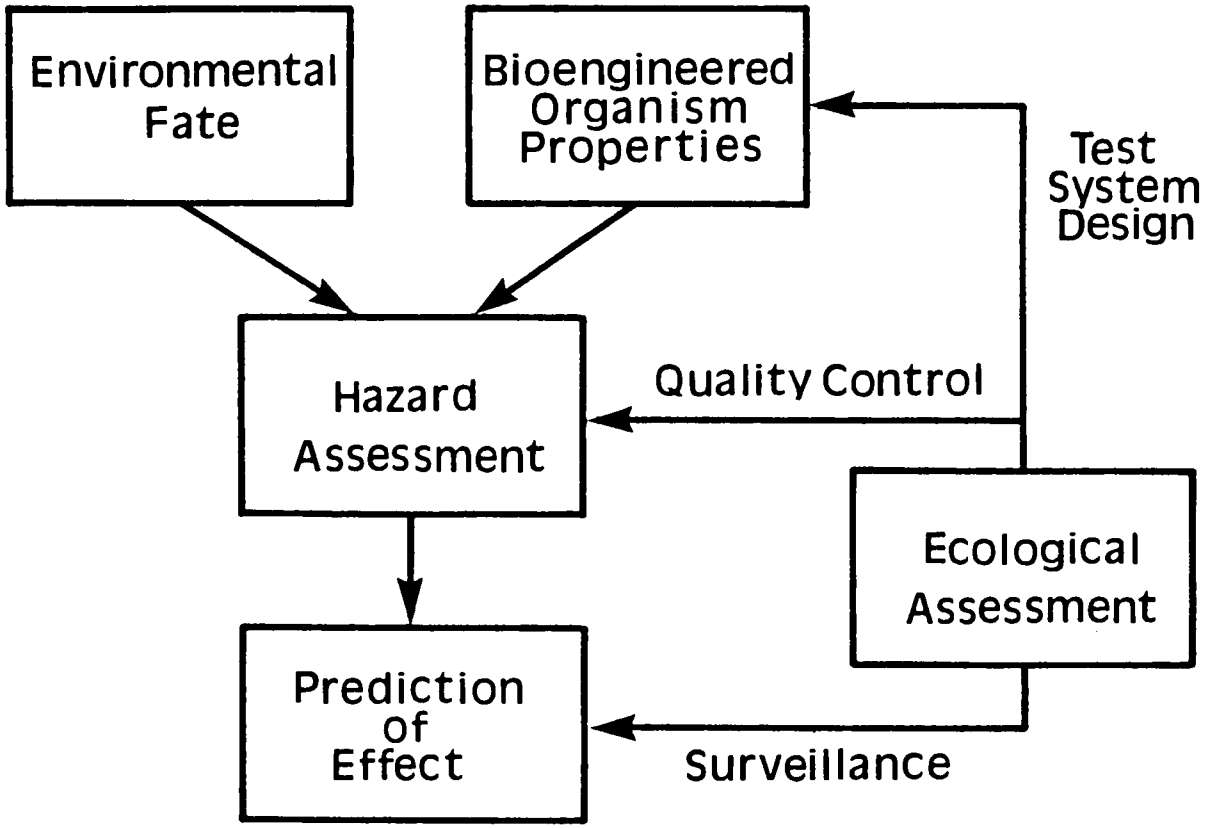

FIG. 6-1. Risk assessment scheme for assessing risk from environmental release of genetically-engineered microorganisms.

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