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METHODS FOR STUDYING BACTERIAL GENE TRANSFER IN SOIL BY CONJUGATION AND TRANSDUCTION

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16. ABSTRACT

The purpose of the this document is to provide a series of protocols by which a trained technician can conduct studies on the transfer of genetic information by conjugation or transduction in soil, with emphasis on bacteria containing recombinant DNA. The level of the document is geared to technicians with some background and experience in standard laboratory methods used in microbiology but who have limited knowledge of, and experience in, bacterial genetics, molecular biology, and microbial ecology, especially in soil. Many of the specific techniques described in this document for studying gene transfer in soil were developed by the authors during the past few years. The primary motivation for these studies was the need for information on the survival of, and gene transfer by, genetically engineered bacteria that could be used in risk assessment of the release of genetically engineered microorganisms to the environment.

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EXECUTIVE SUMMARY

The purpose of this document is to provide a series of protocols by which a trained technician can conduct studies on the transfer of genetic information by conjugation or transduction in soil, with emphasis on bacteria containing recombinant DNA. The level of the document is geared to technicians with some background and experience in standard laboratory methods used in microbiology but who have limited knowledge of and experience in bacterial genetics, molecular biology, and microbial ecology, especially in soil. Consequently, Section I discusses soil as a habitat for microorganisms and stresses the importance of the physicochemical and biological characteristics of soil in the activity, ecology, and population dynamics of microorganisms in this habitat, which, in turn, influence the transfer of genetic information. Section II briefly discusses the molecular aspects of gene transfer by conjugation and transduction and reviews the relevant literature on such transfer in soil. Section III introduces the concepts of microcosms as surrogates for the field and emphasizes that microcosms differ in complexity of design; this Section is not intended to be an exhaustive review of microcosms, as detailed protocol documents on microcosms are available and are referenced. Section IV provides general guidelines on the selection, preparation, maintenance, and storage of soils. More specific guidelines on these aspects are presented in Sections V and VI. Sections I through IV are intended to bring the novice rapidly to a level of understanding that will enable the intelligent and thoughtful conduct of "hands-on" studies of gene transfer. Section V provides details on how to study the conjugal transfer of chomosomal- and plasmid-borne genes. Section VI provides details on how to study gene transfer via transduction by bacteriophages. Both Sections V and VI provide details on how to conduct these studies *in vitro*, which is necessary not only to verify the bacterial strains, novel genes, and phages used, but also to learn the techniques, to estimate the frequencies of gene transfer, and to establish base-line data for comparison with *in situ* results. Once the techniques have been mastered *in vitro*, it

will be possible to conduct studies on conjugation and transduction in soil, employing the techniques developed specifically for use in soil. Section VII provides details on how to identify, characterize, and confirm any presumptive recombinant bacteria that are obtained, using the techniques of both classical microbiology, such as selective and differential media, and molecular biology, such as DNA "fingerprinting", plasmid profiles, DNA-DNA hybridization with DNA probes, and serology. Section VIII provides guidelines for obtaining the types of information that are necessary for quality assurance and quality control. An extensive bibliography, with approximately 200 references, an Appendix containing recipes for media and antimicrobial agents commonly used in studies of conjugation and transduction, and examples of results of studies on gene transfer in soil are included.

Many of the specific techniques described in this document for studying gene transfer in soil were developed by the authors during the past few years, in part with support from the U.S. Environmental Protection Agency, especially in the form of cooperative agreements (CR812484, CR813431, CR813650) with the Corvallis Environmental Research Laboratory (CERL). The primary motivation for these studies was the need for information on the survival of, and gene transfer by, genetically engineered bacteria that could be used in risk assessment of the release of genetically engineered microorganisms to the environment. The support, encouragement, and suggestions provided by Dr. R. J. Seidler, J. L. Armstrong, and other members of CERL are gratefully acknowledged.

Methods for Studying Bacterial Gene Transfer in Soil

by Conjugation and Transduction

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I. Introduction

Microhabitats in soil

Soil is unique among microbial habitats, as it is a structured environment with a high solid: water ratio (Stotzky, 1974, 1986). Inasmuch as all microbes are aquatic creatures, their metabolism in soil is restricted to microhabitats wherein there is a continual supply of available water. Hence, their distribution is essentially restricted to microhabitats that contain clay minerals, as sand and silt do not retain water long against gravitational pull. Clay minerals, because of their surface activity, retain water against this pull, as the water adjacent to these active surfaces and coordinated with charge-compensating ions on the clays becomes sufficiently ordered to form a quasi-crystalline structure (i.e., the strong attraction of water molecules to the negatively and positively charged surfaces of clay minerals and their charge-compensating ions enhances the hydrogen bonding of adjacent water molecules). The ordering of this clay-associated water decreases with distance from the clay surface until a distance is reached at which the water is no longer under the attraction of the clay and is susceptible to gravity.

Clay minerals do not exist free in soil but are present as coatings, or cutans, on larger sand and silt particles or as oriented clusters, or domains, between these particles. The clay-coated particles and domains cluster, primarily as the result of electrostatic attraction between the net negatively charged faces and the net positively charged edges of clays, into microaggregates, that, in turn, cluster to form aggregates that can range from 0.5 to 5 mm in diameter and are stabilized by organic matter and precipitated inorganic materials. These aggregates retain water, the thickness and permanence of which depend on the type and amount of clay and organic matter within the aggregates, and this water may form bridges with the water of closely adjacent aggregates. These aggregates or clusters of aggregates, with their adjacent water, comprise the microhabitats in soil wherein microbes function. The space between the microhabitats constitutes the pore space, which is filled with air and other gases and

volatiles (see front and back cover pages).

As the result of the discreteness of microhabitats in soil, the probability of genetic exchange is markedly less than the probability in ecosystems wherein water is continuous. Except for periods when soil is saturated with water (e.g., after a heavy rain, snow melt, or when irrigated), individual microhabitats are isolated by the surrounding pore space, and movement of bacteria, transducing bacteriophages, and transforming DNA among the microhabitats is limited to areas where water bridges between microhabitats may occur. Even when the pore space is saturated, movement between microhabitats may be restricted, as the surface tension of the ordered water around aggregates may be too great to allow passive movement of bacteria or even active movement by flagellated cells. (There is no convincing evidence that bacteria are flagellated in soil, even though they may have the genetic capability to produce flagella when isolated from soil and cultured in liquid media or on agar.) However, filamentous fungi appear to be able to bridge pore spaces between microhabitats, even when the pore spaces are not filled with water, as these fungi grow apically from mycelia that have a food and water base in a microhabitat and, therefore, are independent of the nutrient and water conditions surrounding the growing mycelia. Moreover, the extending mycelia probably have a surrounding water film in which bacteria or bacteriophages may be transported from one microhabitat to another.

These conditions in soil differ markedly from those in sediments of aquatic systems. Although clay minerals in sediments also occur as domains and as cutans on larger particles and in aggregates, water-dependent microhabitats do not exist as they do in soil, as the water in sediments is essentially continuous from one aggregate to the next. Moreover, microbes in sediments appear to colonize primarily sand and silt particles, rather than clays, as water surrounds these particles, and the need to overcome the electrokinetic repulsion between net negatively charged clays and microbes is eliminated. Hence, care must be exercised in extrapolating observations on gene

transfer in sediments to soil and vice versa.

Gene transfer in soil

The majority of studies on the transfer of genetic information in soil, especially with genetically engineered microorganisms (GEMs; i.e., microorganisms whose genetic information has been deliberately altered), have been conducted with bacteria, wherein DNA can be transferred *in situ* by conjugation (cell-to-cell contact), transduction (via a bacteriophage), and transformation (uptake of "naked" DNA by an intact cell). Although these phenomena have been demonstrated in a wide spectrum of gram-positive and gram-negative bacteria in the laboratory (i.e., in pure culture), there is sparse information on their occurrence in soil and other natural habitats (Levy and Miller, 1989; Stotzky, 1989; Stotzky and Babich, 1986; Trevors *et al.*, 1987).

The first studies on the transfer of genetic information in soil were conducted only in sterile soil (Graham and Istock, 1978; Weinberg and Stotzky, 1972), as methods for conducting such studies in nonsterile soil had not yet been developed. Studies conducted in sterile soil have little relevance to what occurs in nonsterile soil. Hence, extrapolations from results obtained in sterile soil to what presumably occurs in nonsterile soil should be viewed with skepticism, as should studies conducted in soil extracts, wherein microhabitats have been disrupted and physicochemical characteristics altered. Nevertheless, studies in sterile soil can be valuable, when coupled with parallel studies in nonsterile soil, as techniques for subsequent use in nonsterile soil can be developed, and the effects of the indigenous microbiota and of surfaces and some other physicochemical characteristics on survival, establishment, growth, and gene transfer can be estimated.

Many of the early studies on gene transfer in soil were conducted with *Escherichia coli*, which is not an autochthonous member of the microbiota of soil. There were numerous plausible reasons why *E. coli* was used as the model bacterium in these studies: e.g., 1) the genetics of *E. coli* were better defined than those of other bacteria;

2) numerous strains with a spectrum of chromosomal alterations or containing different plasmids were readily available; 3) there had been extensive genetic engineering of E. coli strains for use in a variety of industrial applications; 4) because of the successful experience with genetic engineering for industrial applications, it appeared reasonable to assume that strains of E. coli engineered to perform specific functions would be used initially for releases to the environment; 5) E. coli has been shown to transfer plasmid-borne genetic information to over 40 genera of gram-negative bacteria and even to some gram-positive bacteria; and 6) although primarily an inhabitant of the gastrointestinal tract of many, but not all, animals, E. coli is increasingly found in fresh and estuarine waters and in soils in urban and agricultural areas, probably as the result, in large part, of the presence of human beings. The tendency now is to conduct studies on survival, growth, establishment, and gene transfer with bacterial species that are autochthonous in soil, especially as the genetics of these species become more defined.

A major impetus for these studies has been the concern about the release of GEMs to the environment, especially to soil, for agricultural (e.g., enhanced nitrogen fixation, biological pest control, reduction in frost damage) and other (e.g., degradation of recalcitrant organic pollutants) purposes. The concern is that these released GEMs will not just perform the functions for which they have been engineered but that they will somehow perturb the homeostasis of soil and cause unanticipated and undesirable changes in the activity, ecology, and population dynamics of the soil microbiota.

The survival, perpetuation, and efficacy of recombinant DNA in microbes in soil or in any natural environment depend on: 1) the nature of the microbial host and the vector (e.g., a plasmid or a bacteriophage), if involved, carrying the DNA; 2) the survival, establishment, and growth of the host-vector system; 3) the maintenance, replication, and segregation of the DNA; 4) the frequency of transfer of the DNA to other microbes; 5) the adaptability of the host-vector systems, either the original or the subsequent new ones, to their ecological "niches"; and 6) the selective advantage or disadvantage

conferred on the original or subsequent hosts by the DNA. These phenomena are affected by the physicochemical and biological characteristics of soil and other natural habitats (Table 1).

The relative importance of individual environmental characteristics varies with the specific habitat (e.g., electromagnetic radiation is probably relatively unimportant in soil, whereas it is extremely important in aquatic habitats), and their effects are usually greater on introduced than on indigenous microbes. Moreover, none of these characteristics exerts its influence individually but, rather, in concert with other characteristics. Although the influence of one or a few characteristics may predominate in a specific habitat, these influences have indirect, but cascading, effects on other characteristics. Consequently, an alteration in one environmental factor may result in simultaneous or subsequent changes in other characteristics and, ultimately, in the habitat, and therefore, in the ability of both introduced and indigenous microbes to survive, establish, grow, and transfer genetic information (Stotzky, 1974, 1986; Stotzky and Krasovsky, 1981). Because the possible permutations of interactions between these environmental characteristics are vast, the relative success of microbes containing new genetic information to transfer this information in soil cannot be easily predicted.

Biological factors

For detectable gene transfer to occur in soil or other natural environments, the population densities of the donors and recipients of the gene(s) must be sufficiently high. This is especially true in soil, where the spatial separation of microhabitats reduces the probability of transfer. Consequently, for a bacterium containing a novel gene to transfer this genetic information at a detectable frequency, it must be able to establish (i.e., colonize) and grow to sufficiently high densities within the microhabitat, especially if it is a recombinant that has resulted naturally from transfer *in situ*. If the recombinant bacterium is introduced into soil, even at high densities, it will have difficulty colonizing and growing, as the microhabitats are already filled with organisms

Table 1. Factors affecting the activity, ecology, and population dynamics of microorganisms in natural habitats.

Carbon and energy sources
Mineral nutrients
Growth factors
Ionic composition
Available water
Temperature
Pressure
Atmospheric composition
Electromagnetic radiation
pH
Oxidation-reduction potential
Surfaces
Spatial relations
Genetics of the microorganisms
Interactions between microorganisms

that are highly adapted to these microhabitats after eons of selection. Even in the gastrointestinal tract, an environment that is probably less complex than soil, especially in terms of the number of species present, the introduction of billions of cells of E. coli K12 containing small, nonconjugative plasmids, such as pBR322 and pBR325, did not result in their colonization of the tract unless the indigenous microbiota, of which E. coli is a major component, was suppressed by antibiotics or starvation or the tract was essentially devoid of indigenous microbes, as in gnotobiotic or neonatal animals (see Stotzky and Babich, 1986, for references).

It must be emphasized that "gene transfer" in soil and other natural environments implies not only the transfer of genes, but also their expression, as most methods used initially to detect transfer rely on expression of the genes (i.e., phenotypic characteristics). Hence, the transfer of genetic information in soil may be more frequent than is actually detected.

The apparent lack of high frequencies of detectable gene transfer in soil may be the result not only of insufficient population densities of appropriate donors and recipients, but also of a reduction in the fitness and competitiveness of the recombinants in some microhabitats and of barriers that reduce the successful transfer and expression of genes. In pure culture, the yield of plasmid-containing transconjugants is apparently decreased by approximately the square of the parental densities below 10^8 cells/ml; e.g., the number of transconjugants is reduced by approximately 4 orders of magnitude if the parental densities are only 10^6 cells/ml (Curtiss, 1976). However, this dependence on parental densities was not observed in sterile freshwater (O'Morchoe et al., 1988). Comparable studies have not been conducted in soil, but plasmid transfer is greatly reduced when the survival of exogenously added parentals, especially of the donors, decreases to levels below approximately 10^4 cells/g soil (Devanas and Stotzky, 1988b). Moreover, if the foreign DNA does not confer some selective advantage to the recombinant, the energy and precursors necessary for the replication of the additional

DNA and for the synthesis of the products specified by this DNA, whether functional or not, may reduce the competitiveness of the recombinant (Curtiss, 1976), especially in soil microhabitats, which are usually oligotrophic.

There are numerous cellular barriers that can prevent or reduce the transfer and expression of genetic information. Among these are: 1) restriction/modification systems, in which restriction endonucleases of the recipient cleave incoming exogenous DNA; 2) incompatibility of the exogenous DNA with replicons in the recipient, including insufficient sequence homology for recombination; 3) inability of the recipient to provide the proteins, including the RecA protein, necessary for the establishment and replication of nonself-sufficient exogenous DNA; 4) absence in the recipient of factors necessary for transcription of the exogenous DNA; 5) absence in the recipient of appropriate enzymes for post-translational modification of the gene product into a functional entity; and 6) fertility inhibition, in which the exogenous DNA prevents the expression of transfer genes, especially those that code for the production of pili (Miller, 1988). Despite these numerous barriers, which usually do not occur simultaneously in a recipient, none is probably sufficiently absolute to prevent gene transfer and expression in soil if the number of donors containing the novel DNA is high enough (which it probably will be in the case of planned releases) and the residence time of the novel DNA is long enough (which it may be if the DNA survives). Morever, many of these barriers can be surmounted by alternate strategies of DNA transfer and recombination and if the recipients are stressed (Miller, 1988), which they undoubtedly will be in soil.

It would seem, therefore, that the combination of physical separation of donors and recipients, poor survival (which will reduce the population levels of donors and recipients), cellular barriers to gene transfer and expression, and decreased fitness and competitiveness of the recombinant would reduce greatly the probability of successful transfer and expression of genetic information in bacteria in soil. However, even an extremely low probability of transfer would eventually result, and probably has, in the

successful establishment of recombinant bacteria in soil in situ. Bacteria have existed in soil for millions of years, and some transferred DNA undoubtedly conferred some selective advantage to and, thereby, increased the fitness and competitiveness of the recipient. Moreover, periodic fluctuations in the microhabitats, e.g., introduction of nutrients, changes in pH, E_h, and other physicochemical characteristics, may reduce the levels of the adapted indigenous microbiota sufficiently to allow a recombinant bacterium to colonize and increase its population density before the microhabitat returns to "normal" and again enables the autochthonous microbiota to proliferate. Such periodic alterations in the environmental conditions within microhabitats appear to be involved in the establishment in soil of fungal pathogens of plants and humans introduced naturally into soils in which they were originally absent (see Stotzky, 1974, 1986). Furthermore, the presence in soil of an antimicrobial agent to which a recombinant bacterium has gained resistance (e.g., antibiotics, heavy metals) or of a compound that only it can metabolize (e.g., a recalcitrant pesticide) will increase its survival, establishment, and growth and, hence, the probability of gene transfer. The balance between the factors that mitigate against and those that encourage the transfer and expression of genetic information in soil will not be known until extensive studies are conducted with a spectrum of recombinant bacteria introduced into a wide variety of soils in different geographic locations.

Effects of biological and physicochemical environmental factors on gene transfer in soil

The fate of recombinant DNA in soil is ultimately dependent on the survival, establishment, and growth in soil of the microbial hosts that house the genetic material. The survival, establishment, and growth of the hosts are, in turn, dependent on their genetic constitution and on the biological and physicochemical characteristics of the recipient soil (Table 1). Detectable transfer of genetic information, regardless of the mechanism of transfer, usually requires sufficiently high populations of donors (whether a bacterium, a transducing bacteriophage, or transforming DNA) and recipients. Moreover,

the ability to predict the fate of introduced GEMs and the potential transfer of their novel genetic information to indigenous microorganisms in various types of soil would be enhanced if the knowledge of the effects of different biological and physicochemical characteristics could be related to the actual characteristics of the recipient soil. Unfortunately, insufficient studies have been conducted on the effects of biological and physicochemical factors on the activity, ecology, and population dynamics of microbes and on their ability to transfer genetic information in soil and other natural habitats (see Curtiss, 1976; Freter, 1984; Stotzky, 1974, 1986, 1989; Stotzky and Babich, 1986; Stotzky and Krasovsky, 1981).

Microbial competition. On the basis of numerous studies conducted in vitro, in vivo, and in situ, it is apparent that the survival, establishment, and growth of introduced microorganisms, whether containing recombinant DNA or not, are usually reduced when other species of microorganisms are present. This is especially true in natural habitats, such as soil, wherein the indigenous microbial populations are usually not only better adapted to the habitat, but exert competitive, amensalistic, parasitic, and predatory pressures on the introduced organisms. For example, the survival and growth of bacteria that were not genetically engineered were significantly reduced when they were inoculated into nonsterile environments (e.g., soil, water, sewage) in which they were not natural residents, whereas they survived, and even grew, in the same environments when these were sterilized (e.g., the numbers of Salmonella typhimurium, Agrobacterium tumefaciens, and Klebsiella pneumoniae increased by 1 to 2 orders of magnitude in sterile soil, whereas there was a reduction in numbers when inoculated into nonsterile soil) (Liang et al., 1982). There are numerous other examples in the literature of the apparent lower survival and growth of introduced microbes in nonsterile than in sterile environments (see Stotzky and Babich, 1986), and this relationship appears intuitively to be valid. However, most of these studies did not consider the possibility of the "viable but nonculturable" phenomenon (see below). Both genetically engineered and

nonengineered bacteria introduced into soil and other natural habitats could become so debilitated or otherwise altered that they would not be recovered from these habitats, especially on selective media, even though they could actually be surviving and growing in their new environment. Because of the implications of such alteration to monitoring the fate of GEMs introduced into soil and other natural habitats, it must be clearly established that the apparent lack of survival and growth of an introduced GEM was not an artifact of the experimental procedures.

Energy sources. The presence of available energy sources appears to enhance the transfer of genetic information, as these sources apparently increase the population densities of the donor and recipient and the subsequent growth of the recombinant bacterium. This effect is considerably less pronounced in nonsterile than in sterile soil, as the indigenous microbiota quickly mineralizes added carbonaceous substrates. If the donor or recombinant organism contains a gene that enables the metabolism of a carbonaceous compound that can not be metabolized by other members of the indigenous microbiota (e.g., a recalcitrant pesticide), the organism may have a temporary selective advantage, as the result of its ability to utilize the compound (e.g., Chatterjee et al., 1981). Nevertheless, this advantage is probably lost when the specific compound is mineralized or transformed to an unavailable form. Moreover, the concentration of such recalcitrant compounds in soil is usually low and less than the concentration of natural organic materials that are available to essentially all the indigenous microbes. Nevertheless, the additio . to soil of a compound that only an introduced GEM can utilize should enhance the survival of that GEM in soil, and no further addition of the compound after it has been degraded should reduce the level of the GEM. This may be an efficient method with which to restrict and control the population densities of some GEMs in soil.

Temperature. Temperatures near the optimum growth temperature appear to be necessary for the efficient in vitro transfer of genetic information in laboratory strains of bacteria, although this is not always the case (see Stotzky and Babich, 1986). For

example, maximal conjugal transfer of the R-plasmid, R1drd-19, in strains of E. coli decreased progressively as the temperature was decreased from 37 to 17 C, and no transfer was detected at 15 C (Singleton and Anson, 1981). Transfer of pRD1, derived from a clinical isolate of Pseudomonas and maintained in the laboratory, from an E. coli K12 donor to an E. coli K12 recipient decreased progressively as the temperature was reduced from 37 to 15 C; when Erwinia herbicola was the recipient, transfer occurred even at 12.5 C (Kelly and Reanney, 1984). Plasmids pWK1 and pWK2, that conferred resistance to antibiotics and mercury, were transferred optimally in vitro from a species of Citrobacter and a species of Enterobacter isolated from soil to E. coli at 28 C, and frequencies were greatly reduced at 15 and 37 C. In contrast, the transfer of a plasmid conferring resistance to kanamycin from a strain of Proteus vulgaris, isolated from the human urinary tract, to E. coli was about 5 orders of magnitude higher at 25 than at 37 C (Terawaki et al., 1967). The in vitro transfer frequency of a plasmid conferring resistance to streptomycin and tetracycline from an E. coli isolated from sewage to an E. coli isolated from creek water was highest at 25 C and lowest at 35 C, with frequencies at 15, 20, and 30 C being intermediate. The frequency of transfer in raw sewage in situ was also higher at 22.5 than at 29.5 C (Altherr and Kasweck, 1982).

Plasmids present in enterobacteria isolated from human beings, fecally-polluted rivers, and sewage treatment plants were differentiated on the basis of their thermosensitivity: "thermotolerant" plasmids were transferred equally well at 22 to 37 C, whereas "thermosensitive" plasmids were transferred at high frequencies at 22 or 28 C but at low frequencies at 37 C. Only 3.1% of 775 conjugative antibiotic-resistance plasmids evaluated were thermosensitive (Smith et al., 1978).

The maintenance of some plasmids by their host cells in vitro is also dependent on temperature, and many plasmids are lost above the optimum growth temperature (see Stotzky and Babich, 1986). For example, the Ti plasmid of A. tumefaciens (Watson et al., 1975) and the nodulation plasmid, pW22, of Rhizobium trifolii (Zurkowski and Lorkiewicz,

1979) were lost when the host cells were grown at 37 C. In contrast, the survival of E. coli J5(RP4) and JC5466(pRD1) was reduced to a greater extent in soil maintained at 20 than at 4 C (Schilf and Klingmüller, 1983).

There have been few controlled studies on the effects of temperature on the transfer of genetic information in soil. It appears, however, that conjugal transfer of both plasmid- and chromosomal-borne genes occurs in both sterile and nonsterile soil at temperatures lower than those necessary for optimal transfer in vitro; e.g., transfer in E. coli in soil occurred at temperatures between 15 and 27 C, considerably below the requisite temperatures in vitro (e.g., Krasovsky and Stotzky, 1987; Trevors, 1987b; Weinberg and Stotzky, 1972), and in Bacillus subtilis at 15 as well as at 27 C (van Elsas et al., 1987). Transduction of E. coli occurred in soil at 25 to 27 C (Germida and Khachatourians, 1988; Zeph et al., 1988). Transformation of B. subtilis in sterile soil occurred at 37 C (Graham and Istock, 1978), in a simulated sterile marine system containing sea sand at 23 C (Aardema et al., 1983; Lorenz et al., 1988), and in suspensions of montmorillonite at 33 C (Stotzky and Golard, unpublished). Unfortunately, no studies have been conducted to determine the effects of temperature on transduction and transformation in soil.

Temperature in soil can not be conveniently controlled in situ. However, the range in fluctuations in soil temperature may be an important consideration when constructing a GEM for introduction into soil.

pH. The hydrogen ion concentration is an important physicochemical characteristic of soil that is amenable to control in situ but whose effect on gene transfer in soil has been insufficiently studied. Conjugal transfer of plasmids in E. coli was restricted in vitro to pH 6 to 8.5 (Curtiss, 1976). In soil, both intra- and interspecific plasmid transfer was not detected until the bulk pH was adjusted to 6.8 with $CaCO_3$ (Devanas and Stotzky, unpublished), and conjugal transfer of chromosomal genes was also detected only at pH values near neutrality (Krasovsky and Stotzky, 1987; Weinberg and

Stotzky, 1972) (pH values above neutrality were not evaluated in these studies). Transduction of E. coli by phage P1 was higher in a soil with a pH of 7.9 than in a soil with a pH of 6.8 (Germida and Khachatourians, 1988). However, as these soils also differed in texture, organic matter content, and other physicochemical characteristics, differences in the transduction frequencies cannot be attributed solely to these differences in pH. The effect of pH on transformation in soil has apparently not been studied.

The pH of soil can affect gene transfer both directly (e.g., survival and growth of the parentals) and indirectly (e.g., growth of the competitive and amensalistic indigenous microbiota; adsorption of transducing phages, transforming DNA, and DNase on soil particles). For example, the adsorption of bacteriophages on clay is pH-dependent (Lipson and Stotzky, 1987; Stotzky et al., 1981), and the adsorption of DNA on sea sand increased as the pH was increased from 5 to 9 (Lorenz and Wackernagel, 1987). The pH determines the sign of the net surface charge of amphoteric materials (e.g., enzymes, bacteria, bacteriophages) and the negative charge of ionizable nonamphoteric materials (e.g., DNA). Therefore, the adsorption of these materials on soil particles, especially on most types of clay minerals, which have a pH-independent negative charge, will be influenced by the pH of soil, which, in turn, will influence the persistence and activity of these materials and the transfer of genetic information (see Stotzky, 1986). Consequently, controlled studies on the effects of pH on genetic transfer in soil should be conducted.

Water content. The few data that are available on the effects of water content on the transfer of genetic information in soil indicate that transfer frequencies are higher when the soil water tension is near or at the optimum for microbial growth (i.e., the -33 kPa tension). For example, plasmid transfer and survival of the donors (E. coli J5(RP4) and JC5466(pRD1)) and the exconjugants (Enterobacteriaceae strain 1(pRD1) and Pseudomonas fluorescens (pRD1)) were higher in soil maintained at 16% water than when

allowed to dry to 4% (Schilf and Klingmüller, 1983). Similarly, plasmid transfer in sterile soil was greater between strains of B. subtilis at a water content of 20 to 22% (equivalent to the -33 kPa water tension) than at a water content of 8% (van Elsas et al., 1987); between strains of E. coli in sterile soil at 80 than at 20% of the water-holding capacity (WHC) (Trevors, 1987a); and between E. coli and other enterobacteria and Pseudomonas aeruginosa in nonsterile soils at their -33 kPa tension (24 to 26% water) than at 16% water (Devanas and Stotzky, unpublished). No studies appear to have been conducted on the effects of water content on transduction and transformation in soil. Inasmuch as microbial growth in soil is optimal at the -33 kPa water tension, genetic transfer, regardless of the mechanism of transfer, is probably maximal at this tension, which is easily controlled by irrigation.

Oxygen and E_h. At soil water tensions above -33 kPa, oxygen will become progressively more limiting, and the E_h will be reduced (see Stotzky, 1974). The effects of oxygen tension and E_h on the transfer of genetic information in soil has apparently not been studied, and results from the few studies conducted in vitro are contradictory: e.g., the conjugal transfer of chromosomal genes between E. coli was similar under aerobic and anaerobic conditions (Stallions and Curtiss, 1972); the expression of antibiotic resistance by 45 different plasmids in E. coli was the same under aerobic and anaerobic conditions, although the formation of sex pili was reduced under anaerobic conditions (Burman, 1977); the frequency of conjugal transfer of R-plasmids by two donor strains of E. coli isolated from human feces was reduced 10- to 1000-fold under anaerobic conditions (Moodie and Woods, 1973).

Ionic composition. The effects of the ionic composition of the soil solution, which affects the activity of water (a_w), as well as surface interactions among and between soil particles, especially clay minerals, and bacteria, bacteriophages, DNA, and proteins (see Stotzky, 1986), on genetic transfer requires study. The transfer, in vitro, of plasmid RIdrd-19 in E. coli was apparently stimulated by concentrations of NaCl that are present

in estuaries (Singleton, 1983). The survival of antibiotic-sensitive fecal coliforms was reduced in seawater, whereas that of E. coli strains containing R-plasmids was unaffected (Smith et al., 1974).

Electromagnetic radiation. Although light probably affects only microbes residing on the surface of soils, this physicochemical factor can be important in arid and semi-arid soils, where photosynthesis in algal crusts, both procaryotic and eucaryotic, may be the major source of primary productivity (e.g., Skujins, 1984). Hence, the transfer of genes conferring resistance to ultraviolet radiation may enhance the survival of microbial surface dwellers (Marsh and Smith, 1969). The importance of electromagnetic radiation on gene transfer in soil has apparently not been studied.

Surfaces. The effects of surfaces, especially those of clay minerals, on transfer of genetic information in soil appear to have been studied to a greater extent, albeit also insufficiently, than those of other physicochemical characteristics of soil. Montmorillonite appears to enhance conjugal transfer of both plasmid- and chromosomal-borne genes in both sterile and nonsterile soil, whereas kaolinite appears to have essentially no effect (e.g., Devanas and Stotzky, 1988a; Krasovsky and Stotzky, 1987; van Elsas et al., 1987; Weinberg and Stotzky, 1972). In contrast, colloidal montmorillonite reduced the in vitro transfer of plasmid RIdrd-19 in E. coli by several orders of magnitude (Singleton, 1983). This apparent paradox between in vitro and in vivo effects of clay minerals may be the result of free clay particles in vitro blocking sites on the bacterial surface necessary for gene transfer, whereas few free clay particles exist in soil, as they are relatively stably maintained in cutans and domains.

Surfaces, especially those of clay minerals, can exert both direct and indirect effects on microbial activities, including transfer of genetic information, in soil. However, the mechanisms by which some clays enhance conjugal gene transfer in soil are not clear. Montmorillonite has been shown to stimulate the growth of bacteria, in part by maintaining a suitable pH for sustained growth in the microhabitats, and to reduce the

grow ... of fungi, in part by complexing siderophores necessary for iron transport (see Stotzky, 1986). Van Elsas et al. (1987) suggested that the enhanced transfer of a plasmid in B. subtilis in soil amended with montmorillonite was not the result of an effect of the clay on pH but of "a modification of the physicochemical soil environment, possibly modifying cellular physiology or promoting cell-to-cell contact" or because the "clay apparently protected the recipient population". Unfortunately, no details were presented on the mechanisms of such modifications and protection. Moreover, it is also not clear which physicochemical properties of clays (e.g., cation- and anion-exchange capacity, specific surface area, surface charge density, nature of the charge-compensating cations) are responsible for their effects on conjugation and survival of parentals and exconjugants in soil.

The frequency of transduction in soil was not affected by montmorillonite, although the survival of the transducing bacteriophage, P1, was increased (Zeph et al., 1988). Transduction by phage P1 was greater in a sandy (8% clay) than in a silty clay loam (21% clay) soil (Germida and Khachatourians, 1988), but as these soils also differed in pH, organic matter content, and other characteristics, and the types of clay present were not described, the differences in transduction frequencies cannot be attributed solely to differences in clay content in these two soils. The effects of surfaces on transformation in soil have not been studied. However, both sea sand (Aardema et al., 1983; Lorenz et al., 1988) and montmorillonite (Stotzky and Golard, unpublished) reduced the in vitro frequency of transformation in B. subtilis.

Interactions between factors. The value of studies conducted on the effects on gene transfer of one physicochemical factor of soil at a time may be limited, as a change in one factor can result in changes in numerous other factors, and several factors can interact to affect gene transfer. For example, the in vitro conjugal transfer of plasmid R1drd-19 in E. coli was inhibited more by deviations in pH from the optimum of 6.9 when the temperature was simultaneously decreased from 37 to 17 C (Singleton and Anson,

1983). Studies on the effects of interactions between multiple physicochemical characteristics of soil are needed not only with respect to the transfer of genetic information, but on all aspects of microbial ecology in soil. Nevertheless, even the few studies that have been conducted on the effects of these characteristics individually on gene transfer in vitro and in soil and other natural habitats indicate that in vitro studies of conjugation, transduction, and transformation, which are usually conducted under standardized and optimal growth conditions, are not always adequate predictors of gene transfer in natural habitats, wherein these characteristics fluctuate continually and in concert.

Conclusions

There is insufficient information on the frequency of transfer, whether by conjugation, transduction, or transformation, and on the survival and activity of recombinant bacteria in soil and other natural environments that contain high numbers of other microorganisms not involved in the transfer. The relatively few studies that have been conducted in soil - and it must be emphasized that even these studies have been conducted primarily in the laboratory in microcosms of varying degrees of complexity or under greenhouse conditions, as few field releases have been authorized - indicate that transfer can occur in soil. However, insufficient information is available on how transfer and survival are affected by the physicochemical and biological characteristics of soil; on the numbers of donors and recipients necessary for transfer; on the numbers of recombinant bacteria that can be accurately detected; on the probability that low levels of recombinant bacteria, especially below the level of detection, can multiply sufficiently to become a significant portion of the soil microbial population; and on numerous related questions.

The major lack of knowledge, however, is in the area of the potential effects that recombinant bacteria could have on the structure and function of soil and other natural habitats; e.g., what kinds of genes need to be transferred and how many recombinant

bacteria need to be functioning per unit volume of soil to result in detectable changes in the activity, ecology, and population dynamics of microorganisms in soil? Even if a GEM introduced into soil survives and transfers its novel genetic information to indigenous microbes, there should be little cause for concern unless the novel genetic information, either in the introduced GEM or in an autochthonous recipient to which it has been transferred, results in some unexpected and untoward impacts. Unfortunately, it is difficult with existing knowledge and methodology to study and predict the occurrence, extent, and severity of such impacts.

Preliminary studies on the effects of adding high concentrations (e.g., 10^8 cells/g soil) of various strains of E. coli, Enterobacter cloacae, Pseudomonas aeruginosa, and Pseudomonas putida, with and without plasmids carrying antibiotic-resistance genes, to soil have not shown any consistent and lasting effects on the gross metabolic activity (as measured by CO_2 evolution), transformations of fixed nitrogen, activity of soil enzymes (phosphatases, arylsulfatases, dehydrogenases), and species diversity of the soil microbiota (Doyle et al., 1988; Jones et al., 1988). However, in other preliminary studies, the introduction into soil of a strain of Streptomyces lividans that contained a plasmid carrying a lignin peroxidase gene from the chromosome of Streptomyces viridosporus enhanced the rate of mineralization of soil carbon (as measured by CO_2 evolution) during the 30-day incubation, especially when the soil was amended with lignocellulose (Wang and Crawford, 1988). More such studies are needed, especially with GEMs that have been engineered to perform specific enzymatic functions in soil.

It is obvious, based on the few studies that have been conducted on the transfer of genetic information in soil, that more research is necessary to determine the frequency, the location, and the effects of physicochemical and biological factors on gene transfer in soil and other natural habitats. The attainment of this knowledge will not only be of immense academic interest to microbial and macrobial ecology and evolutionary theory,

but also to risk assessment and regulation of the release of GEMs to soil and other natural habitats.

II. Literature Review of Conjugation and Transduction in Soil

CONJUGATION

Introduction

Conjugation is the transfer of DNA from one bacterium to another by direct cell-to-cell contact and is mediated by transfer (tra) genes. For example, the self-transmissible fertility (F)-factor in the gram-negative bacterium, E. coli, is a plasmid that has 15 to 25 tra genes that encode its transfer, i.e., enzymes and the appropriate F-pili. The F-factor may exist autonomously in the cell, or it can be integrated into the bacterial chromosome, where it is replicated and segregated to progeny cells with the other genes on the chromosome, similar to a prophage in lysogenic bacteria. In E. coli, which has been more extensively studied than other gram-negative and gram-positive bacteria, the DNA moves from the donor F^+ cell to the recipient F^- cell through a special surface pilus called an F-pilus, the synthesis of whose structural proteins are directed by the tra genes. The F-pilus of the donor attaches to the recipient cell at specific surface receptors and retracts until the surfaces of the donor and recipient are touching. At the point of contact on the recipient, a channel is made through its cell envelope, and the DNA is transferred, presumably through the pilus, into the recipient cell. A bacterial cell in which the F-factor is integrated into the chromosome is called a "High frequency of recombination" (Hfr) cell. The integrated F-factor can mediate transfer of part of the F-factor and part of the donor chomosome (an Hfr conjugation) (Freifelder, 1987; Lewin, 1977; Vandemark, 1987).

Gram-positive bacteria can also transfer DNA by conjugation, but no sex pili have been demonstrated. The recipient cells synthesize specific diffusible proteins called pheromones. When these pheromones contact a donor cell, it synthesizes surface proteins with adhesive properties that cause the donor and recipient cells to form aggregates. The sex pheromones then induce the transfer of the plasmid to the recipient,

but the exact mechanism of transfer is not known (Clewell, 1981; Freifelder, 1987).

Transfer of chromosomal genes

Transfer of the bacterial chromosome from an Hfr cell to an F^- cell begins at the 3'-end of the origin of transfer (oriT) site within the integrated F-factor, where a tra-encoded endonuclease nicks and opens one of the two DNA strands. The free 5'-end of the open single-stranded DNA attaches to a site on the cytoplasmic membrane, and the 3'-end acts as a primer for chain elongation, using the closed, intact, circular strand as a template. The open DNA strand, after its release from the cytoplasmic membrane, serves as a template for the synthesis of its complementary DNA and moves, 5'-end first, into the recipient through a conjugation bridge (presumably the F-pilus in E. coli). This is an example of the unidirectional "rolling circle" mechanism of replication. The entire single-stranded DNA from the donor chromosome and the newly synthesized complementary DNA strand must be moved to the recipient for complete transfer of all the genes on the chromosome, including the distal end of the F-factor, and to produce a complete functional integrated F-factor upon reannealing and ligation in the recipient. This process is estimated to require about 100 min in E. coli. This is a relatively long time for bacteria to stay physically attached, especially in soil and other natural habitats, and interruption of conjugation usually occurs after only 10 to 20% of the chromosome has been transferred (Freifelder, 1987; Lewin, 1977). Therefore, the entire F-factor is rarely transferred from an Hfr cell, as the remaining F-factor genes are at the opposite free 3'-end of the nicked chromosome. Genes that show a high frequency of recombination are those nearest the oriT site, and the frequency of transfer of genes decreases proportionately with their distance from this site.

An F-factor that has been integrated into the chromosome may excise itself, by expressing the appropriate enzymes coded on its DNA, by a reverse recombinational event and return to an autonomous, extrachromosomal state. Precise excision, i.e., nicking at the exact sites that were ligated to the chromosomal DNA on integration, will

restore the original autonomous F-factor. Imprecise excision may result in an autonomous F-factor that includes chromosomal genes (possibly introduced recombinant genes) from one or both sides of the integration site of the F-factor. This modified F-factor, sometimes referred to as an F'-plasmid, can transfer these genes to another cell.

Conjugative plasmids

Autonomous F-factors, F'-plasmids, and R-plasmids (conjugative plasmids similar to an F-factor that carry genes coding for antibiotic resistance) are self-transmissible. Hence, they may direct their transfer to other bacteria by expressing their tra genes. Transfer of conjugative plasmid DNA also begins at the oriT site within the tra genes, as it does in Hfr strains. However, as the length of the DNA sequences (i.e., the number of genes) in a plasmid is significantly smaller (1 to 10%) than the length of a bacterial chromosome, the entire plasmid is usually transferred. As in chromosomal transfer, the plasmid DNA is replicated during transfer (i.e., a copy remains in the donor cell), and the recipient cell then expresses the tra genes and becomes a donor cell. Consequently, in the presence of appropriate selection pressure, a plasmid may spread rapidly through a bacterial population, even though plasmid-containing cells may originally have been an insignificant part of the population. This rapid conversion of plasmid-free recipient cells to plasmid-containing donor cells appears to be responsible for the rapid increase in antibiotic-resistant nosocomial infections.

Nonconjugative plasmids

Autonomous, extrachromosomal, and self-replicating plasmids that do not have tra genes are nonconjugative. Consequently, these plasmids, which may code for a variety of functions (e.g., resistance to antibiotics and other antimicrobial agents, catabolic enzymes, toxin production), can not direct their own transfer. However, nonconjugative plasmids may be dispersed to appropriate recipient cells by coinhabiting conjugative plasmids that can "move" them (mobilization) to other cells, or there is recombination and integration into a plasmid that is self-transmissible (cointegrate formation).

Mobilization. If a plasmid is deficient in DNA sequences that code for mobility (mob) (e.g., for endonucleases that nick the oriT region) or transfer (e.g., F-pilus production) functions, the plasmid will not be transferred, even if all other functions are present, and it will persist as an autonomous, self-replicating element. However, such nonconjugative plasmids may be transferred by conjugation if the missing functions are supplied gratuitously (in trans.) by another plasmid present in the same cell. For example, the nonconjugative plasmid, ColEl, can be mobilized and transferred when an F-factor present in the same cell supplies the missing pilus and transfer apparatus (Freifelder, 1987).

The mobilization of nonconjugative plasmids under environmental conditions has been reported. A nonconjugative plasmid, pHSV106, coding for resistance to ampicillin and thymidine kinase from herpes simplex virus, was mobilized by plasmids present in both laboratory and indigenous wastewater strains of bacteria in a laboratory-scale wastewater treatment facility (Mancini et al., 1987). In sterile soil, triparental matings (i.e., mating of a cell carrying a nonconjugative plasmid with a cell carrying a conjugative plasmid and then transfer of either the nonconjugative or both plasmids to a third cell) mobilized nonconjugative plasmids between strains of Streptomyces (Rafii and Crawford, 1988). Transfer of a 2.8-megadalton (Mda) plasmid, pFT30, from B. cereus to B. subtilis occurred in sterile and nonsterile soil when a 29.5-Mda plasmid was present in B. cereus (van Elsas et al., 1987).

Cointegrate formation. When two plasmids coexist in a cell and there is a region of homology between them, e.g., a transposon or an insertion sequence, the two plasmids may fuse to form a single plasmid termed a cointegrate plasmid. If one of the plasmids is conjugative, the cointegrate plasmid, containing genes from both plasmids, can be transferred. Once in the recipient, the cointegrate plasmid may separate into the original two plasmids. Components of an infectious R-plasmid (i.e., the RTF region, containing transfer and replication genes, and the R-determinant, containing drug-

resistance genes) have been shown to form cointegrates with themselves and other R-plasmids and then to separate into new species of plasmids (segregants) of various sizes in the recipient (Freifelder, 1987; Lewin, 1977).

Mobilizing elements

Transfer of DNA that is not self-transferable can also be facilitated by mobilizing elements, e.g, insertion sequences (IS), a transposon (Tn). These transposable elements are specific segments of DNA that have the ability to move autonomously as discrete units to other sites in DNA. The IS are a special type of transposable element containing 800 to 1400 base pairs with inverted repeat sequences that are 16 to 41 base pairs long at each end. The IS encode only those genes necessary for their own transposition, including a gene for an enzyme called transposase, which mediates recombination at special sites in the terminal inverted repeat sequences. Several distinct IS have been detected and characterized in procaryotic and eucaryotic genomes.

The Tns are composite units with terminal IS or short inverted repeat sequences that flank antibiotic-resistance or other genes, as well as the gene for transposase, and do not require homologous recombination genes for movement. These mobilizing elements serve as regions of homology for recombinational events that can: 1) produce cointegrates of transferable and nontransferable plasmids; 2) transfer copies of genes from sites in the chromosome to sites on other replicons, e.g., plasmids; 3) cause insertional mutations within genes; and 4) provide a detectable marker after incorporation into the genome (Freifelder, 1987).

Spectrum of conjugative bacteria

Some genera of gram-negative bacteria that have been shown to be capable of participating in the transfer of chromosomal- and/or plasmid-borne genes by conjugation are: Enterobacter, Escherichia, Salmonella, Shigella, Providencia, Vibrio, Proteus, Klebsiella, Pseudomonas, Serratia, Rhizobium, Bradyrhizobium, Bordetella, Neisseria, Rhodopseudomonas, Azotobacter, Caulobacter, Agrobacterium, Erwinia,

Chromobacterium, Acinetobacter, Rhodospirillum, and Flavobacterium. The gram-positive genera that are known to transfer genes by conjugation include: Staphylococcus, Clostridium, Bacillus, Streptococcus, Lactobacillus, Nocardia, Actinomyces, and Streptomyces.

Conjugal transfer of plasmids occurs in both gram-negative and gram-positive bacteria, and plasmid transfer from E. coli to various gram-positive bacteria has been demonstrated in vitro (Trieu-Cuot et al., 1987).

Survival of introduced genes in situ

When the heterologous DNA is present in a plasmid, the survival and stability of the DNA is dependent not only on the survival of the bacterial host, but also on the maintenance and faithful replication of the plasmid during the cell cycle and the partitioning of the plasmid to the progeny. Low copy number plasmids (1 to 2 per chromosome) are usually stably maintained and stably inherited by a partitioning mechanism, whereas high copy number plasmids (greater than 5 per chromosome) may be inherited by a random distribution mechanism of stochastic segregation, although some form of active partitioning may also occur with high copy number plasmids (Sherratt, 1982). For example, when sterile soil was inoculated with E. coli K12 strain x1666, the number of colony-forming units (CFU) increased rapidly to a maximum population level, the increase being greater when inoculated in Luria Broth (LB) than in saline. When the cells of x1666 contained plasmid pES019, which is a 4-Mda construct of the multicopy, tetracycline (Tc)-resistant plasmid, pBR328 (Covarrubias et al., 1981), engineered to contain gentamycin (Gn)-, chloramphenicol (Cm)-, and streptomycin (Sm)- resistance markers (W. Watkins, personal communication; see Levin, 1982), the numbers of CFU also increased in sterile soil. However, segregation of the plasmid apparently occurred, as the numbers of CFU recovered on the nonselective medium, MacConkey agar (MAC), were significantly higher than the numbers recovered on the medium selective for pES019 (MAC + 25 μg/ml Cm). This difference in numbers between the plasmidless and the

plasmid-containing populations was not the result of an artifact (e.g., a "viable but nonculturable" phenomenon), as the same difference occurred when the CFU recovered on MAC were subsequently transferred to MAC + Cm. The segregation occurred shortly after the addition of the host-vector system to soil, and the plasmid-free population then outgrew the plasmid-containing population. Nevertheless, despite the segregation, the population of ∝1666(pES019) increased by 1 and 3 orders of magnitude when inoculated in saline or LB, respectively, and persisted near these levels throughout the 27-day incubation (Devanas et al., 1986; Devanas and Stotzky, 1988a).

When E. coli ∝1666 and ∝1666(pES019) were inoculated in saline into soil containing a natural microbial community (i.e., nonsterile soil), the numbers of indigenous total bacteria and total gram-negative bacteria remained essentially the same throughout the 20-day study. However, ∝1666 declined rapidly and was not detected 6 days after inoculation, whereas ∝1666(pES019) declined more gradually to undetectable levels after 13 days. In contrast to the results obtained in sterile soil, there was no faulty segregation of the plasmid to produce a plasmid-free population, and the presence of the plasmid enhanced the survival of the host-vector system. The lack of partitioning of the plasmid in nonsterile soil was apparently the result of little or no growth of the host-vector system, as when nutrients (LB) were added on days 1 and 3, both growth and segregation occurred. The possible partitioning of plasmids should be considered if GEMs are added to soils containing sources of available carbon and energy (e.g., fresh plant residues or degradable organic pollutants), as growth of the GEMs may result in the eventual loss of the plasmid-containing population.

Although it has been suggested that the size of the resident plasmid can affect its maintenance (e.g., Hakkaart et al., 1985), and large plasmids appear to prolong the generation time of hosts in vitro (Godwin and Slater, 1979; Zund and Lebek, 1980), the host strain and not the size of the plasmid appeared to be important in the persistence of E. coli host-vector systems in nonsterile soil. There was little difference in the rates of

decline in nonsterile soil of hosts that contained small, multicopy, nonconjugative plasmids (2.6 and 8.0 Mda) or large, low copy number, conjugative plasmids that ranged in size from 26 to 64 Mda, regardless whether they were added in LB or saline (Devanas et al., 1986).

Moreover, the survival of E. coli strains χ1666 and PRC487 was greater when they contained plasmids (pES019 and pACYC175 (8 Mda), respectively) (Devanas et al., 1986). The survival of χ1666(pBR322) in the human gastrointestinal tract was also greater than that of the plasmidless parental (Levy et al., 1980), as was that of the highly debilitated E. coli strain χ1776 when it contained pBR322 (Levy and Marshall, 1979). Furthermore, the survival of plasmid-containing strains of E. coli was similar to that of homologous plasmidless strains in freshwater (Grabow et al., 1975), conventional sewage treatment systems (Sturtevant and Feary, 1969), and seawater (Smith et al., 1974), even in the absence of the selection pressure of the antibiotics to which the plasmids conferred resistance. Although most in vitro studies, usually conducted in chemostats under nutrient-limiting conditions, indicate that plasmid-free cells have a more rapid growth rate and quickly displace plasmid-containing cells of the same species, this is not always the case, as these studies show. Even in chemostat studies, the growth of E. coli that contained either the cos DNA fragment from phage lambda (Edlin et al., 1984), the transposable element; IS50 (Hartl et al., 1983), derivatives of pBR322 that expressed resistance to Tc (Lee and Edlin, 1985), or were lysogenic for phage lambda (Edlin et al., 1975) was faster than that of the homologous hosts not carrying these DNA sequences. The reasons for these observations are not known. Although it is tempting, in the case of the greater survival of plasmid-containing bacteria in soil, to attribute this to the antibiotic-resistance genes carried by the plasmids, there is no convincing evidence that antibiotics are produced in soil, at least not at detectable levels (see Stotzky 1974, 1986). Regardless of the reasons involved, these observations indicate that the presence of plasmids, even if they confer no known advantage to their hosts, is not always

detrimental to the competitiveness of the bacteria and that results obtained *in vitro*, despite their apparent logic, can not always be extrapolated to soil *in situ*.

There appeared to be a more rapid decrease in the numbers of some plasmid-containing *E. coli* strains when recovered from nonsterile soil on selective media (e.g., MAC + 25 μg/ml Tc + 5×10^{-5} *M* mercury (Hg) as $HgCl_2$) than on nonselective media (e.g., MAC), suggesting a loss of the plasmids. However, this was an artifact, and there was no loss of the plasmids: when cells from colonies recovered on nonselective media were transferred to selective media, the numbers of CFU that subsequently developed on the selective media were the same as those recovered on the nonselective media (Devanas *et al.*, 1986). The cells of some host-plasmid systems were apparently sufficiently debilitated in soil to prevent their recovery directly on selective media with the added stress of an antibiotic and/or a heavy metal, and resuscitation on less stressful nonselective media was necessary for subsequent growth on selective media.

This phenomenon of "viable but nonculturable" has also been reported in other environments with bacteria not containing recombinant DNA: e.g., *E. coli*, *Vibrio cholerae* (Xu *et al.*, 1982), and *Salmonella enteritidis* (Roszak *et al.*, 1984) in natural waters and *E. coli* and *Streptococcus faecalis* in chlorinated water (Bissonnette *et al.*, 1975; Camper and McFeters, 1979; Zaske *et al.*, 1980) (*see* Roszak and Colwell, 1987). The mechanisms responsible for this phenomenon have not been definitively established. It has been suggested that the cell membrane may be damaged (Zaske *et al.*, 1980) and, hence, not be capable of using an electrochemical gradient to generate high-energy intermediates for sustained survival (Sjogren and Gibson, 1981) or that the cells may be restricted in obtaining and retaining energy sources for endogenous metabolism and maintenance (Morita, 1982). Regardless of the mechanisms involved, the reduced viability or recoverability of host-plasmid systems introduced into stressful environments, such as soil, may result in erroneous data on the survival of GEMs in such environments.

Even DNA foreign to bacteria appears to be maintained in soil when introduced into bacteria on a plasmid. For example, studies on the survival and maintenance of a 0.9-Mda cDNA fragment that codes for a yolk protein in Drosophila grimshawii and inserted into plasmid pBR322 showed that the presence of the chimeric plasmid (C357) had little effect on the survival of the host (E. coli HB101) in sterile soil (Devanas and Stotzky, 1986). When E. coli HB101(pBR322) and HB101(C357) were added in LB to nonsterile soil, the presence of the insert of Drosophila cDNA reduced somewhat the stability of the plasmid during the 27-day incubation. When the host-plasmid systems were added in saline, the rate of decrease of the system containing the chimeric plasmid was similar to that of the system containing only pBR322, and the decrease of both was greater than when added in LB. The presence of foreign genetic material has been shown to reduce the stability of plasmids when the host-vector systems were grown in a chemostat (Warnes and Stephenson, 1986), and other studies have indicated that pBR322 is unstable in nutrient-limited chemostat cultures (Jones et al., 1980; Jones and Melling, 1984). However, when E. coli HB101(C357) was added in either LB or saline to nonsterile soil, there was no selective loss of the chimeric plasmid, as determined by the isolation of HB101(C357) on a selective medium and colony hybridization with a ^{32}P-labeled DNA probe specific for the cDNA (Devanas and Stotzky, 1986). Nevertheless, HB101(C357) appeared to be less able to cope with conditions of starvation and competition in nonsterile soil, as the addition of LB on day 16 to soil that received the inoculum in saline did not elicit growth similar to that of HB101(pBR322). Although E. coli HB101 is used extensively for cloning, it is a poor choice for studies in soil, as it is auxotrophic and lactose-negative. Hence, an important phenotypic marker (i.e., pigment production) is not available, and it is difficult to distinguish E. coli HB101 from gram-negative indigenous soil bacteria, which are usually also lactose-negative, on MAC.

The survival of P. fluorescens, resistant to kanamycin (Km) and Sm, and B. subtilis, resistant to Tc, in two soils of different texture and planted with wheat was

studied in situ (van Elsas et al., 1986). During 120 days, P. fluorescens decreased more slowly in the silt loam soil (a reduction of approximately 2 orders of magnitude) than in the coarser loamy sand (a reduction of approximately 5 orders of magnitude), whereas B. subtilis declined rapidly in both soils within the first 20 days and then stabilized, with the population being present primarily as spores. These results were similar in both rhizosphere and root-free soil. Although this is one of the few studies with engineered bacteria that has been conducted in soil in situ, it should not be concluded that the survival of engineered gram-negative bacteria is greater than that of gram-positive bacteria in soil, as the bacteria were altered by two very different mechanisms (i.e., P. fluorescens by Tn5 mutagenesis of the chromosome and B. subtilis by protoplast transformation with plasmid pFT30).

In a current in situ study, the survival of root-colonizing Pseudomonas aureofaciens 3732RNL11, that contains the lacZY genes from E. coli K12 (encoding β-galactosidase and β-galactoside permease, respectively) and genes conferring resistance to nalidixic acid (Nx) and rifampicin (Rf) on the chromosome (Drahos et al., 1986), in the rhizosphere is being investigated (E.L. Kline, H. Skipper, and E.J. Brandt, personal communication). The genetically engineered bacterium, in aqueous suspension, was applied to seeds of winter wheat planted at a depth of approximately 2.4 to 3.8 cm, and its survival was monitored by periodically plating dilutions of rhizosphere soil on a minimal medium containing lactose, Nx, and Rf, checking for fluorescence under ultraviolet light, and enumerating colonies that showed β-galactosidase activity (i.e., they turned blue when they cleaved the X-gal reagent, 5-bromo-4-chloro-3-indolyl-β-D-galactoside). The level of detection is approximately 1 to 10 CFU/g soil or root. The numbers of bacteria decreased (after colonization of the emerging roots) from approximately 10^6 to 10^2 CFU/g root during the 31-week growth period (seeds were planted in November, and the crop was harvested in June). The survival kinetics of the host strain that did not contain the lacZY genes were

similar to those of the recombinant bacterium. After harvest of the wheat crop, the levels of the bacteria fluctuated between 10^2 CFU/g root and undetectable levels on soybeans that were subsequently planted in the same site without tillage. Survival of the bacteria was not detected in root-free soil. No apparent transfer of the lacZY genes to indigenous fluorescent pseudomonads occurred, as determined by dot blot hybridization of a ^{32}P-labeled DNA probe (a 502-base pair nucleotide sequence of the Tn7 used to insert the genes) with DNA extracted from more than 5,000 fluorescent bacteria isolated from the rhizosphere (third wash of roots) and nonrhizosphere soil.

The survival of engineered genes, introduced in GEMs, in soil and other natural habitats may be increased by their transfer to indigenous bacteria that are more fit for survival in these habitats (Stotzky, 1989; Stotzky and Babich, 1986).

Conjugation in situ

Transfer of chromosomal genes. Conjugal transfer of chromosomal genes from prototrophic to auxotrophic strains of E. coli occurred in both sterile (Weinberg and Stotzky, 1972) and nonsterile soils (Krasovsky and Stotzky, 1987; Stotzky and Krasovsky, 1981) inoculated with these strains. The frequency of transfer was significantly higher in sterile than in nonsterile soils, indicating that the presence of the indigenous microbiota interfered with conjugal transfer.

The physicochemical characteristics of the soil affected the frequency of transfer of chromosomal genes. For example, the addition of the clay mineral, montmorillonite, with a high cation-exchange capacity and surface area, enhanced the frequency, whereas the addition of the clay mineral, kaolinite, with a low cation-exchange capacity and surface area, had no effect. The enhancement by montmorillonite was apparently the result of the increased growth of the donors and recipients in the microhabitats, which probably resulted in increased conjugation because of the larger number of donors and recipients. Montmorillonite has been shown to increase the growth and metabolic activity of bacteria, primarily by maintaining the pH of microhabitats at levels

conducive to sustained growth (see Stotzky, 1974, 1986). The effect of pH on the transfer of chromosomal genes was further demonstrated in studies that showed that both the survival of the donors and recipients and the frequency of gene transfer increased as the pH of the soil was raised to neutrality (Krasovsky and Stotzky, 1987; Weinberg and Stotzky, 1972).

Transfer of plasmid genes. Numerous studies have demonstrated the transfer of plasmids from bacteria isolated from soils and waters to recipient strains of the same and different species in the laboratory (see Stotzky and Babich, 1986). However, few studies have demonstrated such transfer in soil and other natural environments. For example, when a strain of *Enterobacter cloacae*, isolated from the rhizosphere of *Festuca heterophylla* and that did not fix nitrogen and was sensitive to antibiotics, was mated, *in vitro*, with *E. coli* containing the plasmid, pRD1, which carried genes for nitrogen fixation and resistance to three antibiotics, the exconjugants were able to fix nitrogen and were resistant to the antibiotics. However, many of the exconjugants contained plasmids that were smaller than pRD1 or contained no plasmids, indicating that all or part of the pRD1 had become incorporated into the chromosome of *E. cloacae* (Kleeberger and Klingmüller, 1980). When *E. coli* JC5466 containing the broad-host-range plasmid, pRD1, was added to nonsterile soil, the frequency of transfer of the plasmid to indigenous bacteria was approximately 1×10^{-9} exconjugants/recipient, whereas the *in vitro* frequency of transfer to mixed bacterial populations isolated from soil ranged from 7.1×10^{-8} to 4.5×10^{-6} (Schilf and Klingmüller, 1983). The survival of *E. coli* strains J5(RP4) and JC5466(pRD1), Enterobacteriaceae strain 1(pRD1), Enterobacteriaceae strain 2(RP4), and *P. fluorescens* (pRD1) (the latter three being exconjugants of soil isolates obtained by *in vitro* matings) was also reduced in nonsterile soil, at either 4 or 20 C with a soil water content of 16% or at 20 C with a water content of 4%, presumably as the result of either a loss of the plasmids or a selective decrease in the host-plasmid systems when they were exposed to nonselective conditions in soil. In contrast, the survival of a

chromosomal transconjugant that resulted from conjugation in soil of E. coli strains χ493 (Hfr, prototrophic) and χ696 (F^-, auxotrophic) was greater in nonsterile soil, when reinoculated after isolation from soil and growth on laboratory medium, than that of the parentals (Krasovsky and Stotzky, 1987).

Pertsova et al. (1984) reported the transfer in nonsterile soil of plasmid-borne genes that coded for the degradation of 3-chlorobenzoate from P. aeruginosa and P. putida to indigenous strains of Pseudomonas that were taxonomically different from the donors. Transfer of antibiotic-resistance plasmids among strains of Klebsiella in a sandy loam soil sown with radish seeds (the seeds were inoculated with plasmidless strains and the soil with strains containing the resistance plasmids) was detected on about 24% of the radish seedlings after one week, but not thereafter, at frequencies of 10^{-7} to 10^{-6}, whereas in an aqueous suspension of redwood sawdust, transfer was detected in about 30% of the donor-recipient combinations at frequencies of 10^{-8} to 10^{-5}, and in broth, transfer was detected in about 60% of the combinations at frequencies of 10^{-6} to 10^{-3} (Talbot et al., 1980).

Plasmid transfer also appears to occur among gram-positive bacteria in soil, although this has not been studied as extensively as with gram-negative bacteria. Both conjugative and nonconjugative plasmids were transferred (the latter by triparental matings) between strains of Streptomyces in sterile soil, although less frequently than on agar (Rafii and Crawford, 1988). The transfer of a 2.8-Mda plasmid, pFT30, carrying a Tc-resistance gene, from B. cereus to B. subtilis occurred in sterile soil at a frequency of 7×10^{-8} (van Elsas et al., 1987). Transfer was increased to a frequency of 1.6×10^{-6} by the addition of nutrients and bentonite (montmorillonite), but it was lower at 15 C and a WHC of approximately 20% than at 27 C and a WHC of approximately 60%. Detectable transfer of plasmid pFT30 (frequency of 9×10^{-8}) occurred in nonsterile soil only when it was amended with bentonite, and the clay also enhanced the survival of the recipients. Although not stated, the transfer of this 2.8-Mda plasmid was probably facilitated by the

presence of a 29.5-Mda plasmid in the donor. Bacilli isolated from soil samples collected in hog and cattle feedlots throughout the United States contained plasmids that showed a high degree of homology with plasmids from clinical isolates of Staphylococcus aureus, suggesting the occurrence of natural intergeneric plasmid transfer (Polak and Novick, 1982).

The transfer of nonconjugative plasmids by mobilization, either directly or by cointegrate formation, by conjugative plasmids has been studied extensively in pure culture, but there have been few studies on the frequency of such transfer in soil and other environments. Transfer of nonconjugative plasmids by triparental mating has been demonstrated in E. coli in a laboratory simulation of a sewage treatment plant (Gealt et al., 1985; Mancini et al., 1987; McPherson and Gealt, 1986) and in S. lividans in sterile soil (Rafii and Crawford, 1988). Nonconjugative plasmids, as a result of their smaller size and, hence, lower "burden" on the host, especially if present in low copy number, may be retained longer than larger conjugative plasmids in bacteria in soil, and GEMs that will be released to soil will undoubtedly carry their heterologous DNA on nonconjugative plasmids, if not on the chromosome. Consequently, extensive studies should be conducted on the mechanisms and frequency of transfer of nonconjugative plasmids in soil.

In sterile soil, the conjugative plasmids, pDU202 (64 Mda) and pRR226 (26 Mda), which were derepressed for transfer, were transferred between strains of E. coli (Fig. 1) only when nutrients (LB) were added, and the donors and recipients increased to high numbers, and when the soil had an optimum water content (i.e., -33 kPa water tension; no detectable transfer occurred and survival of the donors and recipients was reduced at lower water tensions) (Devanas and Stotzky, 1988a). Similar results were obtained in sterile soil with E. coli containing a 60-Mda plasmid (Trevors, 1987a; Trevors and Oddie, 1986). However, in nonsterile soil at the optimum water tension, no transfer of plasmids pDU202 and pRR226 was detected between the same strains of E. coli, even when

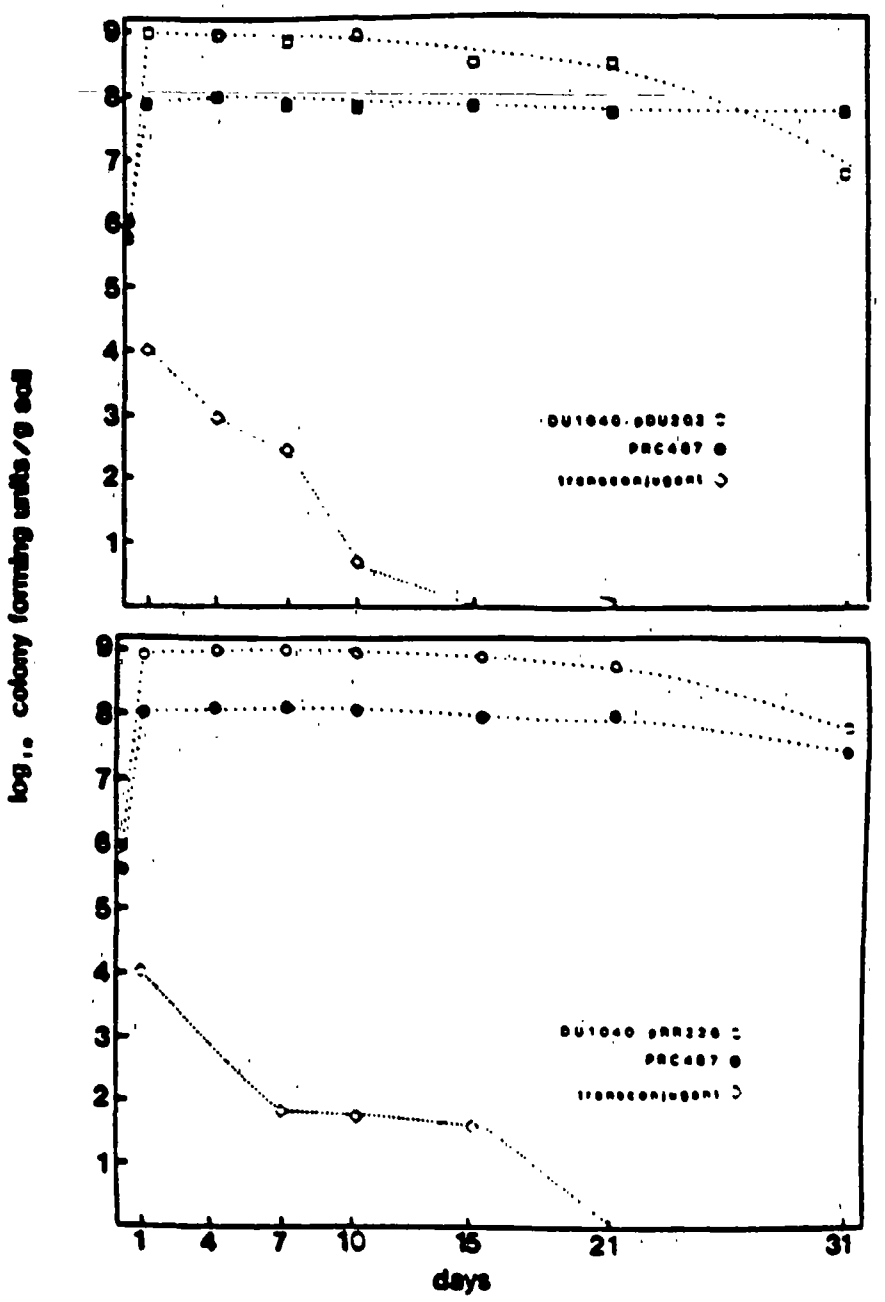

Figure 1. Survival and transfer of plasmids pDU202 and pRR226 from Escherichia coli DU1040 to E. coli PRC487 in sterile soil. Cells were inoculated in Luria Broth (LB). Total numbers of bacteria were enumerated on MacConkey Agar (MAC): strain PRC487 was lactose-positive strain and strain DU1040 was lactose-negative. Cells containing plasmid pDU202 were enumerated on MAC containing 25 µg/ml chloramphenicol (Cm) and those containing plasmid pRR226 on MAC containing 25 µg/ml tetracycline (Tc) (Devanas and Stotzky, 1988a).

nutrients were added initially and during the incubation, which resulted in the growth of the donors and recipients (Fig. 2) (Devanas and Stotzky, 1988a). The soil microbiota apparently interfered in some manner (probably biochemically and not physically) with the transfer of the plasmids. In contrast, Trevors (1987b) reported that a 60-Mda plasmid was transferred between strains of E. coli in nonsterile soil at 22 C and at 20 or 80% of the WHC, albeit at low frequencies, when the donor to recipient ratios were 1: 1 (frequencies of 5×10^{-9} and 3.2×10^{-8} at 20 and 80% of the WHC, respectively) or 1: 10 (5×10^{-9} at 80% of the WHC), but not when they were 1: 100 (at 80% of the WHC).

However, the broad host-range plasmid, RP4, was transferred from E. coli to not only another strain of E. coli and related Enterobacteriaceae (e.g., Enterobacter aerogenes, Klebsiella pneumoniae, both autochthonous soil bacteria, and Proteus vulgaris (Fig. 3)), but also to P. aeruginosa in both sterile and nonsterile soils (Figs. 4 and 5) (Devanas and Stotzky, 1988a, 1988b). In sterile soil, there was growth of the donor and recipients during the first few days after inoculation, especially when inoculated in LB, and then the numbers remained essentially constant. Exconjugants were usually detected 2 h after inoculation (the first time measured) and throughout the incubation at levels ranging from 10^4 to 10^6 CFU/g soil (oven-dry basis).

In nonsterile soil, the numbers of some recipients increased during the first few days after inoculation and then either remained relatively constant or decreased by 1 to 2 orders of magnitude to 10^5 to 10^7 CFU/g soil, depending on the recipient (Figs. 3 and 5). In contrast, the numbers of the donor decreased, sometimes after an initial increase, to either undetectable (less than 10^2 CFU/g soil) or low (e.g., 10^4 CFU/g soil) levels. The numbers of exconjugants detected generally depended on whether the donor and recipients were inoculated in saline or with nutrients (LB), on the type and concentration of clay minerals with which the soil was amended, and, in part, on the species of the recipient and the survival of the donor. For example, the numbers of exconjugants of the Enterobacteriaceae remained at about 10^4 CFU/g soil throughout a 21- to 28-day

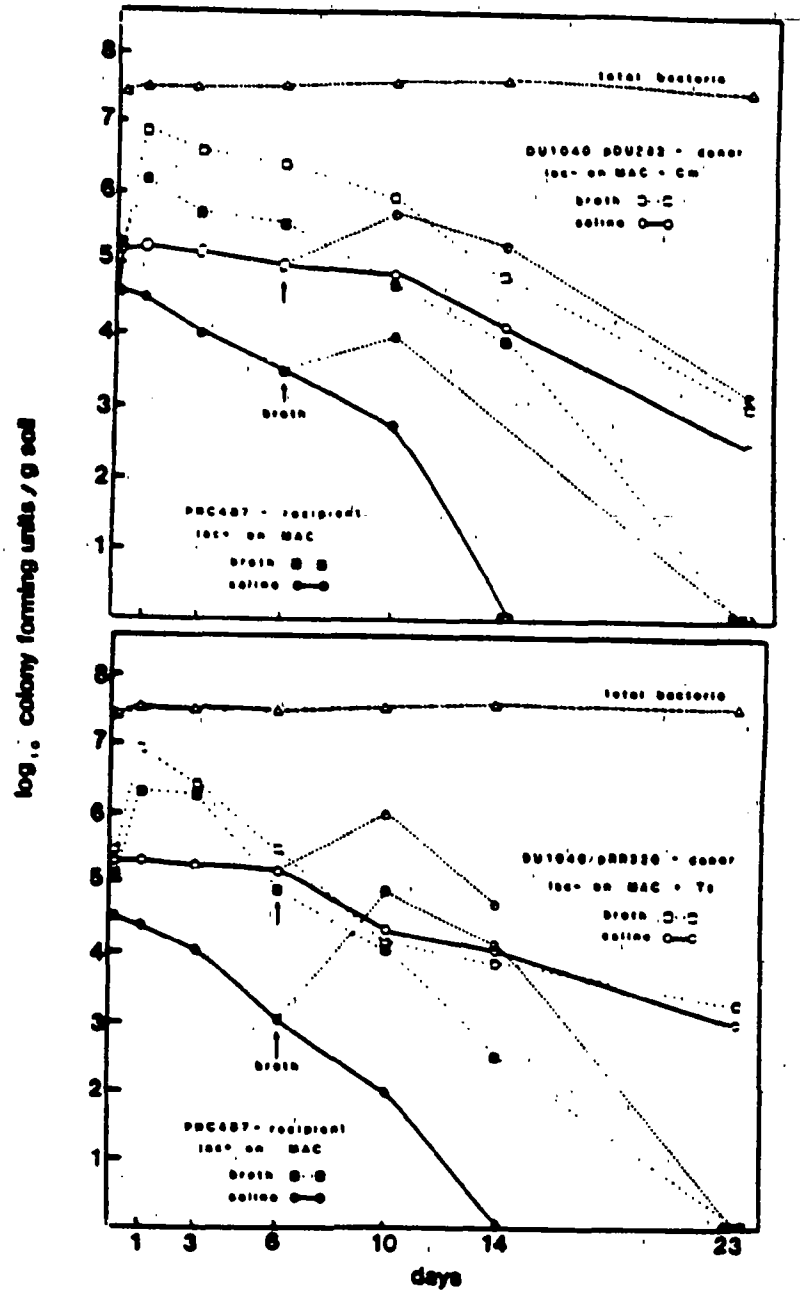

Figure 2. Survival and transfer of plasmids pDU202 and pRR226 from Escherichia coli DU1040 to E. coli PRC487 in nonsterile soil. Cells were inoculated in LB or saline. Total bacteria were enumerated on MAC, bacteria containing pDU202 on MAC containing Cm, and bacteria containing pRR226 on MAC containing Tc (see Figure 1 for details) (Devanas and Stotzky, 1988a).

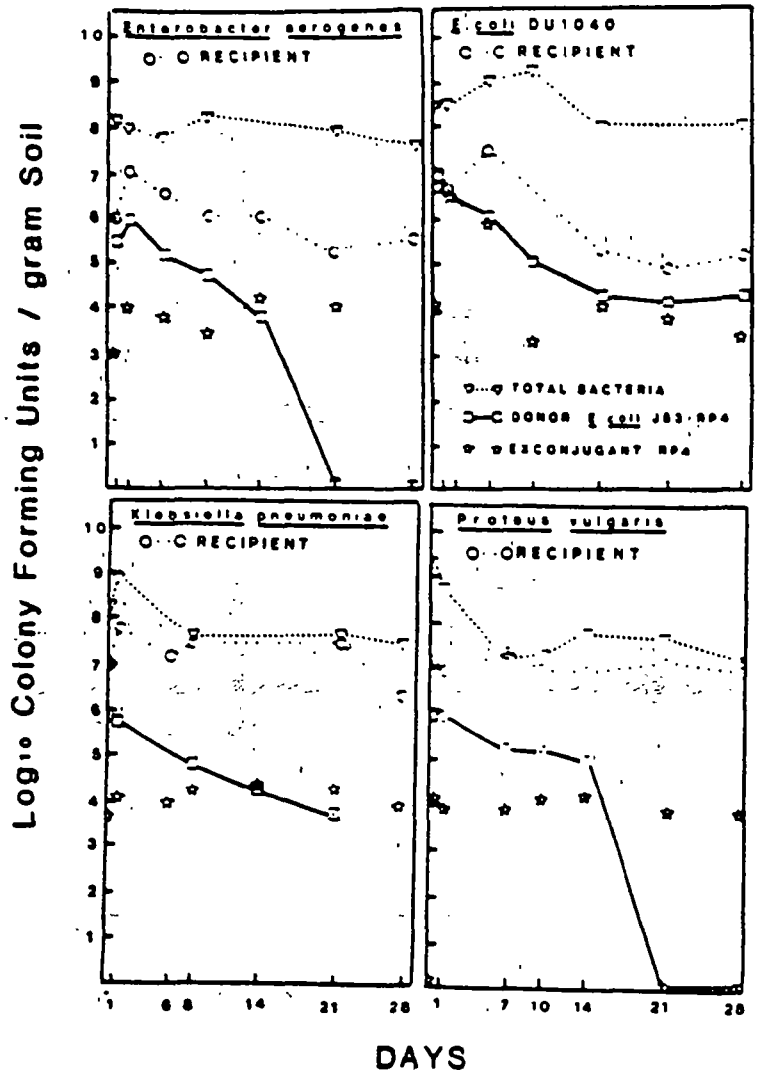

Figure 3. Survival and transfer of plasmid RP4 from Escherichia coli J53 to Enterobacter aerogenes, E. coli DU1040, Proteus vulgaris, and Klebsiella pneumoniae in nonsterile soil. Cells were inoculated in saline. Total bacteria were enumerated on Luria Agar (LA); E. coli J53(RP4) on MacConkey Agar containing tetracycline (Tc); E. aerogenes, E. coli DU1040, P. vulgaris, and K. pneumoniae on LA containing streptomycin (Sm); and E. aerogenes, E. coli DU1040, P. vulgaris, and K. pneumoniae carrying RP4 on LA containing Sm and Tc (Devanas and Stotzky, 1988a).

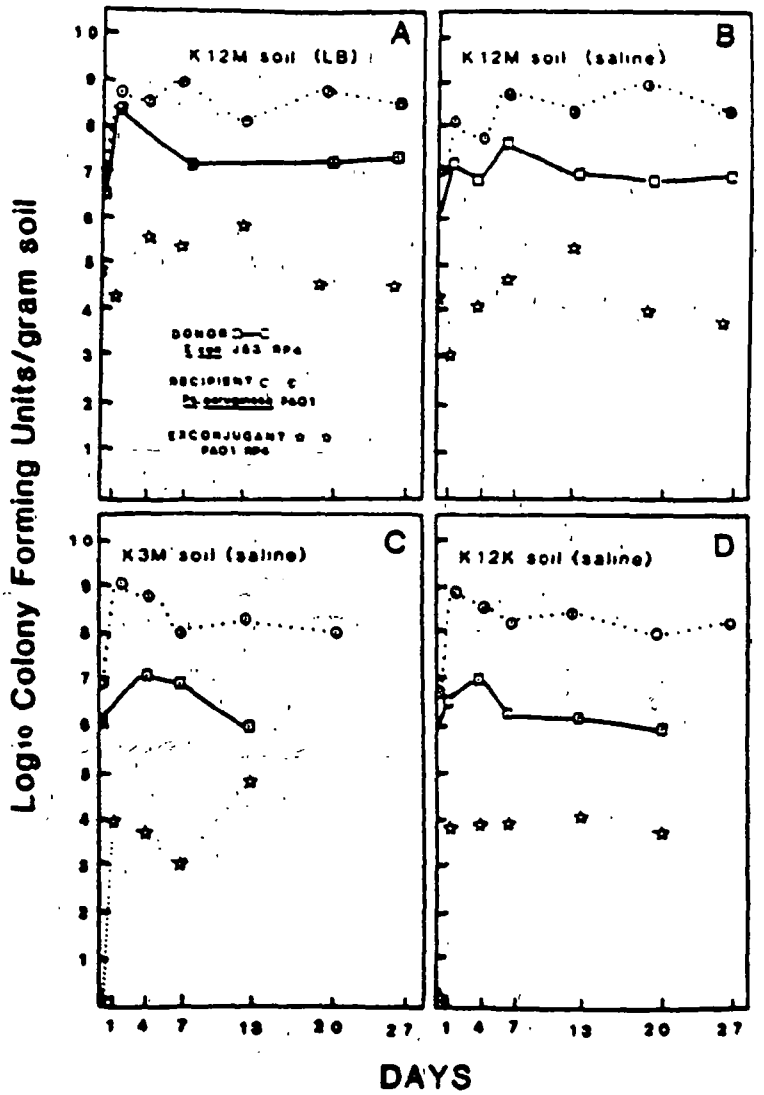

Figure 4. Effect of clay minerals on survival and transfer of plasmid RP4 from Escherichia coli J53 to Pseudomonas aeruginosa PAO1 in sterile soil. Soil (Kitchawan, K) was amended with 3 or 12% (v/v) montmorillonite (M) or 12% (v/v) kaolinite (K). Cells were inoculated in saline or Luria Broth (LB). Total bacteria were enumerated on Luria Agar, J53(RP4) on MacConkey Agar containing tetracycline (Tc), PAO1 on Pseudomonas Isolation Agar (PIA), and PAO1(RP4) on PIA containing Tc (Devanas and Stotzky, 1988a).

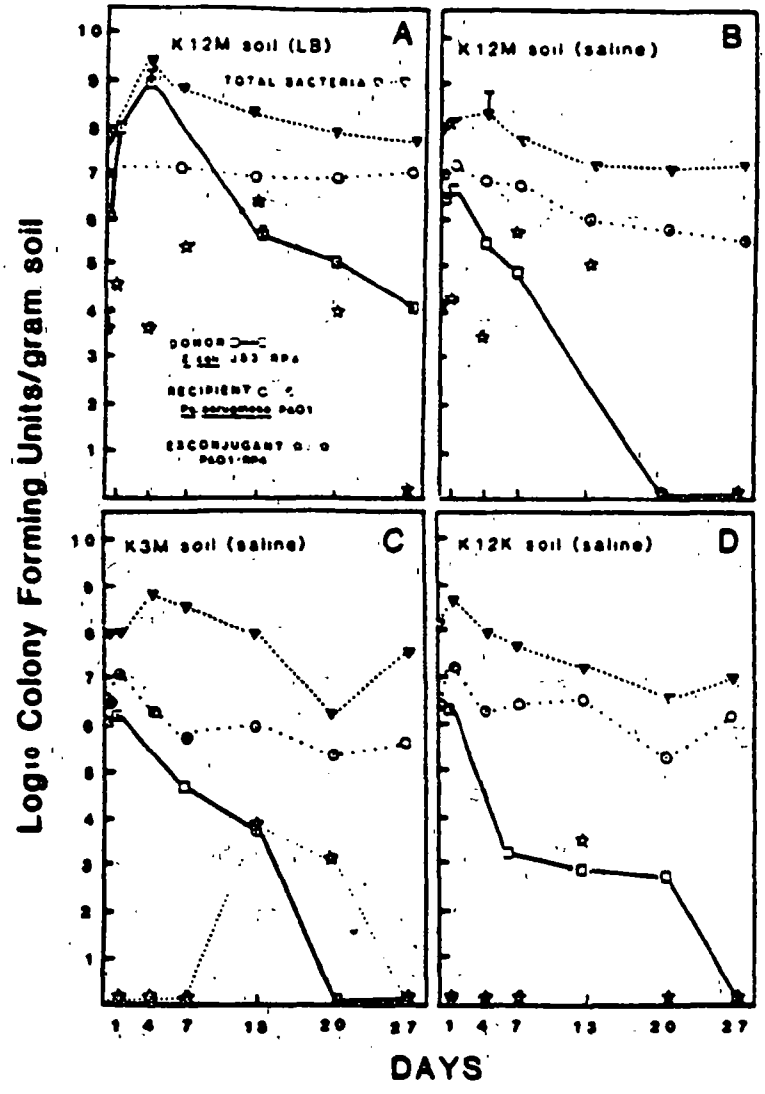

Figure 5. Effect of clay minerals on survival and transfer of plasmid RP4 from Escherichia coli J53 to Pseudomonas aeruginosa PAO1 in nonsterile soil (see Figure 4 for details) (Devanas and Stotzky, 1988a).

incubation, even though the donor and recipients were inoculated in saline and the numbers of the donor decreased by several orders of magnitude or, with E. aerogenes and P. vulgaris as the recipients, to undetectable levels (Fig. 3). In contrast, when P. aeruginosa, as the recipient, was inoculated in saline, its numbers decreased slowly by about 1 order of magnitude, but the numbers of the E. coli donor, also inoculated in saline, decreased to an undetectable level after 20 to 27 days (Fig. 5). The numbers of exconjugants (P. aeruginosa(RP4)) detected differed with the type and amount of clay present in the soil, but in all soils, the numbers of exconjugants decreased to undetectable levels after 20 to 27 days, similar to the decrease in the donor. When the donor and recipients were added in LB, the numbers of recipients remained essentially constant, total bacteria increased initially and then slowly decreased, the donor increased by about 3 orders of magnitude during the first few days after inoculation and then decreased rapidly by about 5 orders of magnitude, and the exconjugants, which were initially detected 2 h after inoculation of the parentals, increased to approximately 5 x 10^6 CFU/g soil, followed by a rapid decrease to undetectable levels (Fig. 5).

Conclusions

The results of these studies again emphasize the importance of the type of bacteria and plasmids involved and of the physicochemical characteristics of the recipient environment to the survival of, and genetic transfer by, GEMs. For example, plasmids pDU202 and pRR226 were not transferred even between strains of E. coli in nonsterile soil, whereas the broad host-range plasmid, RP4, was transferred both intra- and intergenerically in nonsterile soil. P. aeruginosa survived better than E. coli in nonsterile but not in sterile soil, reflecting the relative competitiveness of these species, the former being an autochthonous soil inhabitant. Similar survival data for these species in soil (Klein and Casida, 1967; Zechman and Casida, 1982) and in soil extracts (Walter et al., 1987) have been reported. The presence of nutrients generally enhanced survival and gene transfer in nonsterile soil but had essentially no effect in sterile soil,

and suboptimal water tensions inhibited transfer. Montmorillonite, especially at high concentrations, apparently enhanced the frequency of transfer of both plasmid- and chromosomal-borne genes.

It is not known whether the fluctuating survival patterns of the recombinant bacteria reflected continuous conjugation or the growth and survival of recombinants formed early in the incubation, as the recombinants were not inoculated but resulted from gene transfer in soil. Consequently, the duration of gene transfer in soil may be more apparent than real, as it is not possible to distinguish between continual gene transfer and cell division of recombinant cells formed shortly after introduction of the parentals. Nevertheless, the detection of exconjugants at levels ranging from 10^4 to 10^6 CFU/g soil for at least 20 days after inoculation of the parentals indicated that the novel genes survived. Therefore, there is a possibility that, depending on the host-plasmid system involved, the introduction of GEMs into soil and other natural environments could alter the homeostasis of these habitats.

These results also indicate that more research is needed on the transfer of genetic information by conjugation from GEMs and between indigenous bacteria in soil and other natural habitats in situ. Furthermore, the differences in survival and genetic transfer between the various host-vector systems studied indicate that the survival of, and genetic transfer by, GEMs that may be released to soil or other natural environments must, at this state of knowledge, be evaluated on a "case-by-case" basis.

One aspect that is clear from these studies is that the frequency of transfer of genetic material is higher in sterile than in nonsterile soil, indicating that the presence of the indigenous microbiota interferes with transfer. What is not clear is whether this interference is the result of: 1) a reduction in the population densities of donors and recipients caused by competition for nutrients, water, oxygen, space, etc. or by amensalistic, parasitic, and predatory activities of the indigenous microbiota; 2) the production of molecules by the indigenous microbiota that interfere with transfer (e.g.,

blocking or alteration of receptor sites on the recipients); or 3) other mechanisms. The first possibility is probably not a major factor, as the frequency of transfer in nonsterile soil was reduced even when the population densities of the donors and recipients were high enough to result in transfer in sterile soil (e.g., Figs. 1 and 4). The second possibility is suggested by observations made in studies on the in vivo frequency of transfer of a multiple drug-resistance plasmid from Salmonella typhosa to E. coli in the bladder of healthy rabbits (Richter et al., 1973; Stotzky and Krasovsky, 1981). The frequency was reduced by greater than 1 order of magnitude when Proteus mirabilis and a nonconjugative strain of E. coli (exogens) were also introduced into the bladder. When polystyrene latex particles of approximately the same size (0.8 μm diameter) and at the same concentration as the exogens were introduced into the bladder, the frequency of transfer of the plasmid from S. typhosa to E. coli was not reduced, indicating that the reduction caused by the exogens was not the result of a steric interference but of a chemical interference in the conjugal process. Similar results were observed when these studies were conducted in vitro in urine removed from the bladder or in a liquid mating medium. Whether such interference occurs in soil is not known and requires study. However, the apparent inability of narrow host-range plasmids (e.g., pDU202 and pRR226) to be transferred in nonsterile soil while they were transferred in sterile soil, whereas broad host-range plasmids (e.g., RP4) were transferred in both nonsterile and sterile soil, suggests that the transfer of some plasmids may be more susceptible to chemical interference than the transfer of others.

It is also not known whether the enhancement of the transfer of genetic material by the addition of nutrients (e.g., better transfer when the parentals were added in LB than in saline) was related to this interference. Although the addition of nutrients increased the total numbers of detectable donors and recipients, the numbers initially added, either in LB or saline, were sufficiently high for transfer to have occurred. However, the nutrients may have increased the numbers of parentals in those

microhabitats where transfer was occurring, or the nutrients may have increased the robustness (or reduced the debilitation) of the parentals, which facilitated transfer. This is another aspect that requires study in soil.

The studies on conjugal transfer of chromosomal genes in soil showed that auxotrophic bacteria (in these studies, genetically deficient for the synthesis of some amino acids) can coexist by cross-feeding (syntrophism) in soil and on agar media inoculated directly from soil by the soil replica-plate technique (Stotzky, 1965b) rather than by only genetic recombination (Krasovsky and Stotzky, 1987; Weinberg and Stotzky, 1972). Similarly, cometabolism or "shared detoxification" of inhibitors can contribute to the survival in soil of toxin-sensitive microbes in the absence of genetic recombination (e.g., Daughton and Hsieh, 1977; Senior et al., 1976; Slater and Godwin, 1980; Slater and Somerville, 1979). Furthermore, the transfer of genetic information in soil may be more apparent than real, as the transfer may occur not in soil but on the agar used for the subsequent isolation and enumeration of the donors, recipients, and putative recombinants (Walter et al., 1988a). These aspects, in addition to the "viable but nonculturable" phenomenon, must be considered and controlled in studies on transfer of genetic information in soil.

The apparent higher frequency of conjugal transfer of chromosomal-borne (Krasovsky and Stotzky, 1987) than of plasmid-borne (Devanas and Stotzky, 1988a, 1988b) genes in soil suggests that conjugation may not occur long enough in soil for the transfer of an entire large, conjugative plasmid. This suggestion was supported by preliminary observations that the frequency of transfer of marker chromosomal genes was reduced as their location was more distant from the oriT site. Studies on how plasmid size and the location of chromosomal genes affect the frequency of gene transfer in soil should be conducted, especially as such studies may provide clues for the construction of recombinant DNA that would have a low frequency of transfer.

Although nonconjugative plasmids are used extensively as vectors for the introduction of heterologous DNA into bacteria, the introduced DNA can be moved to the chromosome, e.g., by a Tn, and from there be transferred to another bacterium. Hence, conjugal transfer of chromosomal genes could be an important mechanism for the dispersal of genetic information, e.g., from an introduced GEM to indigenous soil bacteria. However, appropriate genetic manipulation of a chromosome containing heterologous DNA can presumably reduce the probability of transfer in soil (Watrud et al., 1986).

TRANSDUCTION

Introduction

Transduction is another method by which novel genes can be transferred to indigenous soil bacteria after the release of GEMs to the environment. Moreover, bacteriophages may serve as reservoirs of bacterial DNA in soil and other natural habitats, as the packaging of genetic material in a transducing bacteriophage probably represents an evolutionary survival strategy for bacterial genes (Stotzky, 1989; Stotzky and Babich, 1986; Zeph et al., 1988). The survival of a transducing phage that contains the novel DNA could be longer than that of the introduced GEM itself, and these novel genes could reappear long after the GEM can no longer be detected if the phage infects susceptible indigenous bacteria. The probability of the occurrence of this scenario is difficult to predict, as the persistence of, and transduction by, phages in soil and other natural ecosystems has been insufficiently studied.

Two mechanisms of transduction, designated generalized and specialized transduction, have been described in which virions that contain bacterial DNA can be formed after infection of a bacterium by a phage. In generalized transduction, phages, whose DNA is replicated shortly after infection of the bacterial cell (lytic cycle), can package DNA from the bacterium, whose chromosome has been cleaved into small

segments by nucleases, in place of phage DNA during incorporation of DNA into the phage capsid (Lin et al., 1984). Usually, less than 1% of the bacterial genome is packaged into the phage virions. Any single or group of bacterial genes may be incorporated, depending on the size of the phage capsid, and, thus, the designation, generalized transduction. The transducing phages that carry bacterial DNA are capable of adsorbing on and injecting their DNA into new bacterial host cells, but the DNA will be defective for replication of the phages.

In specialized transduction, the bacteriophage genome is integrated into the bacterial chromosome, usually at a specific site, and replicates faithfully with the bacterial chromosome. The integrated phage genome is termed prophage DNA, and the bacteria that contain it are termed lysogenic (Lin et al., 1984). Upon induction of the lytic cycle of phage replication, imprecise excision of the phage genome can result in the packaging of adjoining bacterial genes on the chromosome into the virion with the prophage DNA. The most extensively described specialized transducing phage is phage lambda, which can transduce the genes for galactose catabolism and biotin synthesis that are located on either side of the integration site of this prophage in E. coli. The imprecise excision event occurs at a low frequency, e.g., in about one bacterial cell per 10^6 to 10^7 cells. The ability to transfer only genes that are close to the site of phage integration is termed specialized transduction. A specialized transducing phage may be defective for some of its genes and may require the activity of "helper" phages to replicate in the new host bacterium.

Two types of transducing phages, temperate and lytic, that differ in their mode of replication in the bacterial host, have been described. Temperate bacteriophages are capable of maintaining single or multiple copies of their genome in the host bacterium in a prophage state; the phage DNA is either inserted into the bacterial chromosome through a recombinational event (e.g., coliphage lambda or Mu-1) or remains as an independent and autonomous replicon (e.g., coliphage P1 or phage F116 of P. aeruginosa),

similar to a bacterial plasmid. A temperate phage can initiate a lytic cycle of replication and virion formation (induction), although this occurs at a low frequency under normal conditions (e.g., one in 10^5 to 10^6 DNA replication cycles for phage P1). Exposure of lysogenic bacteria to temperatures above the optimum for growth or to mutagenic agents can result in the induction of lytic reproduction at a higher frequency. Only between 1 to 10% of the infective virions produced after induction *in vitro* will usually establish lysogeny after infection of new host cells, as most infections result in lysis of the cells.

Lytic transducing phages undergo the normal lytic cycle of infection and formation of new virions in the bacterial host, and they are not capable of establishing lysogeny. During replication of the phage DNA, genetic material from the host bacterium, that has escaped degradation by phage-encoded nucleases, can be randomly packaged into intact virions at a low frequency. Hence, lytic transducing phages are all generalized transducing phages. In a typical lysate, about 1% of the virions will contain only bacterial DNA (Stent and Calender, 1978). Consequently, a phage lysate of sufficient titer (i.e., at least 10^6 phages/ml) can be expected to contain at least one virion carrying a copy of each gene on the bacterial chromosome.

Transduction in situ

Gene transfer *in situ* by transduction has been studied to only a limited extent. There have been few studies on transduction in marine or freshwater ecosystems (Baross *et al.*, 1974; Morrison *et al.*, 1978; Saye *et al.*, 1987) and in terrestrial ecosystems (Germida and Khachatourians, 1988; Zeph *et al.*, 1988; Zeph and Stotzky, 1989), even though transducing phages for a variety of bacteria have been isolated from soil. Zeph *et al.* (1988) studied the transduction in soil of bacterial genes that confer resistance to antimicrobial agents by coliphage P1 Cm *cts*, which carries a resistance gene for Cm, and a variant of this phage, P1 Cm *cts*::Tn*501*, which also carries a Hg-resistance gene on the transposon, Tn*501*. A temperature-sensitive mutation (*cts*) results in the

inactivation, at temperatures above the optimum for growth (e.g., at 42 C), of a repressor protein coded for by phage P1 that maintains lysogeny.

Lysates of phage P1 (10^5 to 10^6 plaque-forming units (PFU)/g soil (oven-dry basis)) were added to soil with E. coli (10^5 to 10^6 CFU/g soil), and soil dilutions were periodically plated on selective media to quantitate the numbers of E. coli, phage P1, E. coli transductants (Cm- or Hg-resistant cells lysogenized by phage P1), and total soil bacteria. Significantly higher maximum numbers of E. coli transductants were observed in sterile (approximately 10^8 transductants/g soil) than in nonsterile (approximately 10^6 transductants/g soil) soil (Fig. 6). Amendment of nonsterile soil with nutrients (LB) on day 0 did not significantly affect the numbers or survival of E. coli transductants during the 28-day experiment, even though the numbers of total E. coli increased by 1 order of magnitude in the nutrient-amended soil (Fig. 7). Weekly amendment of the soil with LB also did not result in significant increases in numbers of E. coli, phage P1, or E. coli transductants (Fig. 8) (Zeph and Stotzky, 1988). These results demonstrated that phage P1 was capable of infecting and transducing E. coli under the relatively low nutrient conditions that presumably occur in natural soil. Although the survival of phage P1 was slightly greater in soil amended with the clay mineral, montmorillonite, than with the clay mineral, kaolinite, this improved survival did not result in significantly higher levels of transduction (Fig. 9).

When phage P1 was added to sterile soil in lysogenic E. coli J53(RP4)(P1 Cm cts::Tn501) together with nonlysogenic E. coli W3110 (both at approximately 10^6 CFU/g soil) and nutrients (LB containing 2 mM $CaCl_2$ and 10 mM $MgSO_4.7H_2O$; LCB), phage P1 was released from the lysogenic E. coli and infected E. coli W3110, which resulted in approximately 10^5 transductants/g soil (Fig. 10). In nonsterile soil, using E. coli W3110(R702) as the recipient and E. coli J53(P1 Cm cts) as the lysogen, the number of E. coli W3110(R702)(P1 Cm cts) transductants was significantly lower, as 10^2 transductants/g soil were recovered on day 1 only. The multiplication and survival of the

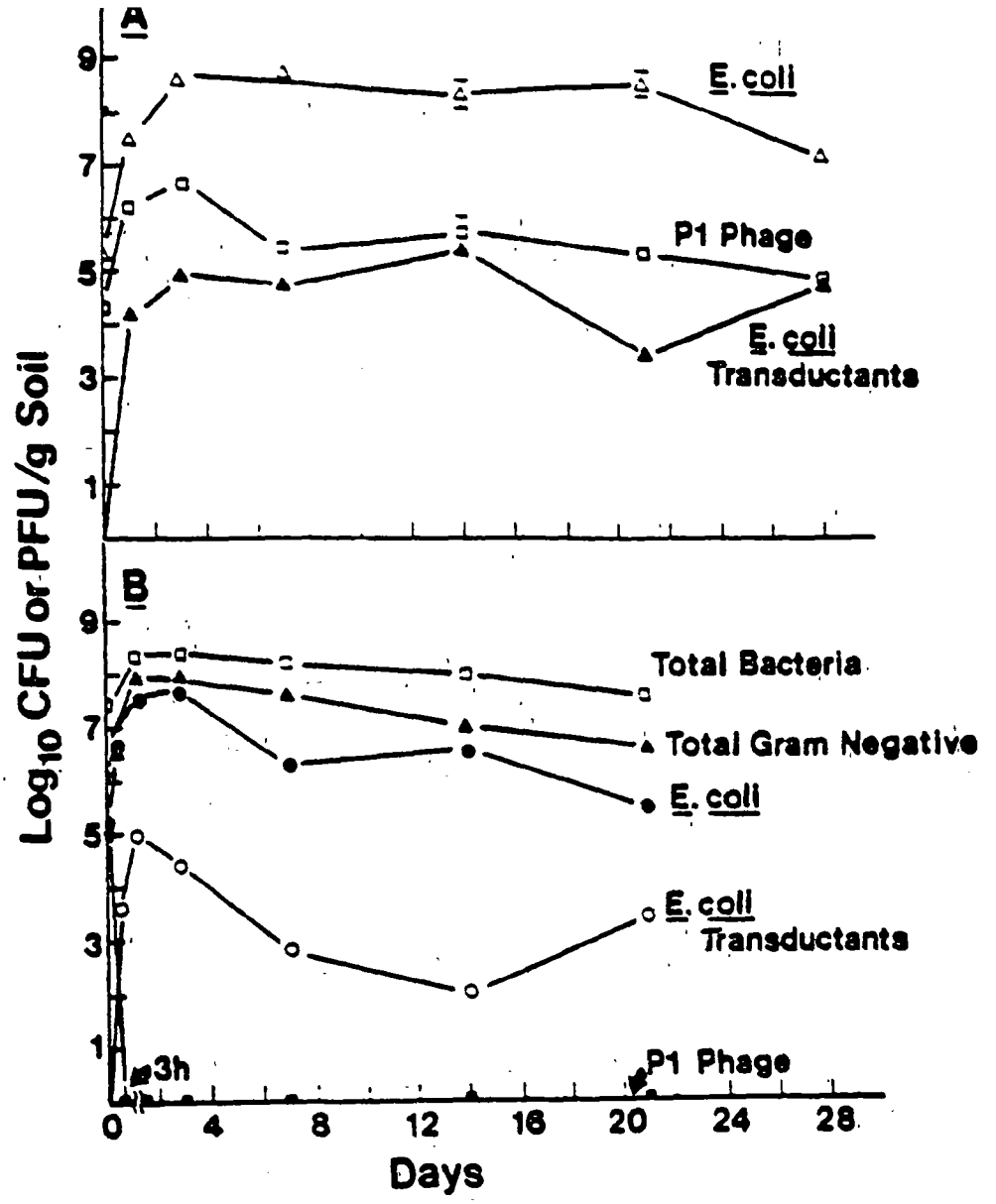

Figure 6. Comparison of transduction of Escherichia coli W3110(R702) by phage P1 CM cts in sterile (A) and nonsterile (B) soil. Bacteria and phage inocula were added in LCB (Luria Broth containing 10 mM $MgSO_4 \cdot 7H_2O$ and 2 mM $CaCl_2$) on day 0. Means ± SEMs, which are shown if larger than the dimensions of the symbols (Zeph et al., 1988).

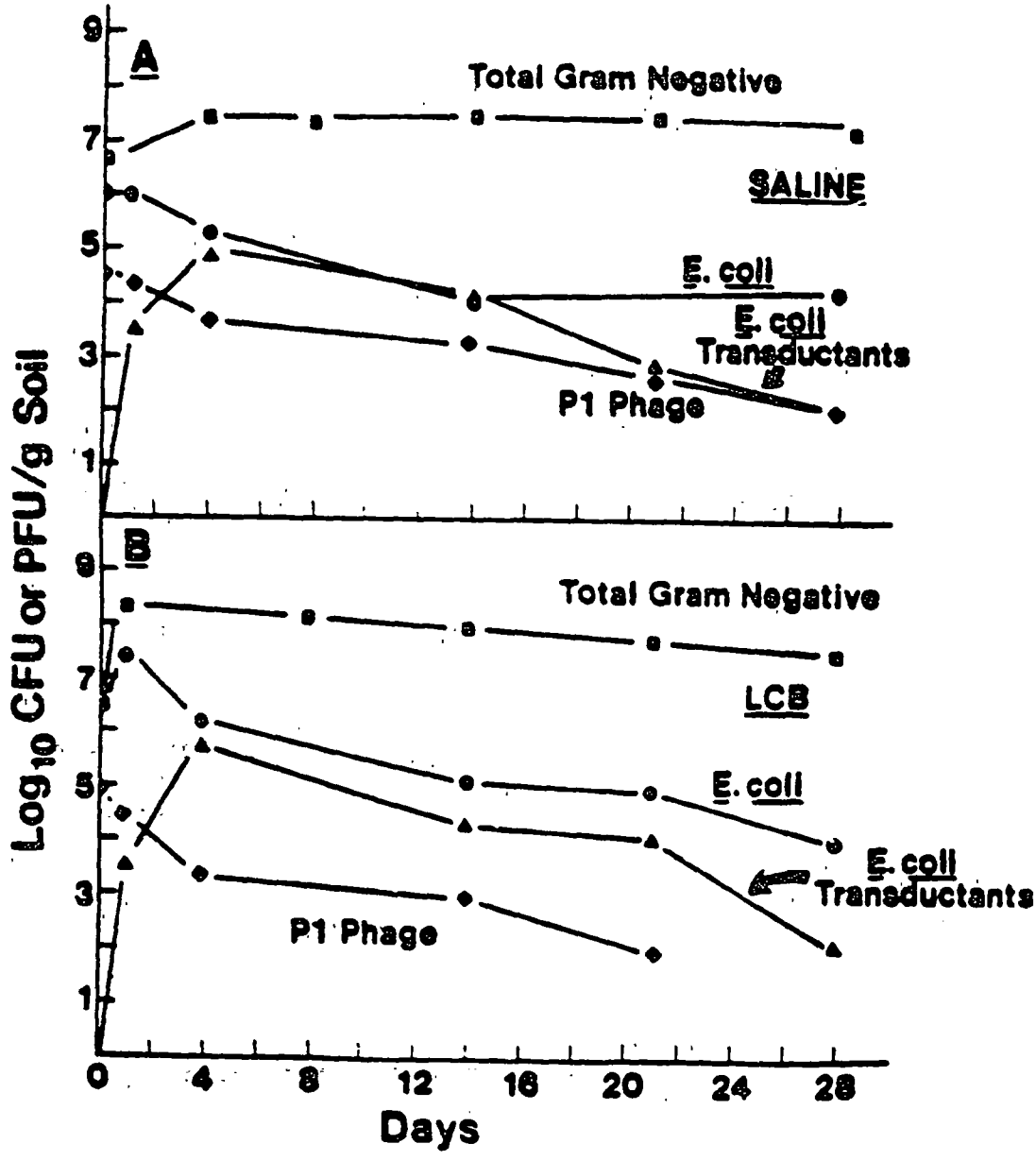

Figure 7. Comparison of the effect of inoculating Escherichia coli J53(RP4) and phage P1 Cm cts::Tn501 in saline (A) or LCB (B) on transduction in nonsterile soil (see Figure 6 for details) (Zeph et al., 1988).

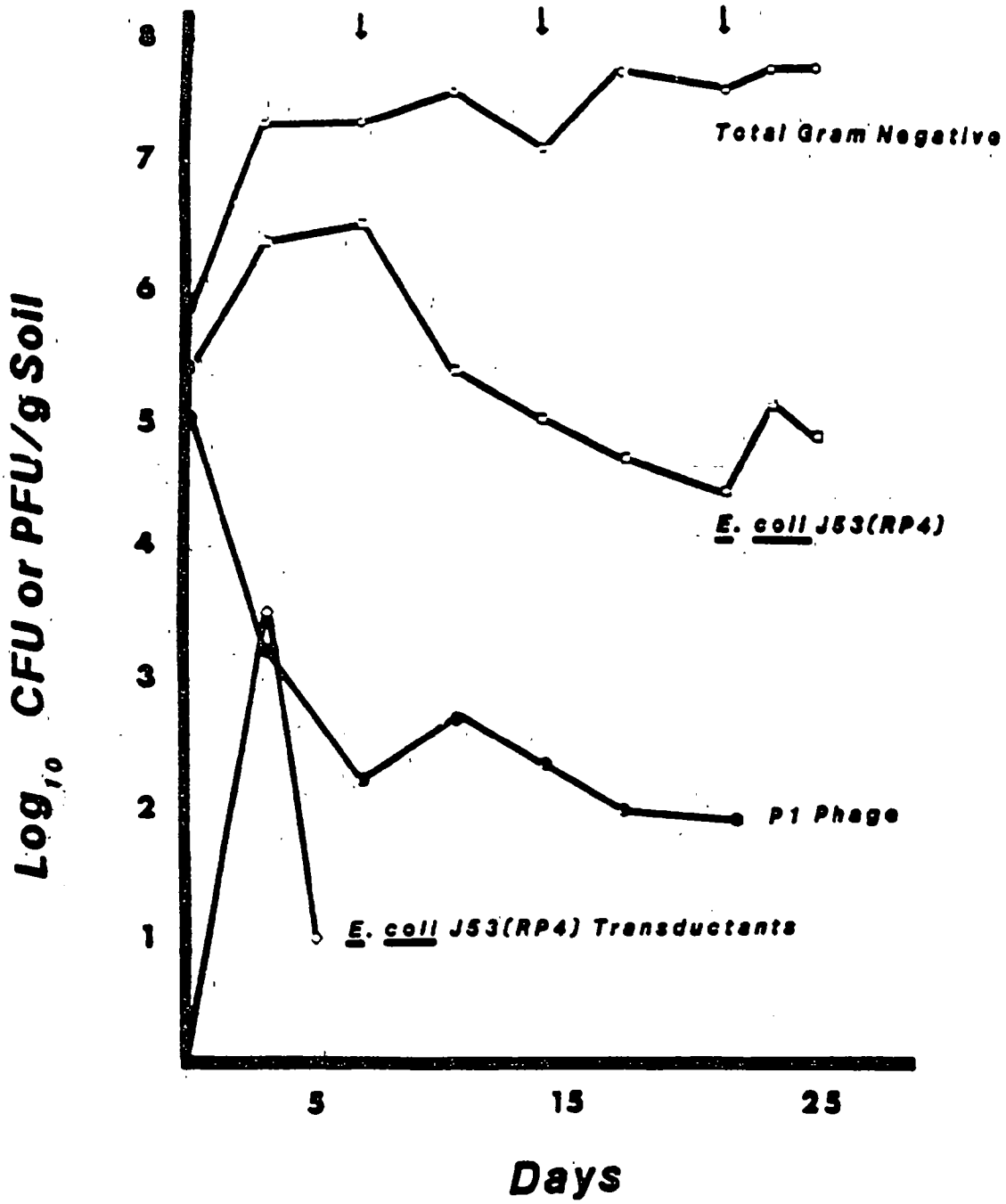

Figure 8. Transduction of Escherichia coli J53(RP4) by P1 Cm cts::Tn501 inoculated in LCB into nonsterile soil. Sterile LCB was added on days 7, 14, and 21, as indicated by the arrows (see Figure 6 for details) (Zeph and Stotzky, 1988).

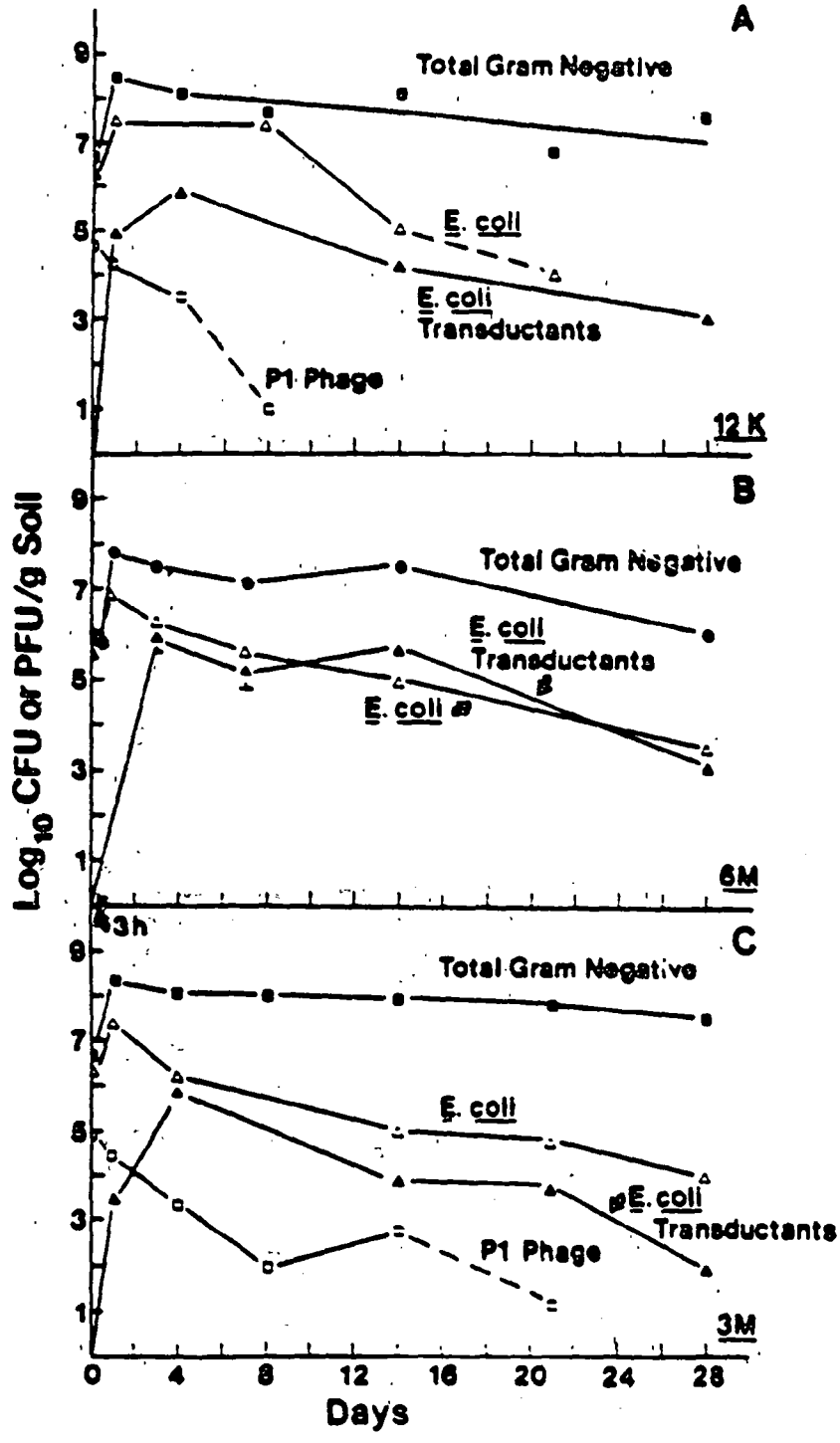

Figure 9. Transduction of Escherichia coli J53(RP4) by phage P1 Cm cts::Tn501 in nonsterile soil amended with (A) 12% kaolinite (12K), (B) 6% montmorillonite (6M), and (C) 3% montmorillonite (3M). Bacteria and phage inocula were added in LCB (see Figure 6 for details) (Zeph et al., 1988).

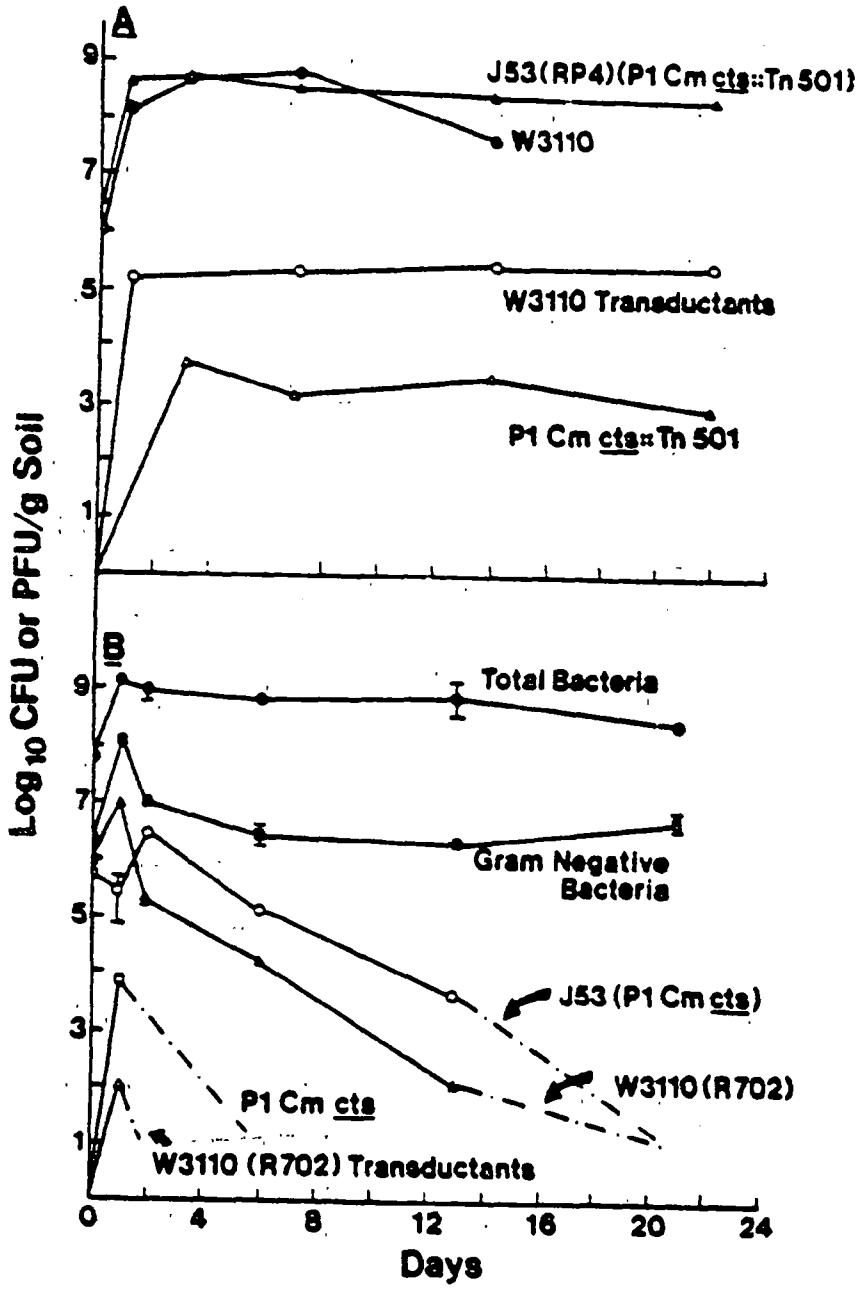

Figure 10. Transduction in sterile (A) and nonsterile (B) soil inoculated with either *Escherichia coli* J53(RP4) lysogenic for phage P1 Cm cts::Tn501 and nonlysogenic *E. coli* W3110 (A) or *E. coli* J53 lysogenic for phage P1 Cm cts and nonlysogenic *E. coli* W3110(R702) (B). Bacterial inocula were added in LCB on day 0 (*see* Figure 6 for details) (Zeph *et al.*, 1988).

lysogenic donor and nonlysogenic recipient E. coli were also significantly lower than in sterile soil (Fig. 10). Despite the lower survival and frequency of transduction in nonsterile than in sterile soil, these studies showed that phage P1 released from one lysogenic E. coli can lysogenize susceptible bacterial cells under conditions similar to those in natural soil.

E. coli transductants isolated from soil were verified by heat-induction of phage P1 and with a biotinylated DNA probe, which demonstrated the presence of phage P1 DNA in the isolates (Zeph and Stotzky, 1989). Approximately 300 isolates of indigenous soil bacteria, isolated on MAC containing 75 μg/ml Cm and inoculated with a 10^{-2} dilution of soil, were negative when subjected to both verification procedures (Table 2), even though the theoretical minimum limit of detection was 10^2 transductants/g soil, and the specificity and sensitivity of the DNA probe was satisfactory. Consequently, few, if any, indigenous bacteria in the soils studied were apparently capable of being transduced by phage P1.

Germida and Khachatourians (1988) studied the generalized transduction by phage P1 of amino acid and Tc-resistance markers on the chromosome of E. coli in a sandy and a silty clay loam soil. The frequency of transduction of these markers to added recipient strains of E. coli was 10^{-6}, which was similar to the frequency obtained in vitro. Thus, conditions in nonsterile soil were apparently not detrimental to transduction by phage P1.

A generalized transducing phage of P. aeruginosa, F116L, has been shown to transfer plasmid RP4 to P. aeruginosa PAO1 in soil (Zeph and Stotzky, 1988). When a lysate of phage F116L (10^7 PFU/g soil), raised in P. aeruginosa PAO1 carrying plasmid RP4, was added to sterile soil with nonlysogenic cells of P. aeruginosa PAO1 (10^8 CFU/g soil), presumed P. aeruginosa transductants resistant to Tc were detected at approximately 10^3 transductants/g soil (Fig. 11). These presumptive transductants were confirmed by the demonstration that their antibiotic-resistant phenotype was the same as the antibiotic-resistance markers present on plasmid RP4 (Km, Ap, and Neomycin

Table 2. Verification by heat induction of lysis and with a biotinylated DNA probe of presumptive transductants of *Escherichia coli* and indigenous soil bacteria isolated on MacConkey Agar containing either $HgCl_2$ (30 μM) or Cm (75 μg/ml).

Presumed transductants	Soil	Heat induction of phage P1[a]	Hybridization with P1 DNA[b]
Indigenous	Nonsterile	0/297	0/297
E. coli W3110(R702)	Sterile	15/15	ND[c]
	Nonsterile	8/8	8/8
E. coli J53(RP4)	Sterile	13/13	ND
	Nonsterile	25/25	7/7

[a] No. of isolates that were positive/no. tested.

[b] No. of colonies or dot blots that were positive/no. tested.

[c] Not determined.

(Zeph and Stotzky, 1989)

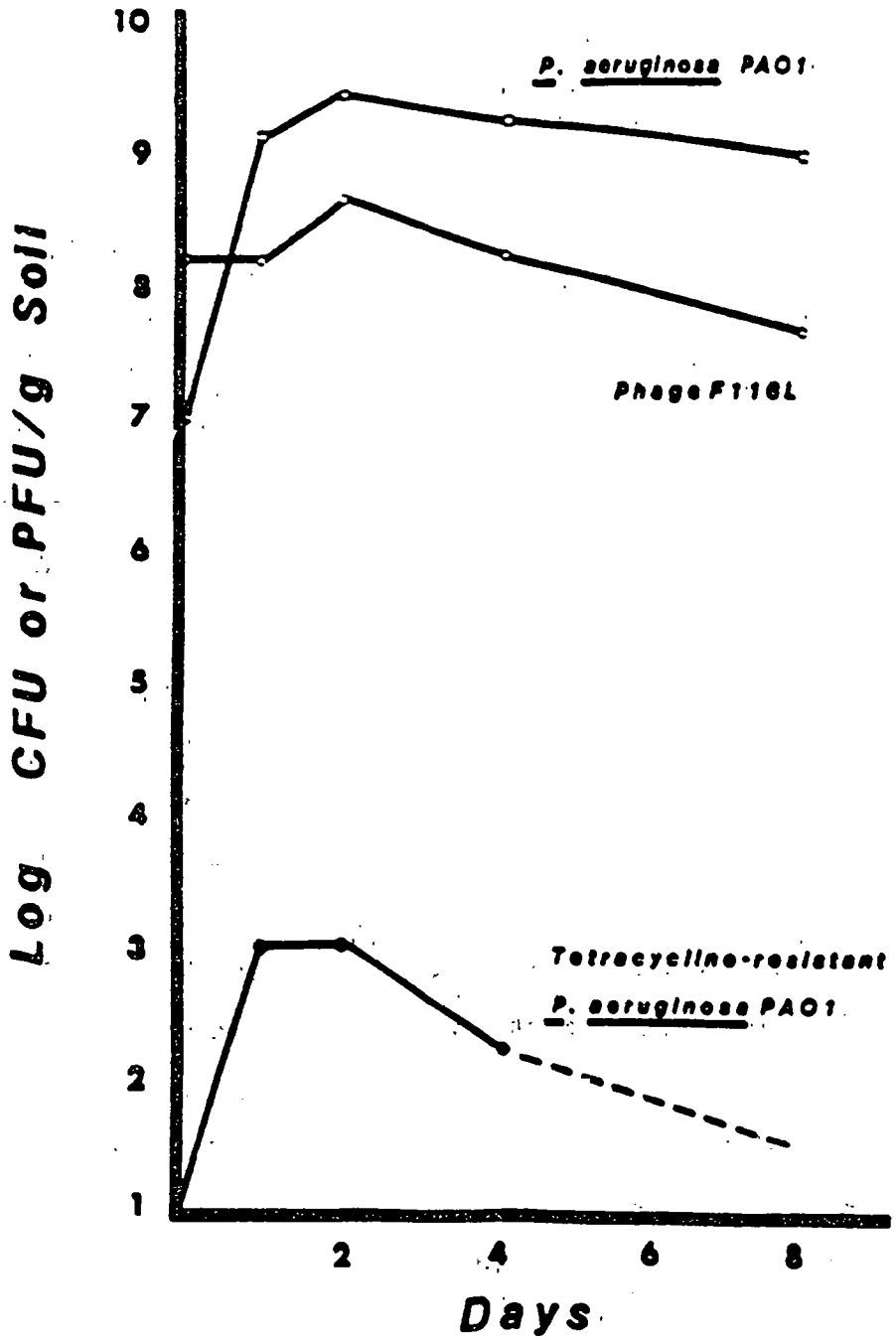

Figure 11. Transduction of Pseudomonas aeruginosa PAO1 by phage F116L in sterile soil. The phage F116L inoculum was raised on P. aeruginosa PAO1 carrying the drug-resistance plasmid, RP4. The bacterium and phage were inoculated in Luria Broth (LB) (Zeph and Stotzky, 1988).

(Nm), in addition to Tc) and by plasmid screening by gel electrophoresis, which showed the presence of plasmid DNA of the same molecular mass as plasmid RP4 (Fig. 12). No RP4 transductants of P. aeruginosa PAT2 or of P. aeruginosa PAT2 lysogenic for phage F116L were detected when these recipient bacteria were added to sterile soil with phage F116L carrying plasmid RP4.

Conclusions

Transduction as a mechanism of genetic transfer may be as, or more, important in soil and other natural habitats as conjugation and transformation. Bacteriophages are capable of multiplying in natural soil, which emphasizes the potential for transducing phages to transfer genes in situ. Furthermore, the survival of viruses, including bacteriophages, in soils and waters is enhanced by their adsorption on clay minerals, especially on montmorillonite, and probably on other particulates. Consequently, DNA in an indigenous bacterium or introduced in a GEM could be incorporated into the genetic material of a phage and could persist in soil longer than in the host bacterium itself. Such persistence would be undetected (i.e., cryptic) in the absence of a bacterial host susceptible to infection and reproduction of the phage. However, if an appropriate host were subsequently infected, the recombinant DNA could be rapidly dispersed among susceptible bacteria; e.g., a single nondefective phage that was able to accommodate the extra DNA and with a burst size of 100 could transfer the recombinant DNA to approximately 10^8 bacteria after only four lytic cycles. Hence, there is the potential for a novel gene to persist undetected and then unexpectedly reappear in soil and other environments if the transfer of the gene occurred by transduction.

Gene transfer to indigenous bacteria by conjugation can theoretically be controlled by engineering the novel genes into the chromosome and through the use of suicide plasmid vectors (Cuskey et al., 1988). However, placing the novel genes in the chromosome will not necessarily decrease the probability of their transfer by transduction, especially as no techniques have been developed for limiting transduction in

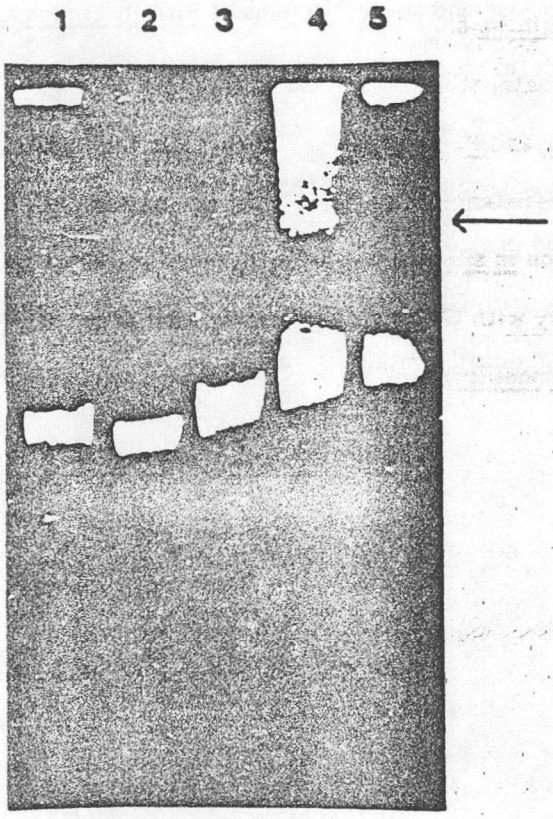

Figure 12. Agarose gel electrophoresis of plasmid and chromosomal DNA extracts of three individual isolates of presumed transductants of Pseudomonas aeruginosa PAO1 isolated from sterile soil that was inoculated with phage F116L and P. aeruginosa PAO1 (see Figure 11). Lanes 1 to 3: P. aeruginosa PAO1 isolates; Lane 4: P. aeruginosa PAO1(RP4); Lane 5: Escherichia coli J53(pDU202); M_r of pDU202 = 64 Mda; M_r of RP4 = 65 Mda (Zeph and Stotzky, unpublished).

situ. Few studies on the frequency of lysogeny in bacteria exist, and it is likely that even these studies underestimate the frequency, as it has been demonstrated that most strains of *P. aeruginosa* and several strains of *P. fluorescens* are lysogenic (Holloway, 1969).

To date, studies on transduction *in situ* have focused on two species of bacteria, i.e., *E. coli* and *P. aeruginosa*, and on a limited number of genetic markers, i.e., genes encoding resistance to antimicrobial agents and amino acid auxotrophy. Studies on transduction *in situ* are needed with more bacterial species and genetic markers, particularly with GEMs that are being used or proposed for environmental releases, e.g., *Rhizobium meliloti* containing recombinant nitrogen-fixation genes.

III. Terrestrial Microcosms

Introduction

Terrestrial microcosms have been defined as analogues of the field, as a field within the laboratory (Pritchard, 1988), or as physical models of the natural environment in which experimental conditions in the laboratory are intended to mimic a field setting (Pritchard and Bourquin, 1984). The extent of such simulations can be as broad as isolating in the laboratory a "piece of the field" that acts ecologically similar to an identical companion "piece" in the actual field (Hicks et al., 1989) or as simple as adding sterile soil to broth in test tubes (Walter et al., 1987). The spectrum of experimental systems that have been called microcosms has resulted in numerous discussions on "what is a microcosm?". Greenberg et al. (1988) stated that "the term microcosm seems to mean all things to all men, ranging, for example, from 'synthetic' to 'natural' systems". The consensus of opinion, regardless of the style or complexity of the microcosm, was that "it is essential that the questions to be asked with the microcosm, and the experimental design, are carefully thought out" and "that those workers who use microcosms should understand the uses, limitations and bias of the particular systems and ensure that people using their results are also aware of these facts". Bull (1980) pointed out that one benefit of the use of microcosms is their replicability in statistical trials; however, they are not exact reproductions of the field but, rather, analytical tools that can be used as the basis for environmental studies.

Simple microcosms

The simplest terrestrial microcosm consists of soil in a container. Sterile soil provides a gnotobiotic environment in which to evaluate GEMs in the absence of the indigenous microbiota. Soil may be amended with nutrients and clay minerals, adjusted to optimum pH values and water contents, and added to test tubes, flasks, or other types of containers. Such simple microcosms have been used to evaluate the survival, growth,

gene transfer, and effects of GEMs in soil in the laborator (Devanas et al., 1986; Doyle et al., 1988; Jones et al., 1988; Krasovsky and Stotzky, 1987; Stotzky, 1965a, 1989; Stotzky and Babich, 1986; Weinberg and Stotzky, 1972) and changes in plasmid frequencies in natural soil microbial populations (Wickham and Atlas, 1988).

Quantities of soil between 2 and 50 g are recommended for simple microcosms, as this facilitates handling of soil samples in the laboratory, enables the initial dilution of the entire soil sample in the same container, and ensures good mixing of the diluted soil sample. Sufficient dilution and mixing of the soil are necessary to prevent aggregation and settling of the soil in pipettes during dilution, which would introduce error and reduce the reproducibility of replicate samples. The soil microcosm used as an example in this document consists of a screw-cap test tube (18 mm x 150 mm), loosely capped and maintained in a high humidity chamber, into which approximately 2 g of soil at the -33 kPa water tension (approximately equivalent to the water content at field capacity) is placed. The container should be large enough to ensure that the surface: volume ratio of the soil sample is sufficiently high to maximize gas exchange between the soil and the head-space of the container (e.g., for large samples of soil, an Erlenmeyer flask is better than a test tube). The selection, preparation, maintenance, and storage of soils is discussed in Section IV.

Master jar studies. The over-all metabolic activity of microbes in soil can be determined with respirometric techniques that monitor either CO_2 evolution or O_2 uptake. These methods, especially when CO_2 evolution is used, probably provide the best and most easily measured index of the gross metabolic activity of mixed microbial populations in soil (see Stotzky, 1960, 1965a, 1974, and Anderson, 1982, for details of the techniques). The "master jar" technique (Stotzky, 1965a) (Fig 13) enables removing subsamples of soil during an extended incubation for various analyses (e.g., transformation of substrates, species diversity, enzyme activities, survival of introduced bacteria, including GEMs and their genes), in addition to continuous measurement of CO_2

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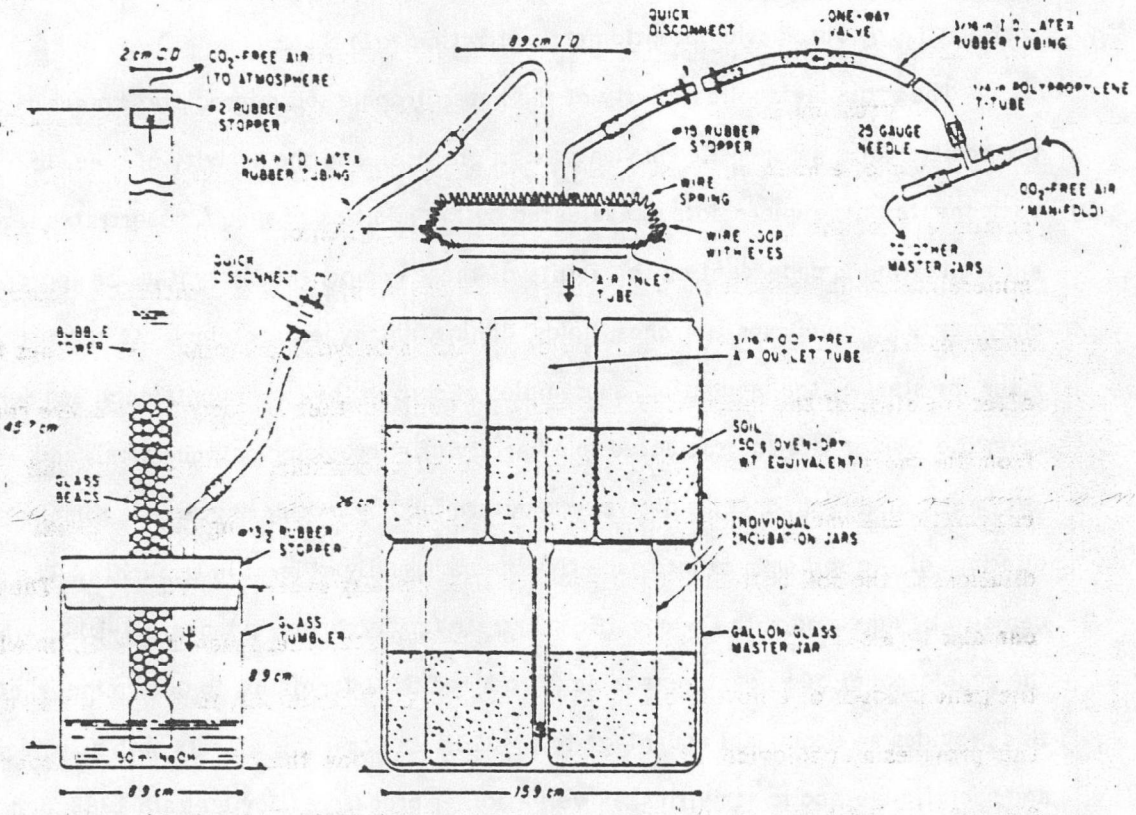

Figure 13. Incubation unit for measuring CO_2 evolution from soil. The unit shown is used when subsamples of soil are removed during incubation. When this is not required, soil is placed directly in the master jar (Stotzky, 1965a).

evolution, without disturbing the remainder of the soil and, thereby, eliminates artifactual peaks in CO_2 evolution that can result from the physical disturbance of the soil. The soils are incubated under controlled temperatures and maintained at their -0.33 kPa water tension by continuous aeration with water-saturated, CO_2-free air. The amount of CO_2 trapped in NaOH collectors is determined, after precipitation of the CO_2 with $BaCl_2$, by automatic potentiometric titration with HCl.

The gross metabolic activity of the heterotrophic soil microbiota is measured by the addition of a nonspecific substrate (e.g., glucose), and the activity of specific components of the microbiota is evaluated by the addition of specific substrates whose mineralization is dependent on the ability of these components to synthesize appropriate enzymes (e.g., celluloses, starches, lipids, lignins, aldehydes, proteins). At various times after the start of the incubation, subsamples of soil, in their own containers, are removed from the master jars and subjected to a variety of microbiological, chemical, and enzymatic analyses. Species diversity is determined by inoculating decade serial dilutions of the soil onto or into selective media, usually at least in triplicate. The soils can also be amended with the specific substrate (e.g., toluene, xylenes, 2,4-D) on which the gene product of a novel gene in an introduced GEM functions, to determine whether this provides an ecological advantage to the GEM and how this affects both nonspecific and specific metabolic activities, as well as other processes (Doyle et al., 1988; Jones et al., 1988).

Soil replica plating. The soil replica plating technique (Krasovsky and Stotzky, 1987; Stotzky, 1965b, 1974; Weinberg and Stotzky, 1972) can be used to determine the growth rates of GEMs and their homologous hosts, the ability of the GEMs to compete with representative soil microbes, and the transfer of genetic information. For example, the GEMs or the hosts are inoculated into the center of petri dishes containing sterile soil, and representatives of the indigenous soil microbiota (bacteria, including actinomycetes, and fungi) are inoculated into equidistant sites around the GEMs. The

plates are incubated in a high-humidity chamber, and replication from the soil plates to selective agars are made periodically with a replicator constructed with stainless steel nails and acrylic plastic and sterilized with alcohol and flaming. The design of the replicator permits numerous replications from the same soil plate without significant disturbance of the soil. The growth of all the inoculated organisms is recorded on maps of the soil plates, and growth rates (in mm/day) are calculated. The technique can also be used with nonsterile soil, but highly selective media must be used to prevent overgrowth of the GEMs by the indigenous soil microbiota (see Section VII).

Complex microcosms

Undisturbed soil microcosms consist of soil cores of varying size that are brought into the laboratory with minimum disturbance of the structure and biotic composition of the soil. The soil sample is removed intact from the coring device, and thereby, the soil system is relatively undisturbed. Disturbed microcosms are those in which the soil ecosystem is reconstituted in the laboratory, e.g., the placement of excavated soil into containers in which seeds or seedlings are planted.

Undisturbed soil cores have been utilized to study, for example, pesticide degradation (Johnen and Drew, 1977; Wingfield et al., 1977), microbial community responses to environmental perturbations (Elliot et al., 1986), soil denitrifier populations under anaerobic and aerobic conditions (Martin et al., 1988), the fate and ecological effects of GEMs (Bentjen et al., 1989), and the effects and mobility of xenobiotics in soil containing plant root systems (Hicks et al., 1989; see Fig. 14). In the latter study, uniform and realistic distributions of the xenobiotics were attained with simulated rain events, and the data on environmental perturbations were consistent with those obtained in parallel field studies. Other studies with intact soil cores have also adequately predicted events subsequently observed in field studies (see Pritchard and Bourquin, 1984). The soil cores can be sampled for the enumeration of microbial populations at

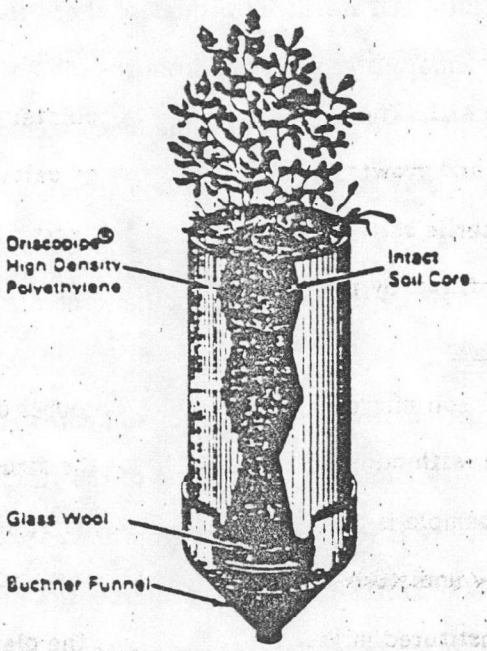

Figure 14. Terrestrial soil-core microcosm test system (Hicks et al., 1989).

different depths through sampling ports inserted in the walls of the soil core cylinder (Ardakani et al., 1973). This also enables the soil sample to be replaced with fresh soil. Examples of various types of microcosms (Atlas and Bartha, 1981; Gillett, 1988; Johnson and Curl, 1972) and guidelines for the use of soil core microcosms, with descriptions of various soil core designs, sampling procedures, and statistical analyses, have been published (Anon., 1987).

With disturbed soil microcosms, it is assumed that biotic and abiotic processes and interactions representative of the in situ situation will be reestablished after suitable incubation or treatment (Pritchard and Bourquin, 1984). Small nonsterile soil cores (5 cm x 2.5 cm deep) were used to study changes in microbial populations and respiration after organic amendment (Elliot et al., 1986). Holben et al. (1988) used a soil-vermiculite microcosm (700 g of this mixture in plastic pots) to study the survival of a model GEM. A coring device was inserted through the side of the plastic pot, a sample of the mixture was removed for enumeration of the GEM, and a test tube was inserted into the hole formed by the coring device. Survival and plasmid transfer have been studied with GEMs added to a rhizosphere microcosm that consists of a peat-vermiculite mixture containing radish or bean plants. The pots or flats containing the simulated soil and plants were maintained within large chambers that enable the control of relative humidity, temperature, and light/dark cycles (Fig. 15) (Armstrong et al., 1987; Gile and Gillett, 1982; Knudsen et al., 1988).

The soil perfusion technique is a highly sensitive, easy to sample, and continuous system for measuring the kinetics of biochemical transformations in soil. Sieved soil is added to a glass column and perfused with water at a cycling rate that achieves the desired saturation and aeration of the soil column. Various amendments can be added to the soil, and the perfusate is periodically analyzed for the biochemical transformations of interests, e.g., nitrogen transformations. This technique has been used to study the impact of GEMs and various environmental factors, e.g., sulfur dioxide, acid

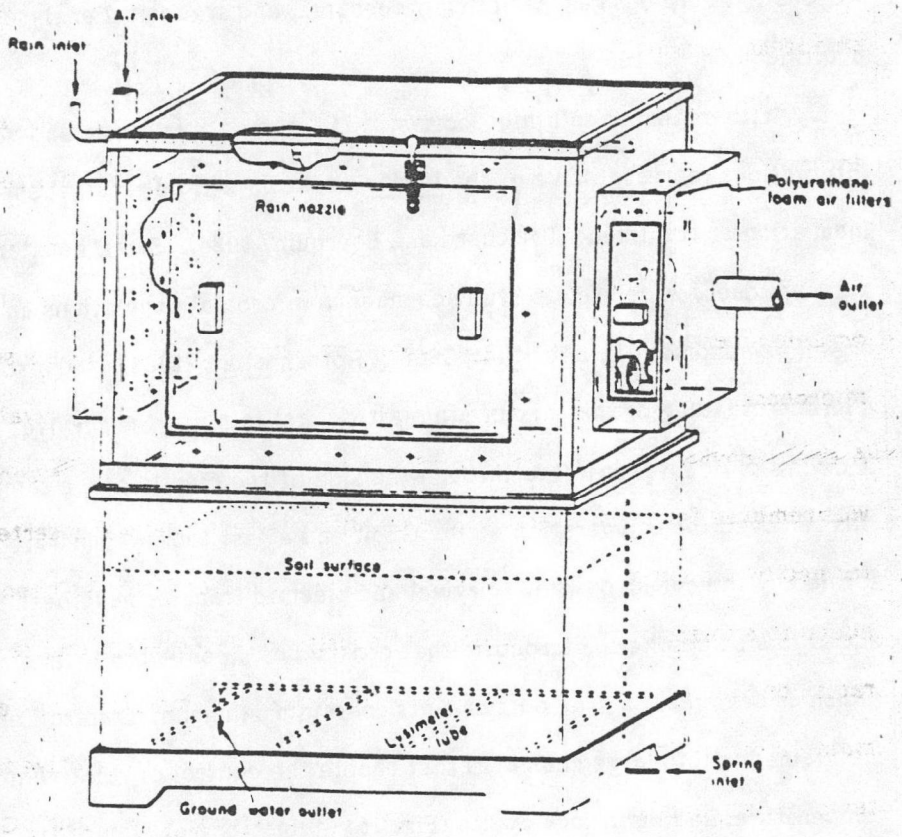

Figure 15. A microcosm used to study the survival, fate, and genetic stability of recombinant bacteria in the rhizosphere and in herbivorous insects. Physical conditions (e.g., temperature, humidity, light/dark cycles) can be controlled in this system (Gile and Gillett, 1982).

precipitation, and heavy metals, on ecologic processes in soil (Doyle et al., 1988; Jones et al., 1988; see Stotzky, 1974, 1986). The enumeration of added GEMs and of the indigenous microbiota and measurement of gene transfer can be accomplished by either sampling or sacrificing individual soil perfusion columns at various times, diluting, and plating the soil dilutions on selective media.

An apparatus for growing plants with the root systems maintained sterile has been described for the collection of root exudates and for measuring root respiration and the release of other volatiles from roots (Stotzky et al., 1962a). The apparatus consists of a growth tube containing the planted seed or seedling, a root chamber containing a solid growth medium (e.g., sand, vermiculite, soil), an irrigation chamber, an irrigation reservoir, an aeration system, a root exudate collecting system, and inoculation ports. The root system is separated from the above ground portion of the plant by a silicone seal, which eliminates the need for enclosing the whole plant. This system could be easily utilized for studying gene transfer in the rhizosphere.

IV. Selection, Preparation, Maintenance, and Storage of Soils

Introduction

Knowledge about the structure, physicochemical composition, and microbial events in microhabitats in soil is sparse and mostly conjectural. What can be assumed with a high degree of certainty is that these microhabitats, and even sites within a single microhabitat, are heterogeneous and differ in the type and amount of clay minerals, organic and inorganic substances, and other physicochemical characteristics and, therefore, in microbial composition and activities, including the transfer of genetic information (Stotzky, 1974, 1986, 1989).

Selection of soils

The selection of soils for studies on gene transfer among bacteria should be based on the relevance of the soils to projected field release sites; for example, recombinant rhizobia should be studied in representative agricultural soils on which legumes are grown commercially. A complete soil analysis should be obtained, to determine the relevance and representativeness of the soil samples to the experimental purpose and design and to correlate the survival of, and gene transfer by, the introduced GEM with the physicochemical characteristics of the soils. The soil parameters determined should include pH, organic matter content, cation-exchange capacity, water content at the -33 kPa water tension (and, preferably, also the water-holding capacity (WHC) and the permanent wilting point (PWP)), elemental analysis (including both plant nutrients and toxic heavy metals), and clay mineralogy (Babich and Stotzky, 1977). This information can most easily be obtained by sending soil samples to the soil testing laboratory of the appropriate State Agricultural Experiment Station for analysis, or analyses can be made using the methods described in Klute (1986) and Page et al. (1982).

The base-line microbiological characteristics of a soil sample that should be obtained include numbers of total bacteria, actinomycetes, gram-negative and gram-

positive bacteria, spore-forming bacteria, nitrifiers, denitrifiers, cellulose utilizers, fungi, and protozoa. The numbers of soil bacteria resistant to specific antimicrobial agents (e.g., antibiotics, heavy metals), especially to those agents to which the GEMs to be studied are resistant, should be evaluated. Other physiological groups of bacteria and the activity of selected enzymes (e.g., acid and alkaline phosphatases, arylsulfatases, dehydrogenases) can also be measured to provide an index of over-all microbiological activity. This information is often useful, as changes in any of these parameters may provide indications of the persistence and ecological effects of the GEMs in soil.

Preparation of soils

The preparation of a disturbed soil sample for use in laboratory studies on gene transfer and the ecology of GEMs begins with particle size separation. The soil is sieved (usually through a 2 mm sieve) to remove stones, gravel, roots, other debris, and large organisms. Hence, the soil particles will not vary significantly in size distribution from sample to sample, and the water content at the -33 kPa tension will be relatively constant. The sieved soil sample may then be amended: e.g., with $CaCO_3$ to raise the pH; with mined clay minerals to adjust the concentration or type of clay; with water to adjust the water tension; with an inoculum of a microbiologically-active soil (e.g., from the field or a flower pot) to provide an active microbiota; with nutrients (e.g., a nutrient broth or glucose plus mineral salts) to establish an active indigenous microbial population; or, usually, with some combination of these. Conditioning the soil by incubating it with a mixed nutrient amendment (e.g., glucose plus mineral salts), in addition to sufficient water to bring the soil to its -33 kPa tension, before initiation of an experiment may be necessary if the soil has dried excessively during storage, which could result in some members of the microbial community declining in number or becoming dormant.

To determine the effects of the indigenous soil microbiota on the transfer of genetic information by the added GEMs, parallel studies should be conducted in sterile

soil. The length of time for soil sterilization and the number of sterilization cycles varies with the size of the soil sample (Johnson and Curl, 1972). Sterilization with moist heat is accomplished by autoclaving the samples at 15 psi and 121 C for 30 min. To ensure sterilization, especially if the soil sample is larger than 50 g, a second autoclaving should be conducted 24 h later. This second autoclaving should kill bacterial spores, which have been heat-shocked by the first autoclaving to germinate into vegetative cells, and microorganisms protected inside soil aggregates and associated with organic matter. A sterility check should be performed by adding a nutrient medium to the soil and, after incubation for 24 to 48 h, the preparation of a streak plate with a 1: 10 soil: water dilution on a nonselective medium to detect the presence of microorganisms. Sterilization with moist heat results in the formation of toxic compounds in soil, such as reduced manganese and various organic and inorganic volatiles. Therefore, the autoclaved soil samples should be maintained, preferably at room temperature and in a high humidity chamber, for several days before inoculation of the GEMs (Stotzky, 1986). Gamma-irradiation is an attractive alternative technique for the sterilization of soil, as it does not result in as many changes in soil (e.g., substantial release of nutrients or toxic chemicals) as does autoclaving (McLaren et al., 1962; Stotzky and Mortensen, 1959). However, it is not as convenient as autoclaving, as it requires a source of gamma rays, and large soil samples cannot be sterilized effectively, as the penetration of gamma rays into soil is poor.

Maintenance of soils

The water content of the soil can be maintained at a constant -33 kPa tension during the experiment by incubating the soil samples in a high humidity chamber or by periodic addition of sterile water on a weight basis. Generally, the water content of the soil sample at the beginning of the study is adjusted to the -33 kPa water tension, as this is the water content at field capacity (i.e., the water retained by soil 24 to 48 h after a rainfall or irrigation) (Cassel and Nielsen, 1986). This water tension, as well as other

critical soil water contents (e.g., WHC, PWP), can be obtained from the State Agricultural Experiment Station, as mentioned above.

Experiments conducted with aerobic microorganisms require adequate aeration. Hence, the soil containers should allow sufficient diffusion of air by placing closures loosely on the container, and the container should be less than one-half full to provide an adequate head space for air above the soil sample. Alternatively, the soil can be aerated with a constant or intermittent stream of water-saturated air, which will provide both sufficient oxygen and water to maintain the soil at its -33 kPa water tension (Stotzky, 1965a).

The effects of nutrient amendment of the soil on survival and gene transfer can be studied by periodic additions of either simple (e.g., glucose) or complex (e.g., cellulose, humic materials) organic compounds or with a specific substrate utilizable only by the GEM, as with GEMs capable of degrading xenobiotics (e.g., toluene, 2,4-D). The effect of nutrient amendments on available water content, pH, and other physicochemical characteristics of the soil must be evaluated.

Sampling of soils

Sampling of soil inoculated with GEMs can involve the sacrifice of individual microcosms or of multiple samplings from the same microcosm. Individual microcosms can consist of test tubes or flasks containing subsamples of the same soil that are sacrificed at different times (see Section III). Usually, triplicate or more replicates are sacrificed at each sampling time. The soil sample is commonly diluted with either sterile tap water, saline (0.85% NaCl), or a phosphate buffer (pH 7) to a 1: 2 (e.g., 10 g soil: 10 ml diluent) or a 1: 10 (e.g., 2 g soil: 18 ml diluent) dilution (see Wollum, 1982, for other diluents), and the subsequent appropriate serial decade dilutions are used to inoculate either spread or pour plates containing selective media. Numerous techniques for dispersal of the microbes in the diluent have been described, and generally, each procedure is selective for a specific portion of the soil bacterial population (Casida,

1968). The efficacy of recovery of inoculated GEMs is usually dependent on the type and ionic strength of the diluent, the amount of mixing, and the temperature at which the sample is mixed (Jensen, 1968).

Methods for multiple samplings from undisturbed soil microcosms, e.g., soil cores, for studying the survival of, and gene transfer by, GEMs are presented in Section III. Multiple samplings from the same microcosms should be performed so that the soil structure of the microhabitats is not significantly disrupted, as this could affect microbial events and subsequent samplings. Microbial counts may vary with the depth in the soil core, and contamination of a soil sample from one depth with soil from other depths must be avoided.

Storage of soils

Soil samples should be stored in bulk, so that repeat experiments can be conducted with the same soil samples without resampling in the field, which could result in variability in samples and microbiological responses. Bulk soil samples can be stored in open plastic bags, usually in metal or plastic garbage cans, and the water lost as a result of air-drying can be replaced, along with desired nutrients, clays, or other amendments, to condition the soil before experimental use, as discussed above. However, the remoistening of air-dried soil can result in increases in microbial populations caused by the release of nutrients, and the soil should be allowed to incubate for at least one week before the initiation of an experiment. Soil samples can also be stored in thin-walled polyethylene bags that prevent the loss of moisture and allow the exchange of oxygen, carbon dioxide, and other gases. However, storage in even such bags can result in significant changes in the composition of the soil microbial community (Stotzky et al., 1962b). Storage at 4 C reduces the loss of water, but such storage probably affects the microbial composition of soil, as this temperature may not be favorable for the survival of some soil microbes and vice versa.

V. Methods for Studying Conjugation

IN VITRO

Conjugal gene transfer in bacteria can be demonstrated in various ways, depending on the location of the genes (i.e., on the chromosome or on plasmids) that will be transferred (Curtiss, 1981). The frequency of transfer (i.e., the frequency of recombination, as this is what is actually measured) of the experimental strains should be determined in standard laboratory conjugation studies, so that the potential for gene transfer by conjugation in these strains, especially when introduced into soil, can be assessed. Moreover, this will enable meaningful comparisons between the experimental strains and well-studied and documented strains, both *in vitro* and *in situ*.

In all studies of gene transfer, both *in vitro* and in soil, appropriate controls must be included to determine the frequencies of spontaneous mutation, as these frequencies may differ *in vitro* and in soil. *In vitro* mutation frequencies are most easily determined during verification of the phenotypes of the donors, recipients, and plasmids. Mutation frequencies in soil are determined by inoculating the recipients and the donors, with and without the plasmids of interest, individually into soil and, after various periods of incubation, plating appropriate soil dilutions on all selective media used to enumerate donors, recipients, and recombinants. For example, a lactose-positive, Cm-sensitive recipient should be plated on MAC containing Cm at the same concentration that will allow the growth of a lactose-negative, Cm-resistant donor and of the resultant recombinants. The frequency of gene transfer must be corrected for the frequency of spontaneous mutation in the parentals.

Chromosomal transfer

A model for the transfer of chromosomal genes in soil that can be used to develop studies with other bacteria is the transfer of prototrophic markers from Hfr *E. coli* K12 donor strains to auxotrophic F^- *E. coli* K12 recipient strains (Krasovsky and Stotzky,

1987; Weinberg and Stotzky, 1972). The mating type and phenotype of the E. coli strains used in these studies were: ∝503, Hfr, prototrophic, streptomycin-sensitive (Sm^S); ∝493, Hfr, prototrophic, Sm^S; and ∝696, F^-, auxotrophic for leucine, proline, and arginine (Leu^-, $ProB^-$, Arg^-), and streptomycin-resistant (Sm^r) (Bachman and Low, 1980; Curtiss and Renshaw, 1969).

Media. The type of medium, i.e, liquid or agar, used for the growth of cultures will depend on the design of the experiment and the information desired. For rapid (e.g., overnight) production of a large number of cells, as for an inoculum, shaken broth cultures are best. A solid medium should be used to distinguish metabolic traits of individual colonies, which is necessary for the enumeration and phenotypic screening of transconjugants. The preferred type of medium for various procedures is described below. Commercial sources of standard media and formulations of specific media are listed in the Appendix.

Cultures should be maintained at 4 C on agar slants, e.g., on Bacto-Penassay Agar (PA), and transferred monthly. Some of the phenotypic markers that are commonly and most easily used to demonstrate gene transfer are unstable or revert and, therefore, can be lost from the gene pool, unless there is selection pressure for the markers. Consequently, to ensure that the resistance markers are not spontaneously lost from the cultures, the strains should be maintained on selective media (see below), or frozen suspensions should be used as the source of cells for each experiment (Lewin, 1977).

The phenotypes of all strains should be verified immediately before use in an experiment. For example, the phenotypes of the E. coli strains described above were verified and maintained on the following media: the prototrophs on Minimal Agar (MA); the auxotrophs on MA + 200 μg Sm, 20 μg L-leucine, 30 μg L-arginine, and 22 μg L-proline/ml; and the recombinants on MA + Sm (Curtiss, 1965; Curtiss et al., 1968; Krasovsky and Stotzky, 1987).

Inoculum preparation. Parental strains (i.e., donors and recipients) are usually grown overnight (ca. 18 h) at 37 C in 125- or 250-ml Erlenmeyer flasks containing 25 or 50 ml of Bacto-Penassay Broth (PB). The period of growth for adequate production of cells depends on the generation time of the strains. The donor and recipient strains are shaken at ca. 120 rpm; however, the donor strain is grown in stationary culture for at least 30 min before mating to minimize the mechanical loss of pili. Both cultures are grown to a density of ca. 10^9 cells/ml, which is determined most easily by spectrophotometry at 520 nm, after correlation and standardization of absorbance units with the colony-forming units (CFU) of each strain. The cultures can be either washed, at least twice, with a suitable mating medium (e.g., Minimal Mating Medium, 3M; LB) by centrifugation at 1,000 g for 20 min at 4 C or just centrifuged to concentrate the cells. The cultures are then resuspended in the required amount of mating medium to yield the desired concentrations of donor and recipient cells.

Mating. An excess of recipient cells is preferable in both in vitro and in situ matings (Curtiss, 1981). Different donor: recipient ratios (ranging from 1: 20 to 1: 1) are obtained by varying the numbers of the recipient and donor cells added. The parental cultures can be mated in liquid or on agar media (Atlas et al., 1988; Curtiss, 1981; Krasovsky and Stotzky, 1987; Miller, 1972; Weinberg and Stotzky, 1972). Conjugation can occur with either type of medium, provided that the medium contains adequate levels of nutrients and energy to support the growth of the parentals and the transconjugants and that no growth-inhibiting substances are present. Schema for mating, for both chromosomal and plasmid transfer, in liquid and on solid surfaces are available (e.g., Curtiss, 1981; Walter et al., 1987) (Figs. 16 and 17).

The selection of either a liquid or a solid medium for the evaluation of rates of transfer of both chromosomal- and plasmid-borne genes requires some knowledge of the type of pili produced by the donor. Rigid pili require a static solid environment, such as that provided by agar, whereas flexible pili usually attach to conjugative partners on a

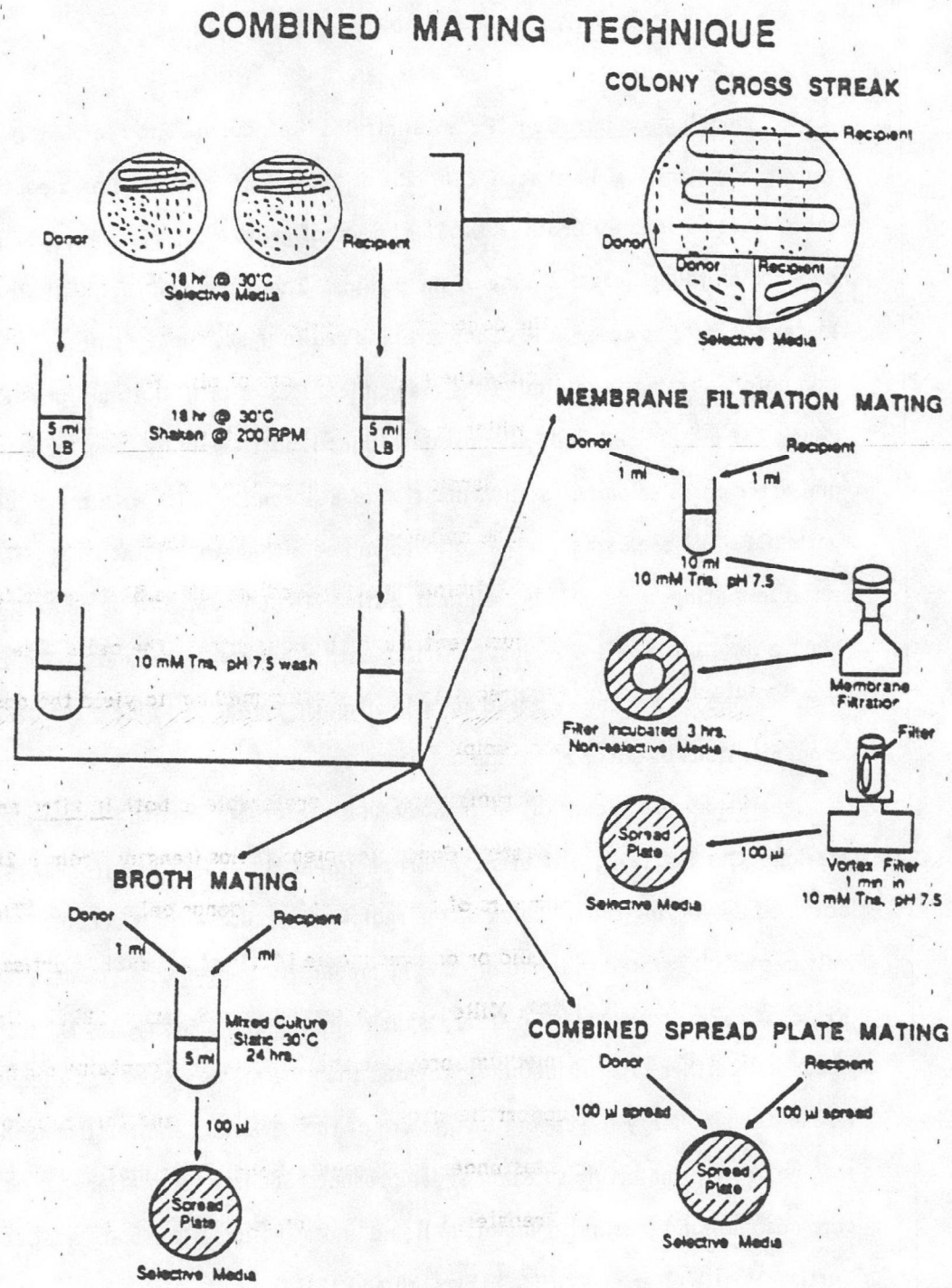

Figure 17. Combined mating techniques for studying the transfer of genes by conjugation *in vitro* (Walter *et al.*, 1987). (LB = Luria Broth)

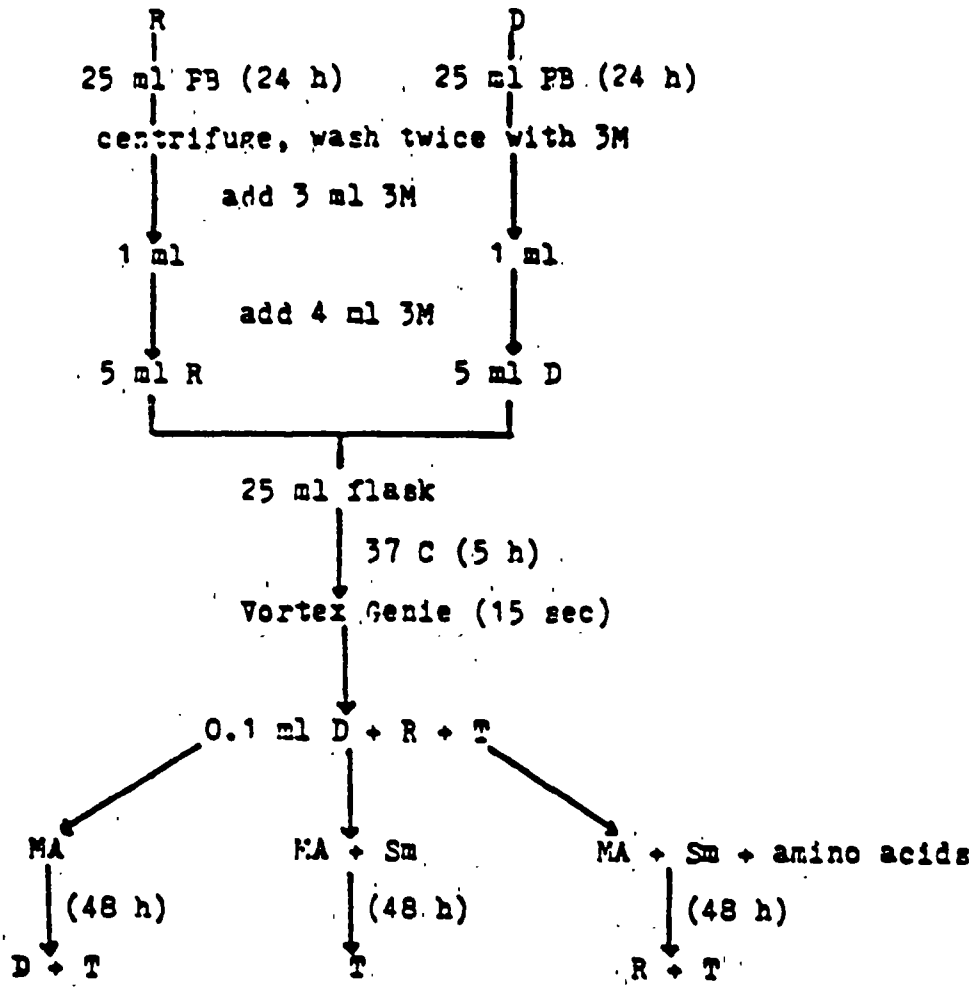

Figure 16. Flow diagram for studying the transfer of chromosomal genes by conjugation <u>in vitro</u> (Krasovsky and Stotzky, 1987). (R = recipient; D = donor; T = transconjugant; PB = Bacto-Penassay Broth; 3M = Minimal Mating Medium; MA = Minimal Agar; Sm = streptomycin)

solid medium and in liquid. The pili produced by the strains of E. coli used herein as examples function adequately both in liquid and on solid media, and frequencies of conjugal transfer are comparable with both types of media.

For broth matings, each parental culture should be mated in at least triplicate. The environmental conditions for mating depend on the system being used, e.g., an F-mediated chromosomal transfer between E. coli should be incubated at 37 C, without shaking, in ca. 10 ml of a nutrient broth contained in a 250-ml Erlenmeyer flask (Curtiss, 1981). The incubation time for chromosomal gene transfer can vary, and sampling at intervals for at least 100 min when using E. coli is recommended. Nutrient content, incubation conditions, and other experimental parameters should be optimized and standardized in vitro before experiments on in situ gene transfer are designed with specific strains.

For plate matings, parental cultures can be streaked separately and sequentially on agar or they can be mixed before placement on a solid medium, to ensure that the parental cells make contact and form conjugative pairs or aggregates (Fig. 17) (Curtiss, 1981; Walter et al., 1987). In the colony cross-streak method, the parental strains are streaked individually at right angles to each other on the surface of the solid mating medium: several streaks of one culture are streaked in one direction across the surface, and the second culture is streaked perpendicular to and crosses the streaks of the first culture. Hence, the parentals are mixed in the area where the two streaks cross. If the medium is selective for the transconjugants, neither parental will grow, and only recipient cells that conjugated and successfully received and incorporated the prototrophic markers will produce colonies. The frequency of gene transfer by this procedure is generally difficult to quantitate, as even if the numbers of each parental streaked and of the transconjugant colonies that develop are known, there is no definitive way to estimate the number of mating pairs that actually occurred in the mix of the cross-streak. If the medium is nonselective, both parentals will grow, and it will be

difficult to quantitate donors, recipients, and transconjugants, as all the cells must be removed from the mating plate, diluted, and then each phenotype enumerated on selective media. Consequently, the colony-streak procedure is not recommended when quantitation of conjugation frequency is required. Conversely, it is a simple method with which to obtain transconjugants for further use in studies in soil. Moreover, it can be useful in the rapid screening of different strains for their ability to conjugate on a solid medium.

In the combined spread-plate procedure, the parentals are mixed as uniformly as possible over the surface of an agar plate. This can be achieved by mixing aliquots of each parental culture in a test tube or flask before spreading the mixture, or the parentals can be uniformly spread sequentially on the plate (Walter et al. 1987). The same limitations in the interpretation of results described above for the colony cross-streak method apply to the combined spread-plate procedure.

In the membrane filter procedure, aliquots of the parental strains are mixed in a test tube or flask, and the mixture is filtered, with vacuum, through a sterile filter membrane. The parental strains are impinged on the surface of the membrane, which increases the probability of the mating pairs being in close proximity. Membranes of nitrocellulose (e.g., Millipore, Bedford, MA) or polycarbonate (e.g., Nucleopore, Pleasanton, CA) with pore sizes smaller than the diameter of the test strains, e.g., 0.22 or 0.45 µm, are commonly used. The membrane is placed aseptically on a nonselective agar medium on which the parentals will grow and conjugation can occur. The membrane is then removed from the agar surface, the cells are resuspended, diluted, plated on selective media, and incubated, and the CFU of each phenotype are recorded (Curtiss, 1981). The limitations in quantitation are similar to those for the colony cross-streak and combined spread-plate methods.

Incubation. The mating mixtures should be incubated at temperatures and for durations that are optimum for conjugation of the strains. Depending on the bacteria

involved, the mating mixture can be either shaken gently (30 to 120 rpm), to enhance aeration, or incubated without shaking in a vessel with a large surface: volume ratio to permit adequate diffusion of oxygen into the medium, e.g., 10 ml of mating mixture in a 250-ml Erlenmeyer flask (Curtiss, 1981; Miller, 1972). The temperature selected for the mating depends on the optimum temperature for growth of the parental strains. For many laboratory strains, 37 C is optimum (Khalil and Gealt, 1987).

For example, for chromosomal transfer between the E. coli K12 strains χ503 and χ696 described above, the mixtures were incubated at 37 C for at least 5 h with gentle shaking at 120 rpm (Krasovsky and Stotzky, 1987). Other strains may require different conditions of time, temperature, aeration, and nutrients. Therefore, optimal conditions for in vitro gene transfer should be determined for each combination of strains (Atlas et al., 1988; Curtiss, 1981; Miller, 1972).

Recovery of donors, recipients, and transconjugants. Conjugation can be interrupted after various periods of mating by mechanical separation of the cells, e.g., on a Vortex Genie for 15 sec or longer (Miller, 1972). The time intervals at which the mating mixture is sampled for the recovery of transconjugants depend on the bacteria being studied. After the appropriate incubation period, serial decade dilutions of the mating mixture are made, and aliquots of appropriate dilutions are spread-plated on selective and nonselective agar media.

Media used for the recovery and enumeration of donor, recipient, and transconjugant populations will depend on the types of markers present in the parental strains. Auxotrophic mutants have been typically used as the recipients, and gene transfer is measured by the frequency at which a specific auxotrophic marker is replaced by the specific prototrophic gene sequence in the donor. Therefore, minimal agar medium, containing an agent to counterselect against the donor, can be used for isolating prototrophic transconjugants; minimal medium can be used for isolating donors (and transconjugants); and minimal medium supplemented with the required nutritional factors

and the counterselecting agent can be used for isolating recipients (and transconjugants). For example, in the mating between E. coli strains x503 and x696 described above, MA was used for the recovery of donors and transconjugants, MA + Sm + amino acids for the recovery of recipients and transconjugants, and MA + Sm for the recovery of transconjugants. Aliquots of the mating mixture were also plated on MA + Sm amended with various combinations of amino acids for which the recipient was auxotrophic, to determine the numbers of partial and complete transconjugants. Other selectable phenotypic characteristics can also be utilized, e.g., catabolic functions, heavy metal- or antibiotic-resistance, for quantifying the frequency of transfer of chromosomal genes (Curtiss, 1981). The markers used for phenotype verification and strain maintenance can usually also be used for the recovery of the parental strains, but recovery of the transconjugant will require specific and unique media to detect the transferred gene(s) of interest.

Syntrophy. A potential artifact that can confound the enumeration of transconjugants in soil is syntrophy, or cross-feeding, a type of mutualism that enables different auxotrophic strains to grow together, but not apart, when the nutrients necessary for the growth of each auxotroph are absent or their concentrations are very low, which is not uncommon in the soil environment. Syntrophy can enable auxotrophic organisms to grow on minimal media selective for transconjugants, as the amino acids, vitamins, and other growth factors required by one auxotrophic strain may be excreted by the other strain, which can result in an exchange of nutrients under conditions that would not support the growth of either auxotroph when cultured alone. Consequently, any isolates that grow on selective minimal media must be verified to be transconjugants, rather than auxotrophic parentals that are cross-feeding on the recovery media, by streaking purified colonies of presumed transductants individually on selective minimal media or by the colony cross-streak method (Krasovsky and Stotzky, 1987; Weinberg and Stotzky, 1972).

Frequency of recombination (FOR). This measure of successful conjugal transfer of chromosomal genes is calculated from the ratio of the numbers of CFU recovered on media selective for the transconjugants to the total numbers of CFU recovered on media selective for the donor (on which the transconjugants will also grow):

$$\text{FOR} = \frac{\text{CFU on TC-medium}}{\text{CFU on D-medium} - \text{CFU on Tc-medium}}$$

where: TC-medium is the selective medium on which only transconjugants will grow; and D-medium is the selective medium on which donors and recombinants will grow.

When an Hfr strain is mated with an F^- strain, the amount of the donor genome and the specific genes transferred depend on the duration of mating and on the location of the integration site of the F-factor (i.e., the oriT site), respectively. By sampling and plating cultures of mating bacteria on selective media at different times, the order of genes on the donor chromosome can be determined by the times at which they appear in the recipient and by their order and relative location (Curtiss, 1981; Miller, 1972). Genes that are located near each other on the chromosome are transferred together with a high frequency. Moreover, the frequency of chromosomal transfer of genes near the oriT site may be greater than that of plasmid transfer. Bacteria in soil may not conjugate long enough to transfer a large plasmid, but they probably conjugate long enough to transfer several genes located close to the oriT site. Conversely, if the marker genes are located too far upstream from the oriT site, the apparent FOR may be low, despite significant conjugation between parentals and the transfer of genes that were not detected. These principles of chromosomal gene transfer must be considered when designing studies to observe the potential for gene transfer by conjugation in the environment.

Plasmid transfer

Many of the procedures used for the study of chromosomal transfer can also be used to study plasmid transfer by conjugation. Host strains that will maintain the plasmid(s) being studied, before and after conjugation, are required. The strains should not contain other plasmids that may interfere with conjugation, either by surface exclusion (i.e., prevention of "superinfection" by plasmids of the same type) or by competing during cell division for membrane-bound segregation systems that provide for maintenance and inheritance (i.e., incompatability) (Freifelder, 1987; Lewin 1977). Ideally, the strains should be devoid of plasmids other than the ones being studied, as shown by phenotypic expression and by the inability to detect other plasmid DNA by agarose gel electrophoresis after extraction of plasmid DNA by standard procedures (Curtiss, 1981; Maniatis et al., 1982; Miller, 1972) (see Section VII). Selective enumeration of donors, recipients, and exconjugants is facilitated if the chromosome of the donor and recipient strains is "tagged", i.e., contains antibiotic-resistance marker(s) or some other easily characterizable phenotype, e.g., the production of a red color by lactose fermenters on MAC (Atlas et al., 1988; Curtiss, 1981; Devanas et al., 1986; Miller, 1972) or a blue color on X-gal agar by cells containing the ZY genes of the lactose operon (Drahos et al., 1986). The lacZY genes can also be used as plasmid markers.

The choice of plasmids to use experimentally will depend on the type of system to be studied and the type of information desired. The enumeration of donors and exconjugants is facilitated if the plasmid encodes catabolic pathways, resistance markers to antibiotics and/or heavy metals, or some other easily characterizable phenotype (Maniatis et al., 1982; Miller, 1972).

Media. The media used for the maintenance of the hosts, with or without plasmids, and for mating and recovery of the donors, recipients, and exconjugants depend on the nutritional requirements of the bacterial strains. Most bacteria will grow adequately on standard laboratory media, such as Nutrient Broth (NB), Tryptone Glucose

Yeast Extract Broth (TGYB), and LB and on agars with the same nutritional composition. Environmental isolates, however, may require growth factors that are not present in standard commercial media. Such fastidious strains can usually be maintained on media supplemented with extracts of soil or with specific nutrients (see Appendix). In contrast, many commercial media may be too nutritious for indigenous soil bacteria and, possibly, also for introduced bacteria after they have adapted to the oligotrophic conditions in soil.

Slower growth and, hence, fewer cells occur on agar than in liquid media, thereby reducing the number of mutants that develop in the cultures and extending the intervals between necessary transfers of the cultures to fresh medium. A solid medium is also better if resistance markers must be maintained by a constant selection pressure, as the destruction or alteration of the selective chemical (e.g., an antibiotic) is less than in liquid media, as the result of the slower rates of diffusion of degradative extracellular enzymes from a resistant colony on agar. Liquid culture is generally better for obtaining large numbers of bacteria in a short period of time (e.g., in 18 h), which is important for the preparation of inocula.

Bacteria will usually not maintain extrachromosomal genetic information that is not used (i.e., that is not induced to transcribe). For example, plasmids that encode resistance to antibiotics (R-plasmids) will not be replicated, segregated, or maintained after several transfers if there is no selection pressure of the respective antibiotic(s) in the medium. Bacterial strains containing R-plasmids are best maintained on a selective agar medium that contains at least one of the antibiotics to which the plasmid encodes resistance. If the plasmid is especially unstable and the loss of plasmid genes is likely, several or all of the compounds (e.g., antibiotics, heavy metals, substrates for degradation) for which the plasmid encodes resistance or utilization should be included in the selective maintenance medium. For example, an *E. coli* strain that carries the plasmid, RP4, is maintained on LA containing 25 μg/ml Tc. When the host is a strain of

Pseudomonas, which are generally more resistant to many antibiotics than most members of the Enterobacteriaceae, the basal resistance level to specific antibiotics of the plasmidless host must be determined and the selective antibiotics (e.g., Tc) added at higher concentrations for both maintenance of the plasmid and selection of exconjugants. For example, P. aeruginosa PAO1 is resistant to Tc at concentrations above 100 μg/ml, and the quantity of Tc necessary for maintenance and selection of plasmid RP4 in this strain is 200 μg/ml.

Inoculum preparation. Strains can be grown in a nonselective broth to obtain large numbers of cells. However, if the plasmid phenotype is unstable and a significant proportion of the cells is likely to loose the plasmid when grown under nonselective conditions, then selective pressures must be maintained. In either situation, the phenotype of the donor and recipient strains must be verified before use. One method to maintain selection for the plasmid markers is to grow the strain overnight on a selective agar medium. For example, cells of E. coli containing plasmid RP4 were grown on MAC + 25 μg/ml Tc, scraped, diluted with saline to 10^4 CFU/ml, of which 0.25 ml was added to 25 ml of LB to yield a density of 10^3 CFU/ml, and grown overnight at 37 C with shaking (Devanas et al., 1986). This produced a suspension of cells that contained the plasmid, as all cells grew on the selective medium. Consequently, loss of plasmids, that could result from their partitioning (segregation) during growth under nonselective conditions, can be minimized. If the donor strain readily looses the plasmid under nonselective conditions, suspensions of cells scraped directly from a selective medium can be used. The concentration, washing, diluting, and inoculation procedures are as described above for chromosomal transfer.

Mating. The conditions selected for mating will vary with the type of system to be studied. For example, F-factors, their derivatives, and other plasmids derepressed for transfer are transferred readily at high frequencies (ca. 100%); other conjugative plasmids have lower frequencies (10^{-2} to 10^{-7}) (Lewin, 1977). Various proportions of donor

to recipient cell numbers should be evaluated, as the optimum donor: recipient ratio for plasmid transfer depends on the species and strains used (Curtiss, 1981; Miller, 1972). This ratio should be determined in preliminary studies. In the model systems of E. coli J53(RP4) as the donor strain and P. aeruginosa PAO1, E. aerogenes, K. pneumoniae, and P. vulgaris as the recipient strains, the overnight broth cultures (usually 10^9 CFU/ml) were diluted with sterile saline to cell densities of approximately 10^6 CFU/ml and then added to the mating medium (LB) in a donor: recipient ratio of 1: 5, which ensured that each potential donor was likely to encounter a suitable recipient (Devanas and Stotzky, 1988). Successful mating and transfer may occur within 1 to 4 h with plasmids depressed for transfer. For other plasmids, longer periods of mating may be necessary. The mating mixture is then diluted, and samples are plated on appropriate selective agar media for recovery of donors, recipients, and exconjugants, as described above for transfer of chromosomal genes.

Calculation of the frequency of conjugation (FOC). The frequency of plasmid transfer by conjugation is calculated by dividing the number of exconjugants by the number of donors in the mating mixture (Curtiss, 1981):

$$\text{FOC} = \frac{\text{CFU of exconjugants}}{\text{CFU of donors}}$$

IN SOIL

Chromosomal transfer

The design of studies to examine the potential for conjugal transfer of chromosomal genes in soil must consider all the variables that affect chromosomal transfer in vitro, as well as the physicochemical factors inherent in the soil environment. The selection of donor and recipient strains and the methods used for their maintenance, culture, and preparation as inocula are essentially the same as those employed for in vitro studies. Consequently, only additional aspects unique to studies of in situ chromosomal gene transfer are described.

Inoculation. The use of donor and recipient cells that are in log phase appears to enhance conjugal chromosomal transfer in soil (Weinberg and Stotzky, 1972). One method for the preparation of log phase cells for inocula uses cells that were grown overnight, washed by centrifugation (2 to 3 times at 1,000 g), followed by resuspension of the cells of each strain in a phosphate buffer, usually at pH 7.

The sequence of inoculation of the donor and recipient cells has been shown to affect their survival and the frequency of conjugation (Krasovsky and Stotzky, 1987; Weinberg and Stotzky, 1972). The survival of both donors and recipients was higher when the recipient cells were inoculated before the donor cells than when the donors were added before the recipients. When the donor and recipient cells were inoculated together (i.e., mixed before addition to soil), their survival and the FOR were greater than when they were inoculated sequentially (Krasovsky and Stotzky, 1987). However, mixing of the parentals before their addition to soil may result in mating pair formation outside of the soil, thereby producing artifactually inflated frequencies in soil. Hence, mixing donors and recipients before addition to soil is not recommended for studies designed to observe the transfer of chromosomal genes in soil.

Incubation. Soils used for *in situ* mating experiments should be incubated under conditions that maintain the moisture and temperature conditions defined for the experiment. To maintain the optimum soil water tension (i.e., -33 kPa), high-humidity chambers can be used (Stotzky, 1986). One to 2 inches of water are placed in the bottom of large glass containers (e.g., chromatography tanks, desiccators) and covered with lids that have a vent that is loosely plugged with nonabsorbent cotton. This arrangement produces a water-saturated atmosphere that maintains the optimum soil water tension. Temperature ranges of natural soils can be simulated by placing the high-humidity chambers in incubators maintained at the desired temperatures.

Sampling. The choice of sampling regimes will depend on the type of microcosm used and on the purpose of the experiment. Simple test tube microcosms, in which the

entire soil sample is "sacrificed", reduce the sampling error associated with repeated replicate samplings from larger batch volumes. However, sampling error can be introduced at the level of preparation and inoculation of the individual tubes. Hence, precise weighing of soil and uniform inoculation are necessary. Different types of soil microcosms and sampling systems are discussed in Section III.

The samples of soil are subjected to serial decade dilution with standard laboratory diluents, e.g., sterile distilled or tap water, saline, buffers, 0.1% peptone. The range of dilutions suitable for donors, recipients, and recombinants will depend on the cell densities added. Dilutions from 10^{-5} to 10^{-7} should be suitable for the enumeration of the indigenous soil microbiota. The types and numbers of indigenous microbes present in the soils being studied should be evaluated before any experimental strains are introduced, so that observations made after introduction of the strains, which may alter the profile of indigenous microbes, can be compared with these baseline measurements. Aliquots of the dilutions of sample are plated on appropriate selective media, incubated, and the CFU recorded. A tared sample of the soil is dried for 24 h at 100 C, and the CFU are expressed and compared on the basis of 1 gram of oven-dried soil.

Recovery of donors, recipients, and transconjugants. The appropriate selective media are based on the phenotypic markers of the donor, recipient, and transconjugant strains, as in *in vitro* studies. In studies with nonsterile soil, the media used for the isolation of donors, recipients, and transconjugants may not be selective enough to eliminate or reduce sufficiently the indigenous soil microbiota to enable isolation and distinction of the introduced strains. To suppress the indigenous populations of fungi, 50 to 200 μg/ml Cy should be added to the recovery media. The concentration of Cy needed will depend on the type of soil and on the numbers and types of fungi present. Indigenous populations of bacteria may be counterselected by the antibiotics used to isolate the parentals or by a combination of antibiotics to which most of the indigenous populations, but not the parentals, are sensitive, e.g., a mixture of Sm, Ap, Nx, and Rf. Media that

suppress indigenous gram-positive bacteria can be formulated based on the phenotype of the experimental gram-negative strains, as in the case of E. coli x503 and x696, which are tolerant of 400 μg/ml eosin and 65 μg/ml methylene blue, whereas gram-positive bacteria are not (Krasovsky and Stotzky, 1987). The concentrations of the various antimicrobial agents necessary to suppress the indigenous microbiota will vary with different soils and should be determined in preliminary studies.

To distinguish between transconjugants that resulted from chromosomal transfer (as well as exconjugants that resulted from plasmid transfer, see below) in soil from those that may have resulted from matings between donors and recipients on the recovery medium, Nx is incorporated into the selective medium, which will inhibit gene transfer by Nx-sensitive donors (Curtiss, 1981; Walter et al., 1988a) (see Section VII).

Calculation of frequency of recombination (FOR). The FOR is calculated by dividing the number of transconjugants by the number of donors isolated from the soil (see FOR above).

Plasmid transfer

Studies to measure conjugal transfer of plasmid genes in soil require careful design and selection of plasmids and of donor and recipient strains, as well as extensive controls for both gene transfer and environmental variables. The nutrient content of the soil, the method of inoculation of the parental strains into the soil, the recovery of donor, recipient, and exconjugants from the soil, and the transfer of the plasmid to indigenous bacteria must also be considered.

Inoculation. The sequential addition of donor and recipient cells may affect the survival of the parental strains and the potential for gene transfer, particularly if one mating partner is not added for some time after the addition of the other partner. For example, preincubation of the recipient, P. aeruginosa PAO1, in soil did not appear to have any significant effect on its survival, whereas the donor, E. coli J53(RP4), declined rapidly after addition to soil. Gene transfer occurred immediately after log phase donor

cells were added to preincubated recipients (Fig. 18), whereas exconjugants were recovered only after 1 to 3 weeks of incubation of the soil when log phase recipients were added to preincubated donors (Fig. 19) (Devanas and Stotzky, 1988b).

The presence of nutrients, whether introduced with the inocula or as amendments during the incubation, affect the survival and potential for gene transfer in soil (Devanas et al., 1986). Studies on survival, recovery, and gene transfer in soil should be designed to include both unamended soil as well as soil amended with nutrients.

Recovery of donors, recipients, and exconjugants. The soil is sampled the same as for chromosomal transfer, and the procedures for the recovery of donors, recipients, and exconjugants are the same as those for in vitro plasmid transfer. However, the recovery of some strains from soil may sometimes be reduced, as the stress of being in soil may sufficiently debilitate these strains and affect their subsequent recovery. This phenomenon of "viable but nonculturable" has been described for bacteria in various aquatic systems (Roszak et al., 1984; Zaske et al., 1980). A similar phenomenon appears also to occur in soil, as shown by the differences between the CFU of some strains recovered on highly selective media (e.g., MAC+Tc) and on less stressful selective media (e.g., MAC) (Devanas et al., 1986). A number (50 to 100) of the colonies recovered on the less stressful media should be transferred to the more stringent selective media to determine if the differences in the proportion of CFU recovered by direct isolation on each medium are real or if the reduced numbers that developed on highly stressful recovery media were the result of a "viable but nonculturable" condition (see Figs. 18 and 19). The stress of being in soils may also retard the rate of growth of cells even on nonselective and nonstressful media. Consequently, repeated observation of the recovery plates, marking the colonies as they appear, and reincubating the plates until no new colonies appear may be necessary.

Transfer to indigenous bacteria. Gene transfer to indigenous bacteria is generally difficult to demonstrate, as selection for gene transfer to these bacteria is based solely

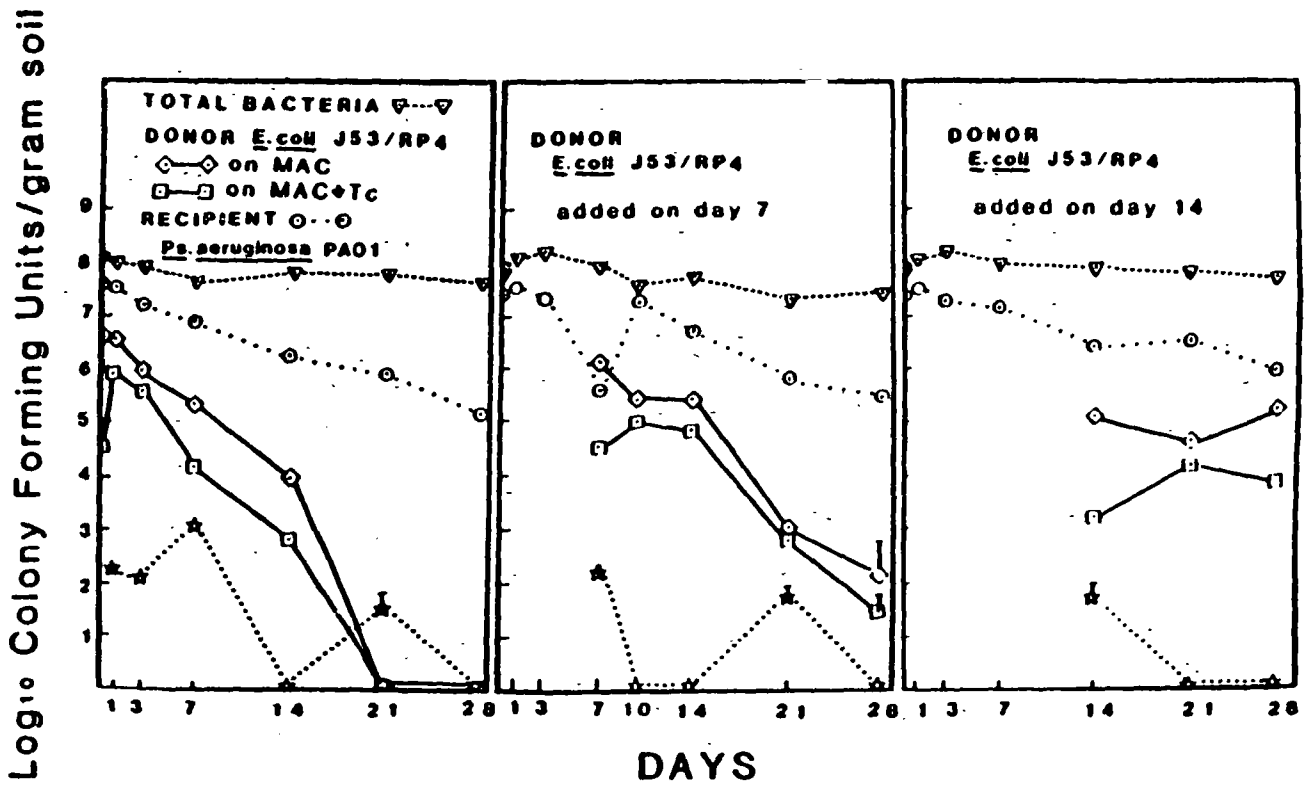

Figure 18. Effect of preincubation of recipient Pseudomonas aeruginosa PAOl cells on the survival and transfer of plasmid RP4 from donor Escherichia coli J53(RP4) cells in nonsterile soil. All cells were inoculated in saline. Donor cells were added immediately (i.e., on day 0) or after the soil had been incubated with recipient cells for 7 to 14 days. E. coli J53(RP4) was enumerated on MacConkey Agar (MAC) containing 25 µg/ml tetracycline (Tc); P. aeruginosa PAOl on Pseudomonas Isolation Agar (PIA); and PAOl(RP4) on PIA containing 200 µg/ml Tc. When the colony-forming units (CFU) of J53(RP4) on MAC + Tc were significantly lower than the CFU of E. coli J53 on MAC, selected colonies from the MAC plates were transfered to MAC + Tc. All transfers grew on MAC + Tc, indicating that the plasmid was not selectively lost from the J53(RP4) population but that the stress n soil produced a "viable but nonculturable" population of J53(RP4) that did not grow on MAC + Tc when isolated directly from oil (Devanas and Stotzky, 1988b).

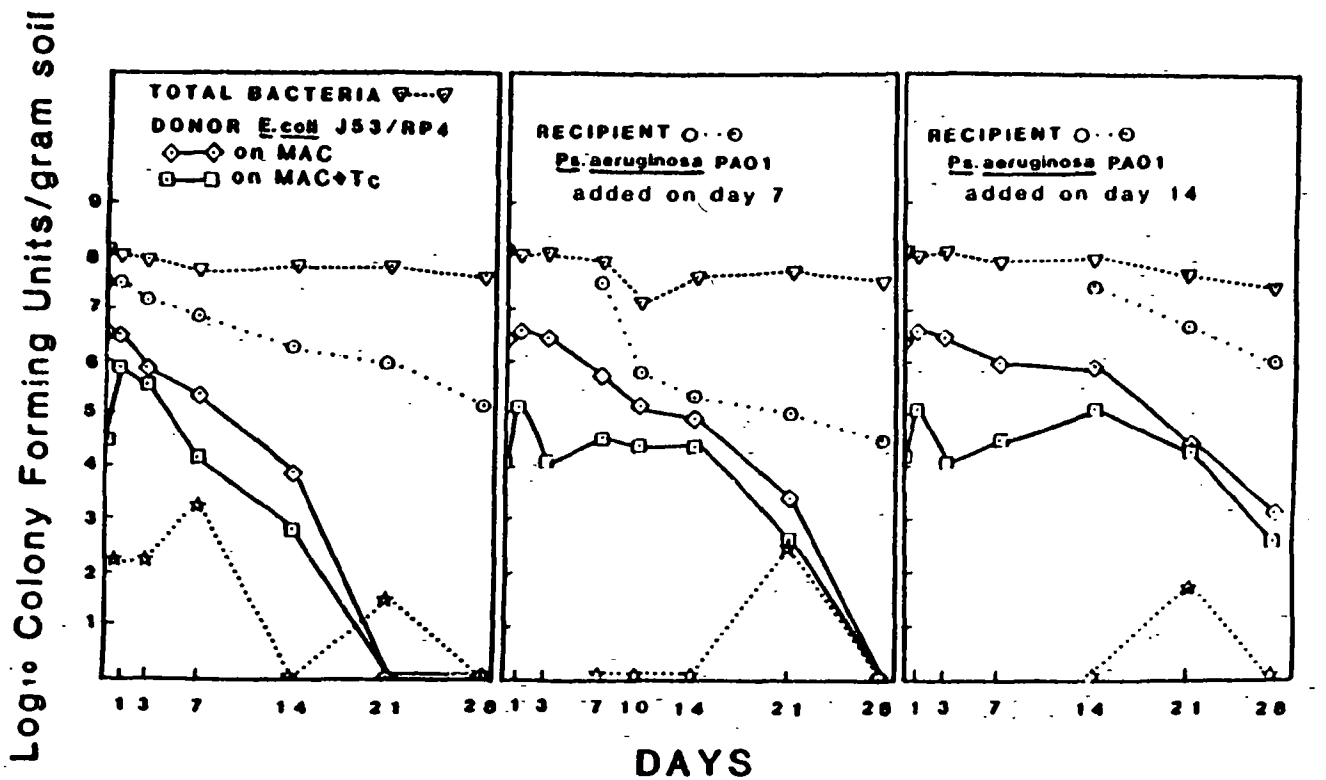

Figure 19. Effect of preincubation of donor Escherichia coli J53(RP4) cells on the survival and transfer of plasmid RP4 to recipient Pseudomonas aeruginosa PAO1 cells in nonsterile soil. All cells were inoculated in saline. Recipient cells were added immediately (i.e., on day 0) or after the soil had been incubated with donor cells for 7 to 14 days. E. coli J53(RP4) was enumerated on MacConkey Agar (MAC) containing 25 µg/ml tetracycline (Tc); P. aeruginosa PAO1 on Pseudomonas Isolation Agar (PIA); and PAO1(RP4) on PIA containing 200 µg/ml Tc. When the colony-forming units (CFU) of E. coli J53(RP4) on MAC + Tc were significantly lower than the CFU of E. coli J53 on MAC, selected colonies from the MAC plates were tranferred to MAC + Tc. All transfers grew on MAC + Tc, indicating that the plasmid was not selectively lost from the J53(RP4) population but that the stress in soil produced a "viable but nonculturable" population of J53(RP4) that did not grow on MAC + Tc when isolated directly from soil (Devanas and Stotzky, 1988b).

on the presence of the transferred marker genes. All colonies that appear on media selective for exconjugants must be tested for the presence of the marker genes by phenotypic (e.g., morphology, gram stain, biochemical characteristics) and physical (e.g., DNA extraction, agarose gel electrophoresis, DNA fingerprinting, DNA probes) methods (see Section VII). Such analyses should clarify whether the exconjugant isolated is the added recipient or an indigenous soil bacterium to which the introduced genes have been transferred. The intrinsic resistance of the indigenous soil bacteria to the antimicrobial agents used to evaluate the transfer of marker genes must be determined by plating appropriate soil dilutions of a control (i.e., uninoculated) soil sample on the various selective media used.

Limits of detection of gene transfer. There are always limits to the detection of microbial populations in any natural environment. These populations may: 1) not grow on any of the recovery media; 2) be present in such low numbers that unrealistically large volumes must be sampled; 3) adhere tightly to surfaces and are not removed for recovery by the diluents in serial dilution; and 4) be obscured or overgrown on the recovery media by faster-growing strains. All these problems occur in soil and make the detection of introduced GEMs and, especially, of any recombinants that may result from gene transfer difficult. The lower limit of the numbers of CFU detectable in soil samples is a function of the size of the soil sample, the ratio of the initial dilution of the soil sample (e.g., 1:10), and the volume of the dilution plated. For example, the recovery of 1 CFU when 0.1 ml of the 10^{-1} dilution of soil is plated represents a lower detection limit of 100 CFU/g soil. The limit of detection of a recombinant bacterium in a sample of soil is generally in the range of 1 to 20 CFU/g soil, and the level of confidence in this range is a function of the number of replicate soil samples evaluated and the number of CFU of the recombinants recovered (Devanas et al., 1986).

Stepwise Summary of Procedures for Studying Conjugation in Soil

- Prepare soil (e.g., sieve; amend with clays, $CaCO_3$, etc.; condition; add to appropriate microcosm).

- Prepare suspensions of donor and recipient cells.

- Wash suspensions by dilution or centrifugation.

- Adjust concentration of donor and recipient cells for appropriate conjugation ratio (1: 1 to 1: 20).

- Add donor and recipient cells sequentially to soil with sufficient water (or a nutrient solution) to bring the soil to its -33 kPa water tension.

- Incubate soil, preferably in a high-humidity chamber.

- Sample soil periodically (either by sacrificing multiple microcosms or from a batch system).

- Prepare appropriate serial decade dilutions of the soil.

- Plate dilutions on selective media suitable for growth of donor, recipient, and recombinant cells, as well as of indigenous soil bacteria.

- Incubate plates, and record numbers of colony-forming units that develop on each medium.

- Verify presumptive donor, recipient, and recombinant cells for phenotype and/or genotype (see Section VII).

VI. Methods for Studying Transduction

Transduction in vitro

Preparation of bacteriophage lysates. Two methods for preparing phage lysates of high titer are commonly used, depending on the phage-host system that is being studied. The first method involves inoculating a liquid culture of a susceptible host bacterium with an appropriate phage or inducing (e.g., by heat, ultraviolet radiation, chemical mutagens) a culture of a lysogenic bacterium. An example of the use of a lysogenic culture is described for the preparation of lysates of phage P1. Examples of other methods for preparing phage lysates in liquid culture can be found in Adams (1959).

Phage P1 Cm cts contains a temperature-sensitive mutation that enables the induction of the lytic cycle at temperatures above (e.g., 42 C) those optimal for growth of the host bacterium, E. coli (e.g., 37 C). E. coli AB1157, lysogenic for phage P1 and stored on LA slants containing 30 μg/ml Cm to maintain the temperate phage P1 DNA in the bacteria, is grown at 30 C in LCB to an optical density of 0.2 (at 600 nm) and shaken at 42 C for at least 2 h until lysis is evident (30 C is used for growth, to reduce spontaneous induction of lytic reproduction as the result of the temperature-sensitive mutation). Chloroform (0.5 ml/100 ml of culture) is added, with mixing, for 1 min to lyse cells and release additional phage virions, and the remaining intact cells are removed by filtration through a 0.45 μm nitrocellulose filter membrane (Millipore). The phage particles are stored in LCB at 4 C. Phage P1 titers between 10^8 to 10^{10} PFU/ml are commonly obtained.

An alternative method, which is more suitable with certain phages for the preparation of lysates of sufficient titer, is the agar-overlay method. An example of this technique, used with the generalized transducing phage, F116L, that infects P. aeruginosa, is described below (Miller and Ku, 1978). Although phage F116L does not contain a temperature-sensitive mutation, many lysogenic bacteria can be induced to lysis by growth at temperatures above their optimum, as the repressor protein that is

involved in the maintenance of lysogeny will be impaired in activity or inactivated by such higher temperatures. Phage F116L (10^5 PFU in 0.1 ml) and P. aeruginosa PAO1 (10^6 CFU in 0.1 ml) are mixed in 2.5 ml of melted LA (top agar), to give a multiplicity of infection (MOI; no. of phages: no. of host cells) of 0.1, and poured on the surface of LA plates (bottom agar). After incubation overnight (ca. 18 h) at 37 C, the top agar layer is removed in 5 ml of LB, the suspension is vortexed for 1 min and centrifuged for 10 min at 5000 g to pellet the bacteria and agar, and the supernatant is filtered through a 0.45 μm nitrocellulose membrane to remove remaining bacterial cells. The phage F116L stocks (10^8 to 10^{12} PFU/ml) are stored at 4 C.

The lysates can be purified of bacterial cell debris and soluble cytoplasmic components by various techniques, e.g., differential centrifugation on CsCl gradients, polyethylene glycol (Miller, 1972). However, the removal of such contaminants is seldom necessary or recommended for studies that are intended to simulate in situ situations.

Preparation of bacterial cultures. The bacterial cultures used as hosts for in vitro transduction should be grown to log phase in liquid culture (e.g., in LCB), and the optimum MOI for the specific host-phage system, determined in preliminary experiments, should be used. For transduction to E. coli of resistance to Cm by phage P1 Cm cts, an MOI between 1 and 5 is typically used with 10^7 to 10^8 CFU/ml of E. coli.

Transduction procedure. Transduction experiments in vitro should be conducted in liquid rather than on solid medium, to ensure adequate mixing of the phage and bacteria during the adsorption period. Phage P1 is allowed to adsorb on E. coli for 30 min at 30 C without shaking in LCB. The presence of Ca^{2+} and Mg^{2+} in this medium promotes phage P1 adsorption on the bacteria. The adsorption period is restricted to 30 min to prevent both the phage from completing a reproduction cycle and initiating a burst and the multiplication of transduced E. coli cells. After the 30-min adsorption period, E. coli transductants are enumerated by plating serial decade dilutions of the bacterium-phage suspension on either LA or MAC containing 30 μg/ml Cm. The E. coli inoculum is also

plated on this medium, to determine the number of spontaneous mutants to Cm resistance.

Quantitation of the bacterial and phage inocula should be conducted to determine the exact MOI in the transduction mixture. Dilutions of the E. coli suspension are plated on LA, and the plates are incubated at 30°C until colonies appear. Phage P1 is titered on LCA bottom and top agar at 42 C, and plaques are counted after 24 h.

The frequency of transduction is expressed as the number of transductants divided by the number of bacteria originally present in the transduction mixture. The number of phages added can be used as the denominator instead of the number of bacteria, to give a transduction frequency related to the total number of phage particles.

Transduction in soil

The design of transduction experiments in soil must consider the complex nature of soil and the fact that studies will be conducted over a period of several days or longer, thereby making it difficult to calculate the frequency of transduction. The major factors to consider are: the MOI; the use of either a phage lysate or a lysogenic bacterium as the source of the transducing phage; whether the recipient bacteria should to be added to soil after washing (e.g., in saline) or with nutrients (e.g., LB); and the methods used to enumerate selectively the added bacteria, transductants, and phages.

Inoculation and amendment of soil. The optimum MOI in soil should be determined before extensive experiments are designed, as it may differ from that in vitro. The number of phages added should be appropriately adjusted to the number of bacteria added to the soil. In studies of transduction by lysates of phage P1 in soil, 10^5 to 10^6 CFU/g soil of recipient E. coli J53(RP4) or W3110(R702) are typically added, to avoid adding unrealistically high numbers of bacteria, and the MOI is approximately 3. E. coli and phage P1 are added individually in 0.1 ml of LCB or saline, so as to bring the final wet weight of the soil to 2 g (Zeph et al., 1988). When lysogenic strains of E. coli are used as the source of phage P1, the same numbers of lysogenic and recipient E. coli cells are

added. Control studies with soil inoculated with the same numbers of only phage P1 or only E. coli donors or recipients must be conducted, to determine both survival and mutation rates.

The addition of nutrients can be achieved most easily by diluting the host and transducing phage in fresh LCB before their addition to soil. Amendment of the soil with LB or other nutrient solutions can be made periodically, to measure the effects of nutrients on transduction and on the multiplication and survival of the phages and host cells. The potential for transduction in the absence of exogenous nutrients can be determined most easily by diluting the host and phage inocula in saline before their addition to soil. For example, inocula of E. coli and phage P1 (10^8 to 10^9 CFU or PFU/ml) raised in LCB are diluted 1: 1000 in saline, and a final inoculum of 10^5 to 10^6 CFU or PFU/g soil is added. Concentration and washing in saline of the host cells can be accomplished simultaneously by centrifugation at 5000 g for 10 min. However, as the viability of the inoculum could be affected by centrifugation, viable counts of the washed suspensions should be determined.

Incubation and sampling. Soil samples containing phage and bacteria should be incubated at approximately 25 C (the average temperature of soils in temperate zones) and maintained at their -33 kPa water tension in a high-humidity incubator and on the basis of periodic weight measurements and the addition of sterile distilled water (sdH_2O) when necessary. Three to four samplings of the soil for the enumeration of recipient and donor bacteria, phage, and transductants should be made during the first week (e.g., on days 0, 1, 3, and 7), and then sampling should be at least weekly until there are no significant changes in these populations. If sufficient personnel and funds are available, the numbers of total bacteria, fungi, protozoa, and other indicators of species diversity should be evaluated concurrently.

In studies on the transduction of E. coli by phage P1 in soil, experiments were conducted for 28 days (Zeph et al., 1988). Individual soil tubes were sacrificed at each

sampling by the addition of 18 ml of sdH_2O to duplicate or triplicate soil tubes that contained 2 g soil (i.e., a 1: 10 dilution), and subsequent serial decade dilutions were spread-plated on selective media. Plates for the enumeration of E. coli donors, recipients, and transductants were incubated at 30 C; for phage P1 at 42 C; and for total and gram-negative indigenous soil bacteria at 25 C.

Enumeration. The efficacy of the selective media used for the enumeration of the various groups of bacteria (i.e., transductants, donors, recipients, and indigenous) in nonsterile soil is an important parameter to establish before the start of an experiment, especially as the numbers of bacteria transduced in soil can be relatively low. In studies on the transduction by phage P1 Cm cts::Tn501 of Cm- and Hg-resistance to E. coli, the resistance markers on the phage genome were utilized to monitor the number of transduced E. coli in soil, and the antibiotic-resistances coded on nonphage plasmids enabled following the fate of the recipient or donor (lysogenic) E. coli populations. E. coli transductants were enumerated on MAC containing Cm (90 μg/ml; MAC-Cm) or Hg (20 or 30 μM; MAC-Hg), as the lactose-utilizing strains of E. coli form distinctive dark red colonies on MAC. Recipient E. coli J53(RP4) and W3110(R702) were enumerated on MAC containing Tc (25 μg/ml; MAC-Tc), resistance to which is encoded on the plasmids.

In studies in nonsterile soil with lysogenic E. coli donors and nonlysogenic recipents, the donor E. coli J53(P1 Cm cts) was enumerated on MAC-Cm, the recipient E. coli W3110(R702) on MAC-Tc, and the transduced E. coli W3110(R702)(P1 Cm cts) on MAC containing both Cm and Tc at the concentrations shown above. The lowest limit of detection of E. coli donors, recipients, and transductants in nonsterile soil was 10^2 to 10^3 CFU/g soil, as growth of indigenous soil bacteria resistant to the concentrations of Cm, Tc, and Hg used interfered with accurate detection of lower numbers of E. coli (Zeph et al., 1988). The problem of interference by indigenous soil bacteria resistant to antimicrobial agents is discussed below.

When lysogenic E. coli J53(RP4)(P1 Cm cts::Tn501) donors and nonlysogenic E. coli

W3110 were added to sterile soil, Hg-resistant transductants of E. coli W3110 (i.e., W3110(P1)) were enumerated on M9 minimal agar containing 30 μM Hg (M9M). The auxotrophic E. coli J53(RP4)(P1) donor does not grow on minimal agar, and it was enumerated on MAC-Tc. MAC was used to enumerate total E. coli [E. coli J53(RP4)(P1), W3110, and W3110(P1)], which enabled the numbers of E. coli W3110 to be calculated from the difference between the total E. coli and the E. coli J53(RP4)(P1) plus W3110(P1) counts.

Auxotrophic recipients can also be utilized to study transduction in soil. For example, a Sm-resistant E. coli K-12 recipient that was auxotrophic for threonine and leucine was used to study transduction by phage P1 in nonsterile soil (Germida and Khachatourians, 1988). Lysates of phage P1 were raised on prototrophic E. coli. The minimal salts agar medium (MSA), which contains Sm, Cy to suppress fungi, and eosin and methylene blue to suppress the growth of bacteria other than enterobacteria, was satisfactory for the isolation of E. coli transductants prototrophic for threonine and leucine and resistant to Sm.

Minimal media can also be used to counterselect an auxotrophic E. coli lysogen, as discussed above (Zeph et al., 1988). Nevertheless, the use of markers for resistance to antimicrobial agents is more efficient for monitoring transduction in soil, as many soil bacteria will often grow more readily on minimal media than on media containing antimicrobial agents. Moreover, when using a minimal agar medium to select for transductants, incubation of the plates for more than 3 or 4 days can result in the syntrophic growth of the auxotroph and the formation of background lawns on the plates. This makes it difficult to differentiate between cells that have been transduced to prototrophy and cells of the auxotrophic recipient.

Detection of transductants in nonsterile soil. The detection of transductants in nonsterile soil is difficult, as there are no efficient methods for the enumeration of low levels of transduced bacteria. Chromosomally-borne genes for resistance to Nx and

other antibiotics or to heavy metals can be exploited in the isolation of a host bacterium containing either or both of these groups of genes. However, isolation of specific hosts based on resistance, either chromosomal or vector-borne, to many commonly used antibiotics (e.g., Sm, Cm, Tc) or some heavy metals (e.g., Hg) has limitations, as many bacteria in many soils now exhibit resistance to these antimicrobial agents. The limit of detection for most methods is, at best, between 10^2 to 10^3 transductants/g soil, and it is often several orders of magnitude higher. In some soils, the "background" resistance to many common antimicrobial agents can be as high as 50% of the isolatable bacteria. Increasing the level of resistance in the host bacterium through mutation and selection or concurrently using a second antibiotic- or heavy metal-resistance marker often results in reduced recovery, as some bacteria are not able to express readily the antimicrobial-resistance phenotype after exposure to the stressful conditions in soil (Devanas et al., 1986; Pettibone et al., 1987). Furthermore, resistance to antimicrobial agents may be lost if the resistance genes are located on a plasmid that may disappear through segregation during the period of the soil study. A "viable but nonculturable" physiological state, in which the cells remain viable but are so debilitated that they will not form colonies on selective media (e.g., Devanas et al., 1986; Roszak and Colwell, 1987), has been suggested as one cause of the poor recovery of some introduced bacteria from soil. However, by initially plating the soil dilutions on nonselective media, to allow "resuscitation" of the stressed bacteria, and then replica-plating to selective media, specific donors, recipients, and transductants can be enumerated (see Section VII).

Recovery of phages from soil. Difficulties can be encountered in the recovery of phages from soil, as adsorption of phages on soil particulates, especially on clay minerals, can result in apparent decreases in phage titers in soil. For example, phage P1 adsorbs strongly on soil components, probably on the clay fraction, as over 95% of the added phage P1 pelleted with the soil particulates after centrifugation of soil amended with this phage (Zeph and Stotzky, unpublished). The usual technique for the recovery of

phage particles from soil, which is filtration of appropriate soil dilutions through a 0.22 or 0.45 μm membrane filter, removes phage P1 from the dilutions, as the phage binds on soil constituents present in the dilutions and impacted on the membrane.

An alternative technique involves the use of nonfiltered soil dilutions and plaque-assay lawns of antibiotic-resistant E. coli grown on media containing the appropriate antibiotic at concentrations inhibitory to soil bacteria (Zeph et al., 1988). For example, 100 μg/ml Tc is added to LCA bottom and top agar, which allows the growth of confluent lawns of E. coli J53(RP4) and the formation of plaques by phage P1. To enumerate free phage P1 in soils inoculated with E. coli lysogenic for the phage, chloroform should be added to a concentration of 2% (vol/vol) to the initial soil dilution for 5 min, with periodic vortexing, to kill the lysogens and to prevent their prophages from forming plaques on the assay lawn. Once the chloroform has settled to the bottom of the initial dilution tube (usually after approximately 1 min), dilutions are prepared for plaque assay of the phage, as described above. The use of chloroform should be restricted only to chloroform-resistant phages, such as phage P1.

As the recovery of some phages from soil can be low, specific eluents have been used to desorb various phages from soil particulates. For example, the substitution of skim milk for dH_2O as the initial diluent increases the recovery of phage P1 from less than 10% (dH_2O) to 60% (skim milk) of the original number of phage particles added to soil (Zeph and Stotzky, unpublished). Other common eluents used to desorb phages from soil are casein, nutrient broth, beef extract, glycine, EDTA, and egg albumin (Bitton, 1980). However, recovery of phage P1 with the eluents so far tested has been less than 100%.

Suppression of fungal growth on selective media. Fungal growth on selective media containing dilutions of nonsterile soil can be suppressed by the addition of Cy to the media. The concentration of Cy to use depends on the population level of fungi in the specific soil and the soil dilutions to be plated. A concentration of 50 to 200 μg/ml

Cy will usually inhibit essentially all fungal growth in the 10^{-1} dilution of nonsterile soil (Tremaine and Mills, 1988; Wollum, 1982).

Transduction frequency. The calculation of transduction frequencies *in situ* is complicated by the fact that studies in soil are usually conducted for periods of days or weeks. Inasmuch as transduced bacteria may multiply in soil and produce cells with the same phenotype, it is not possible to distinguish between multiplication and transduction. Consequently, estimates of transduction frequency are best obtained early in a soil study before significant multiplication of cells occurs. The transduction frequency is expressed as the number of transductants divided by the number of recipient bacteria originally added.

Stepwise Summary of Procedures for Studying Transduction in Soil

- Prepare soil (e.g., sieve; amend with clays, $CaCO_3$, etc.; condition; add to appropriate microcosm).

- Prepare suspensions of cells of recipient and lysogenic bacteria or phage lysate.

- Wash cell suspensions by dilution or centrifugation.

- Adjust suspensions for appropriate multiplicity of infection (MOI) for transduction (MOI = 1 to 5).

- Add cells of recipient and lysogen or phage lysate sequentially to soil with sufficient water (or a nutrient solution) to bring the soil to its -33 kPa water tension.

- Incubate soil, preferably in a high-humidity chamber.

- Sample soil periodically (either by sacrificing multiple microcosms or from a batch system).

- Prepare appropriate serial decade dilutions of the soil.

- Plate dilutions on selective media suitable for growth of recipient, lysogen, and transductant, as well as of indigenous soil bacteria. Plaque assay the dilutions for free phage.

- Incubate plates, and record numbers of colony-forming and plaque-forming units that develop on each medium.

- Verify presumptive recipient, lysogen, and transductant cells for phenotype and/or genotype (see Section VII).

VII. Identification, Characterization, and Confirmation of Recombinants

Introduction

The isolation of genotypically-identical bacteria, whether donors, recipients, or recombinants, for identification is most commonly and easily done by separating individual cells on a solid nutrient medium. Streaking or spreading serial dilutions of soil directly on the surface of the medium or suspending the dilutions in molten agar before making pour plates are means of separating cells so that they produce separate colonies (i.e., aggregates of cells) that presumably arise from each individual cell. However, even the formation of a single colony does not guarantee that the colony is a pure culture, and it is advisable to restreak the colony of interest until colonies are produced that are identical to one another, both macroscopically and microscopically. Even pure cultures can exhibit some variation, e.g., smooth-rough colony morphology, presence and absence of spores, and variability in the gram stain. Microbiology laboratory manuals describe in detail the methods for obtaining and maintaining pure cultures from isolated colonies (Atlas et al., 1988; Ausubel et al., 1987; Benson, 1986).

Selective media

Conventional methods of enumeration and identification depend on the growth and "phenotypic expression" of measurable traits of an organism under conditions that restrict or inhibit organisms without the traits, e.g., selective media. Many methods can be used to select or enrich for specialized, individual phenotypes, and most microbiology laboratory manuals provide descriptions of various selective and differential media and their applications. When sampling soil environments, specialized media will be necessary for the enumeration of typical groups of soil microbes (e.g., nitrifying, nitrosofying, denitrifying, sulfate reducing, and cellulolytic bacteria; fungi; protozoa), if knowledge of the spectrum of soil microbes present is desired. A soil extract medium will be required to recover oligotrophic microbes that are not recovered on more copiotrophic standard

laboratory media (Wollum, 1982) (see Appendix).

Media for the detection and differentiation of GEMs on the bases of their metabolic and cultural requirements are useful for the detection, identification, and enumeration of novel genes in soil. The use of strains that are labeled or "tagged" with genes that enable a particular metabolic activity (e.g., toluene degradation, use of X-gal) or confer resistance to an antimicrobial agent (e.g., Tc or Hg) facilitates the detection of the GEM containing the novel genes. If the strains are to be released to the environment, it is essential to choose antibiotics that are not used for treatment of humans or other animals, as microbial resistance to clinically-useful antibiotics is a major health problem.

Genes that are carried extrachromosomally may be unstable unless there is selection pressure, as the plasmids may be lost through segregation (see Section II). Therefore, to monitor the persistence of introduced novel genes, it is preferable to use genes that are located on the chromosome rather than on a transposable element that readily recombines with other sites or elements, such as transmissible plasmids. It may be beneficial to use a strain with two genetic markers, so that a spontaneous mutation will not eliminate the detectability of the phenotype when it is plated on media containing each selective agent individually, as well as on media containing both agents. The additive effect of a second antibiotic or heavy metal in the selective medium may also reduce the background level of contaminating indigenous microbes, as autochthonous microbes are not usually multiply-antibiotic resistant (see Section V).

The use of microbes tagged with genes that code for metabolic activities that can be linked to a chromogenic substrate in a selective medium is a distinct advantage in their detection and enumeration. For example, the insertion of genes for lactose fermentation into a nonenteric bacterium will facilitate its enumeration on media that are selective for coliforms, e.g., Eosin methylene blue agar, MAC agar, Endo-agar, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) agar, IPTG (isopropylthio-β-D-galactoside,

an analogue of lactose) agar, as the chromogenic substrates aid in the visualization of colonies of tagged organisms (Jain et al., 1988). Various cloning vectors have been developed that use the lacZ (β-galactosidase) gene as a marker, e.g., phage M13 (Messing, 1979) and plasmid pUC (Vieira and Messing, 1982, 1987). These DNA sequences that code for lactose metabolism have been integrated into the chromosome of the host to provide a marker for monitoring the GEM in the environment (Drahos et al., 1986). The xylE gene from the TOL plasmid of P. aeruginosa has been cloned into other genera of bacteria, which then produce a yellow color on catechol-containing media, as the result of the activity of catechol oxygenase (Walter et al., 1988b).

Some strains of Pseudomonas (e.g., P. aeruginosa, P. fluorescens) release fluorescent water-soluble pigments, that are easily visualized under ultraviolet light. Various types of Pseudomonas isolation media are commercially available.

"Breakthrough" of indigenous microbes

Many indigenous soil microbes are inherently resistant to the antibiotics and/or heavy metals routinely used in selective media for the isolation of cells containing novel genes. The use of additional antibiotics in the medium to reduce the growth of indigenous bacteria is sometimes effective. The upper limits of tolerance of the novel genes and the hosts in which they may reside to the selective antibiotics and/or heavy metals must be defined, so that the maximum levels of the antimicrobial agents can be used in the recovery medium to reduce the growth of the indigenous microbiota. Prolonged incubation of plated soil dilutions, even on media containing multiple antibiotics, may also permit the "breakthrough" growth of indigenous microbes, particularily of fungi (Krasovsky and Stotzky, 1987). Hence, shorter incubation periods and the enumeration of small colonies may be necessary to estimate the populations of recombinants before they are overgrown by indigenous microbes. Morphological differences, e.g., in colony size, shape, texture, and pigmentation, between the colonies of the GEM and those of indigenous microbes sometimes enable the distinction between

introduced experimental and autochthonous strains.

The high numbers of indigenous soil bacteria (10^8 to 10^9 CFU/g soil) will sometimes have a "sparing" effect on the antimicrobial action of the selective agents. Under these conditions, the next higher dilution of the soil sample may reduce the numbers of indigenous microbes sufficiently to reduce or eliminate the sparing effect and, thereby, breakthrough.

Indigenous soil fungi may frequently break through the selective recovery medium and obscure or inhibit the growth of the bacterial strains of interest. The antifungal agent, Cy, should be incorporated into the recovery medium at concentrations from 50 to 200 μg/ml, depending on the soil, to curtail fungal growth with no effect on the growth of bacteria.

Viable but nonculturable bacteria

A phenomenon referred to as "viable but nonculturable" was first described for aquatic bacteria that could not be recovered or detected on complex synthetic laboratory media but which retained the ability to produce a virulent infection in laboratory animals (Colwell et al., 1985; Roszak and Colwell, 1987). The inability to detect some introduced GEMs directly from soil on initial isolation media has also been reported (Devanas et al., 1986). If the host-novel gene systems to be studied show a tendency towards "viable but nonculturable", it may be advisable to use initially a less selective recovery medium (e.g., MAC) rather than one that may be inhibitory (e.g., MAC + Tc) to the microbes stressed in the soil environment, even though the phenotype of the host-gene system is for resistance (e.g., Tc^r). Colonies recovered on the less selective medium are then tested on a more stringent selective medium for expression of the resistance phenotype. In other words, a "resuscitation" step is introduced for the "stressed" experimental GEMs, after which the complete resistance profile is expressed (see Section V).

Oligotrophic media may be necessary for the recovery of indigenous soil organisms that are not accustomed to the relatively high levels of nutrients contained in standard

laboratory media. Various oligotrophic media have been suggested for the recovery of microorganisms from soil (Wollum, 1982).

Gene transfer on recovery media vs. in situ

One of the purposes of developing methods for the monitoring, isolation, and identification of GEMs is to detect the transfer of their novel genes *in situ*. If the concentration of parentals in the soil dilutions is high, there is a possibility of cell contact between the parentals on the recovery medium. Consequently, there is the possibility that any gene transfer observed, particularly by conjugation, did not occur in soil but only on the recovery medium. Several methods have been suggested to determine whether the presence of recombinants on the recovery medium is a result of gene transfer *in situ* or *in vitro* (Curtiss, 1981). In one method, the recipient strain is selected to be resistant to Nx, which is incorporated into the medium along with other selective agents, for the recovery of the recombinant. Donor cells will be incapable of participating in gene transfer on this medium, as Nx inhibits the DNA gyrase necessary for the transfer of DNA (Curtiss, 1981; Walter *et al.*, 1987). Cells that grow on the Nx-containing medium and have the selectable phenotype for the novel genes are assumed to be recombinants that resulted from gene transfer in soil.

DNA "fingerprinting" and plasmid profiles

Restriction endonuclease analysis or DNA "fingerprinting" can be used to detect and monitor the fate of specific genes. Restriction endonucleases recognize specific nucleotide sequences in double-stranded DNA and cleave the DNA at a specific sequence that is usually four to eight base pairs long. Different restriction endonucleases recognize different sequences, and depending on the frequency of the recognition sequence, a specific endonuclease will cleave the DNA numerous times, which will result in a number of different-sized DNA fragments that can then be separated according to their molecular mass (M_r) by agarose gel electrophoresis. The M_r of each fragment is estimated by comparing the migration of the DNA fragments to DNA standards of known

M_r. Markers of M_r that are commonly used to produce a standard curve are restriction endonuclease digests of DNA from coliphage lambda or plasmid pBR322, and many DNA fragments and plasmids of known M_r are commercially available. The distribution of different-sized fragments resulting from the cleavage by different endonucleases is unique for each chromosome or plasmid analyzed; hence, a "fingerprint" of the specific DNA is obtained (Ausubel et al., 1987).

The bacterial strains that express the specific phenotype determined by the genes of interest are isolated in pure culture, as described above. The total DNA is extracted from these cultures by any of several procedures (Ausubel et al., 1987; Crosa and Falkow, 1981; Maniatis et al., 1982). Chromosomal DNA is separated from plasmid DNA by linearization of chromosomal DNA by mechanical shearing followed by differential centrifugation techniques, e.g., cesium chloride-ethidium bromide density gradient centrifugation, or by agarose gel electrophoresis. The choice of procedure will depend on the information desired from the isolated DNA. The appropriate DNA containing the gene of interest (i.e., either on the chromosome or on a plasmid) is treated with a series of endonucleases, which are commercially available (e.g., International Biotechnologies Inc., New Haven, CN; New England Biolabs, Beverly, MA; Bethesda Research Laboratories, Gaithersburg, MD) (Ausubel et al., 1987).

Crude but rapid screening of intact plasmid preparations can be used to evaluate hundreds of colonies a day for the presence of plasmid DNA by gel electrophoresis (Kado and Liu, 1981). This is a well-established method for the characterization of plasmids. Plasmid profiles (number and size per strain) have been used to identify bacterial populations and to study plasmid epidemiology in natural environments, including soil (Jain et al., 1988).

DNA probes

Introduction. DNA-DNA hybridization can be used to detect specific gene sequences in GEMs that have been added to soil or other natural environments and in

indigenous bacteria to which the genes may have been transferred. A highly specific and unique DNA sequence in the chromosome of the host or in the vector (e.g., a plasmid or a bacteriophage), preferably in the novel DNA, is isolated and purified, as described above, and labeled with either a radioactive nuclide (e.g., ^{32}P) or a chromogenic agent (e.g., biotin-streptavidin-alkaline-phosphatase-dyes) (Ausubel et al., 1987). DNA probes are often prepared from genes cloned in plasmid vectors so that the probe DNA can be conveniently produced in sufficient quantities. This DNA probe is hybridized with DNA from bacteria (usually those that express the specific phenotype on selective media) that have been isolated from soil and immobilized and lysed on a nitrocellulose or nylon filter membrane. Bacteria whose DNA hybridizes with the probe are then located on the filter.

One advantage of hybridization with DNA probes is the relatively high degree of specificity when compared with classical microbiological plating methods on selective media. Another advantage is that microorganisms can be detected directly in soil without prior growth on selective media. Techniques for DNA hyoridization in studies of microbial ecology include Southern blot, dot or slot blot, and colony hybridization (see below).

Methods that isolate total bacterial DNA from soil and then hybridize this DNA with specific DNA probes may be valuable in: 1) monitoring the survival, establishment, and growth of, and gene transfer by, GEMs in soil (see Holben and Tiedje, 1988; Steffan et al., 1988); 2) the detection of groups of microorganisms capable of performing the same function (e.g., containing nif genes); 3) monitoring gene flow among indigenous bacteria; and 4) the detection of nonculturable microorganisms (Jain et al., 1988). These direct DNA methods, however, have a detection sensitivity of only about 4×10^4 cells/g soil (Holben et al., 1988), whereas some selective isolation methods have a sensitivity approaching 2×10^1 cells/g soil (Devanas et al., 1986). Moreover, direct methods for the detection of novel genes in soil, including DNA hybridization, do not distinguish between viable and nonviable microorganisms. The choice between using DNA hybridization or

classical microbiological methods will depend on the information and the sensitivity required. In all probability, both methods, in various combinations, will be used. Furthermore, DNA hybridization methods that are more sensitive, specific, and rapid, as well as less expensive, will undoubtedly be developed.

Types of DNA probes. The probe DNA must be labeled with a tag that will enable its detection when hybridized to homologous (i.e., target) DNA sequences in the cells being probed. The first labeling technique for DNA probes was the use of radioactive nuclides, e.g., ^{32}P, which were incorporated into the nucleotides of the probe DNA (Ausubel et al., 1987; Maniatis et al., 1982). ^{32}P is preferred to ^{35}S or ^{3}H, as it has a higher specific activity and, therefore, results in the greatest sensitivity. Moreover, the activity of enzymes that act on DNA labeled with ^{32}P is not affected, as ^{32}P causes only small changes in the structure of DNA. After hybridization to its target DNA, the probe DNA is visualized by exposure of the hybridization membrane to X-ray film, which is sensitive to the gamma rays emitted by ^{32}P.

An alternative to autoradiography are chromogenic techniques that use DNA labeled with nonradioactive molecules, such as biotin. The biotin-labeled DNA is visualized by the application of a streptavidin-alkaline phosphatase (Leary et al., 1983) or streptavidin-peroxidase (Syvanen et al., 1986) conjugate, followed by enzymatic conversion of a chromogenic substrate to produce a color, which identifies the target DNA with which the probe has hybridized on the filter.

Preparation of DNA probes. Labeling of the probe DNA with radioactive nucleotide triphosphates can be accomplished by nick translation. DNA polymerase I adds labeled nucleotides to the 3'-OH terminus at nicked sites along one strand of double-stranded DNA. This enzyme also has exonuclease activity and removes unlabeled nucleotides that are then replaced with radiolabeled nucleotides, which results in DNA of high specific activity, e.g., greater than 10^8 cpm/μg of DNA when labeled with ^{32}P (Maniatis et al., 1982). Nick translation is not the only method for radiolabeling DNA for

use as probes. End-labeling of small DNA fragments with (γ-^{32}P)nucleotides, using polynucleotide kinase from coliphage T4, or labeling with (α-^{32}P)oligonucleotides, using random primers and the Klenow fragment of E. coli polymerase I or phage T4 polymerase, can be used to obtain radiolabeled DNA with specific activities that are often higher than those obtained by nick translation (Bentjen et al., 1989). Procedures for the isolation of DNA, probe preparation, hybridization with target DNA, and detection of the hybridization signal are detailed in Ausubel et al. (1987) and Maniatis et al. (1982).

Three different techniques are available for the preparation of nonradioactive DNA probes, i.e., nick translation, chemical labeling, and photolabeling. Commercially-prepared kits are available for all three methods of DNA labeling, which result in nucleotides labeled with biotin.

Nick translation is the most frequently used method of labeling DNA with biotin (Brigati et al., 1983). Kits, in which biotin-labeled dATP or dUTP and enzymes are provided for the nick translation reaction, are available from Bethesda Research Laboratories and Enzo Biochem, Inc. (New York, NY). Procedures outlined by the manufacturer or in Ausubel et al. (1987) and Maniatis et al. (1982) can be used to label DNA with biotinylated nucleotides; as the nick translation reaction is the same as used with radionucleotides.

Chemical labeling of the DNA probe involves the insertion of an antigenic sulfone group into the cytosine moieties of the denatured DNA (Renz and Kurz, 1984). A specific monoclonal antibody is then bound to the sulfone group, followed by the application of an enzyme-anti-immunoglobulin conjugate that catalyzes a chromogenic substrate system and enables visualization of the labeled probe DNA. Kits are available from FMC Bioproducts (Rockland, ME).

Procedures for the direct photolabeling of DNA with biotin have been described by Forster et al. (1985). This is a nonenzymatic method in which the probe DNA is labeled by irradiating photoactivated biotin with a high intensity lamp in a mixture containing

the DNA. Photolysis of an azide group on photobiotin results in covalent bonding to the base group on the nucleic acids. Visualization is achieved by the application of a streptavidin-enzyme conjugate and a chromogenic substrate, as described above. Highly purified DNA must be used, as the highly reactive photoactivated biotin will bind to proteins, tris buffer salts, and polyethylene glycol and cause nonspecific signal development. A commercial kit that supplies the photobiotin and procedures for labeling is available from Sigma Chemical Co. (St. Louis, MO) or Bethesda Research Laboratories.

Hybridization techniques.

Southern hybridization. Hybridization of DNA probes to target DNA that has been separated on agarose gels by electrophoretic techniques and transferred to a nitrocellulose or nylon filter membrane is termed Southern hybridization (Southern, 1975). Details of the procedures used for Southern hybridization are presented in Ausubel et al. (1987) and Maniatis et al. (1982).

Two distinct protocols for isolating the target DNA from soil samples for Southern hybridization have been developed: 1) direct extraction of DNA from soil samples (Ogram et al., 1987); and 2) extraction of the bacterial population from soil, followed by lysis of the bacterial cells (Holben et al., 1988). Direct extraction of nucleic acids from soil is more efficient than the recovery of intact bacterial cells. However, direct extraction suffers from the limitations that a heterogeneous DNA sample is obtained, as eucaryotic and cell-free DNA are also extracted, and that humic acids, among other soil constituents, contaminate the DNA sample, which could result in decreased hybridization of the DNA probe to target DNA. Although the recovery of intact bacterial cells by differential centifugation is a more lengthy process and results in the recovery of only approximately 80% of the total bacteria presumably present in soil (Holben et al., 1988), the DNA sample is not contaminated with humic acids and nonbacterial DNA, and it undergoes less shearing, so that the sample contains DNA of more uniform size than that obtained by direct extraction. Treatment of the purified DNA extract with restriction

enzymes before hybridization enables the comparison of the size of the labeled fragment with a known restriction map of the DNA from the GEM of interest and, thereby, easier identification of restriction fragments that contain the novel gene (Jain et al., 1988). Moreover, restriction analysis of DNA isolated from soil, either directly or indirectly, can detect gene rearrangements that may have occurred in soil (Holben et al., 1988). Gene arrangements and deletions have also been detected in freshwater, suggesting that genetic instability may be enhanced in natural habitats (O'Morchoe et al., 1988).

Slot/dot blot hybridization. Slot or dot blotting techniques of DNA hybridization involve the placement of bacterial cells, viruses, or extracted DNA at specific locations on nitrocellulose or nylon filters. The cells or viruses are then lysed, and the DNA is immobilized on the filter and hybridized with the DNA probe. For example, dot blots are prepared by placing aliquots (5 to 10 μl) of target DNA directly on the filter with a micropipettor, forming a circular dot of bacteria, phage, or DNA for hybridization. Alternatively, slot blot apparatuses are available in which samples are placed in slots (30 to 40 samples per filter are possible) and immobilized on the hybridization filter by the application of a vacuum (Steffan and Atlas, 1988). Slot blot hybridization with radiolabeled DNA probes has been used to detect novel genes in GEMs added to a nonsterile sediment microcosm (Sayler et al., 1986; Steffan and Atlas, 1988), and dot blot hybridization with a biotinylated DNA probe has been used to evaluate presumed E. coli transductants isolated from nonsterile soil that was inoculated with E. coli and phage P1 (Zeph and Stotzky, 1989).

Colony hybridization. The hybridization of a DNA probe to target DNA released from bacterial colonies impregnated into nitrocellulose or nylon filters is termed colony hybridization (Grunstein and Hogness, 1975). Descriptions of the methods employed in colony hybridization with DNA probes can be found in Ausubel et al. (1987) and Maniatis et al. (1982). Colony hybridization techniques have been used to detect specific gene sequences naturally present in soil bacteria (Barkay et al., 1985) and novel genes

introduced into bacteria that had been added to soil (Rafii and Crawford, 1988). Normally, dilutions of soil are plated on selective media, and plates with well separated bacterial colonies are used as the source of colony DNA. The colonies are transferred to a filter membrane for lysis and hybridization. Alternatively, the screening of large numbers of bacteria for the presence of novel DNA can be accomplished if lawns of bacteria (approximately 10^5 to 10^6 cells/plate) are used in colony hybridizations with ^{32}P-labeled probes (Sayler et al., 1985), particularly if the isolation of the organism of interest from discrete colonies is not desired. However, when this procedure is used with biotin-labeled DNA probes, problems can arise with nonspecific signal development during the probe visualization step (see below).

The advantage of the colony hybridization technique is that it often enables a more rapid screening of environmental samples for the presence of microorganisms containing a novel gene(s) than Southern hybridization or slot/dot blot techniques. However, the microbes must grow on the isolation media, to produce sufficient target DNA for hybridization, in contrast to the Southern hybridization method. For some purposes, e.g., monitoring gene transfer from an introduced GEM to the indigenous bacterial population, colony hybridization is the preferred method, as growth of the introduced GEM can be inhibited on selective media, thereby allowing hybridization of the probe with colony DNA from only the indigenous population. Jain et al. (1988) emphasized that preliminary testing of the DNA hybridization protocol is important to avoid false positives when attempting to detect GEMs in environmental samples: a good selective medium must be used to prevent growth of indigenous bacteria that can give false positive hybridization signals; and the specificity of the DNA probe for the novel gene must be ascertained before its use in the monitoring of GEMs in the environment.

Sensitivity and specificity of DNA probes. The sensitivity of radiolabeled DNA probes is generally greater than that of nonradioactive probes. Zwadyk et al. (1986) reported that a ^{32}P-labeled probe was 100 times more sensitive than biotinylated probes

in detecting plasmid pBR322 DNA with Southern hybridization, and a minimum of 22 ng of target DNA was necessary for detection with biotinylated plasmid pBR322 DNA probes. However, when purified target DNA was spotted on nitrocellulose filters, Zwadyk et al. (1986) detected as little as 9.7 pg of target DNA with a ^{32}P-labeled probe and 39 pg with a biotinylated probe prepared with a kit (Bethesda Research Laboratories). Zeph and Stotzky (1989) detected 50 pg of target DNA from phage P1 using the same biotinylated probe kit. Leary et al. (1983) reported greater sensitivity with a biotinylated DNA probe made from linearized rather than from intact plasmid DNA, and they detected 3.1 pg of target DNA by Southern hybridization.

Another expression of sensitivity is the minimum number of cells containing target DNA that can be detected in a background of indigenous bacteria on enumeration plates by colony hybridization. This is useful in comparing DNA probes on the basis of their ability to detect GEMs added to natural environments that may contain high numbers of indigenous bacteria. Sayler et al. (1985) utilized ^{32}P-labeled DNA probes to detect P. putida added to sediment microcosms at a sensitivity of 1 colony of P. putida in a background of 10^6 indigenous bacterial colonies that contained nonhomologous DNA.

The minimum number of cells containing homologous DNA that can be detected is also used as a measure of the sensitivity of DNA probes. For example, Holben et al. (1988) were able to quantitate Bradyrhizobium japonicum, added to nonsterile soil, at levels of 4.3×10^4 cells/g soil, which was equivalent to 0.2 pg of target DNA per 1 μg of total purified DNA isolated from soil bacteria. Frederickson et al. (1988) used the most probable number (MPN) method, combined with DNA hybridization of DNA isolated from individual MPN tubes, to detect P. putida, added to nonsterile soil, at concentrations as low as 10 cells/g soil and Rhizobium leguminosarum at 100 cells/g soil. The application of DNA probe techniques to studies of microbial ecology has been reviewed by Holben and Tiedje (1988).

The relatively low sensitivity of DNA probes in detecting and enumerating bacteria containing homologous DNA in soil can be overcome, in part, through the amplification of the target DNA sequences. The polymerase chain reaction (PCR) procedure has been used to increase probe sensitivity by using the hybridized DNA probe as a primer for chain elongation beyond the target DNA sequence (Somerville et al., 1988; Saiki et al., 1988). After hybridization of the probe to target DNA, DNA synthesis is initiated from the 3'-OH terminus of the probe DNA strand with radiolabeled nucleotides and DNA polymerase. The PCR is repeated 20 to 30 times by remelting the DNA strands and hybridizing with fresh probe DNA, followed by another chain elongation reaction, which results in an enhancement of the sensitivity of the DNA probe by at least 3 orders of magnitude. The specificity of the probe depends on the initial hybridization to target DNA, and the sensitivity is enhanced by DNA synthesis from the free 3'-OH end into adjacent genes (Colwell et al., 1988). Steffan and Atlas (1988) employed the PCR method to increase the sensitivity of a probe for the detection of catabolic genes in Pseudomonas cepacia AC1100 that was added to sediment microcosms.

The use of biotinylated DNA probes has an inherent problem, as contamination of the sample with protein will result in nonspecific color development caused by the binding of the avidin-enzyme conjugate to protein components in the cell lysate, in addition to binding to the biotin moiety on the labeled probe. Consequently, all protein must be removed from the hybridization filters when using biotinylated DNA probes, particularly when whole bacterial cells are lysed on the filter, as in slot/dot blots or colony hybridizations. Treatment of the filter with chloroform, phenol, and proteolytic enzymes will remove significant amounts of cell debris and protein (Haas and Fleming, 1986; Rafii and Crawford, 1988; Zeph and Stotzky, 1989).

In summary, radiolabeled DNA probes have the advantage of being more sensitive in many applications than biotinylated probes, although sensitivity is being improved in the latter. However, biotinylated DNA probes are less hazardous than radiolabeled DNA,

have a much longer storage life (one year or more compared to two weeks for ^{32}P-labeled DNA), and have a lower cost.

Serological techniques

Serological methods, based usually on the expression of specific surface antigens, require the isolation of the bacteria of interest on media that are relatively selective (i.e., that eliminate or reduce the growth of unrelated soil bacteria). The bacteria are purified and then challenged with a spectrum of polyclonal or monoclonal antibodies raised against the bacteria of interest. The reactions between the bacteria and the antisera (usually polyclonal) are then scored as being either: 1) nonreactive; 2) cross-reactive with many sera without distinctive features and common to all isolates; 3) cross-reactive with distinctive features between isolates and a few antisera; or 4) a specific reaction with a single antiserum. Although this method can be highly specific in some instances, the surface antigens of some bacteria change during growth, and they may be different in soil than in the pure cultures that were used to raise the antigens. Moreover, serological data do not always agree with data obtained with DNA probes and with other characteristics of the bacteria (e.g., Schofield et al., 1987).

Although polyclonal antisera are easier to prepare, they are less specific that monoclonal antibodies. The use of labeled monoclonal antibodies provides a highly specific technique for the direct detection of GEMs in the environment. However, there are significant limitations in this methodology: e.g., the presence of the novel DNA in the microbe is only presumptive and must be verified by other methods; the sensitivity is low when employed in soil studies; it is not possible to differentiate between viable and nonviable cells (Colwell et al., 1988). The detection of GEMs in the environment with monoclonal antibody techniques has been reviewed by Omenn (1986).

Heat induction of prophages

Verification of the transfer of novel genes by transduction is possible with some phages by induction of the lytic cycle of replication of the prophage. Zeph et al. (1988)

confirmed by heat induction of lysis that presumptive E. coli transductants, isolated from soil inoculated with E. coli and phage P1 on the basis of the antimicrobial-resistance phenotype coded for on the phage P1 genome, contained the phage (Table 2). The phage P1 strain used had a temperature-sensitive mutation in the c1 gene, which codes for a repressor protein that maintains lysogeny. The presumed E. coli transductants were grown in LCB at 30 C, with shaking, until log phase (optical density of 0.2 at 600 nm), followed by induction of lysis by incubation at 42 C. The presence of phage P1 virions in the culture was demonstrated by spotting aliquots of the heat-induced lysate on lawns of E. coli. It might be possible to use this technique to confirm the presence of any prophage DNA in presumed transductants, as incubation of lysogenic bacteria at temperatures above their optimum for growth should inactivate or impair the activity of the repressor protein that maintains lysogeny. Moreover, other agents that can induce the lytic cycle (e.g., UV radiation, mitomycin C) should be evaluated with lysogens that do not respond to elevated temperatures.

When attempting to demonstrate gene transfer to indigenous soil bacteria by transduction by phage P1, isolates of soil bacteria were grown at 25 C until log phase, exposed to 42 C for 30 min, and then reincubated at 25 C to allow lysis of the bacteria by the phage. Continued incubation was not at 42 C, as most soil bacteria have temperature optima for growth between 20 and 30 C and may not grow at the 42 C used to induce prophage P1.

VIII. Quality Assurance/ Quality Control

Sample representativeness and custody

All soil samples should be contained in separate and well-identified (e.g., labeled with waterproof ink) bags or plastic-lined garbage pails. All bacterial cultures should be maintained at 4 C in pure culture on slants in well-identified test tubes and transferred regularly. Purity and phenotype should be determined after each transfer. Soil stocks should be located in a separate locked room, and stocks of bacteria, phages, media, clays, etc. should be in locked laboratories to which only personnel of the laboratory and appropriate security personnel have access. These stocks should be under the direct supervision of the Principal Investigator.

Sampling procedures

Stocks of soils, clays, bacteria, phages, media, etc. should be thoroughly mixed before each experiment and before subsampling to ensure maximum homogeneity. The number of subsamples will vary from experiment to experiment, depending on results of previous experiments, and should be based on significance between treatments at a probability level (P) of less than 0.05.

Comparability

Microbiological data should be reported as number of CFU or PFU/g soil, oven-dry equivalent. Frequency of recombination is expressed as a per cent of donor, recipient, or donor plus recipient, depending on the study. The denominator used to obtain the percentage must be clearly indicated. All other data (e.g., physicochemical soil characteristics; nomenclature and phenotype of bacteria, plasmids, and bacteriophages; M_r of DNA fragments) should be reported in standardized units.

Calibration procedures and frequency

All calibration activities should be thoroughly documented. Calibration records should be on permanent file in bound notebooks.

Instrumental. Thermometers for monitoring temperatures of refrigerators, freezers, incubators, laboratories, etc. should be verified biannually against a thermometer traceable to the National Institute of Standards and Technology. Buffer solutions for the calibration of pH meters can be obtained from commercial sources (2 to 3 pH values that bracket the working range of pH should be evaluated by this method, in addition to electronic resistance methods). Pipettors and glass pipettes should be calibrated every six months by gravimetry; random samples of disposable pipettes should also be checked periodically for accuracy. Spectrophotometers should be calibrated biannually with decade dilutions of several sources or types of DNA, protein, and bacteria. Calibration of agarose gel electrophoresis is conducted with commercial DNA M_r standards (these standards should be run with all unknown preparations). Microscopes should be calibrated annually with decade dilutions of standard-sized polystyrene latex particles. For instruments used routinely, a check list should be fastened on or near each instrument, and each user should initial and date the list after routine calibrations have been made before and after analysis of samples.

Analytical. ^{32}P- and other DNA probes should be verified for specificity with the cDNA against which they were prepared. Similar verification can be made for probes for gene products. These verification procedures can also serve to test the reactivity of the X-ray films and the efficacy of the film developer solutions, the enzyme color development reaction for nonradioactive DNA probes, and the sensitivity of monoclonal antibodies in serological techniques.

Microbiological. The phenotype of all novel bacteria and the infectivity of the phages should be verified in pure culture before experiments are conducted in soil. Furthermore, studies in sterile soil can provide another level of verification for studies in nonsterile soil. Spectrophotometric calibration of bacterial numbers should be correlated periodically with microscopic and plate counts. The efficacy of the sterilization of soils must be determined by plating on several media, ranging from nutrient-rich to nutrient-poor.

Analytical procedures

Quality assurance must be established by numerous replicates per experiment and repeat experiments. When more than one genetic marker is contained in the novel DNA, direct or replica plating should be to selective media specific for the detection of each marker, either individually or in combination. Furthermore, DNA probes should be used when necessary (e.g., when there appears to be a loss or segregation of the novel DNA). When analytical procedures developed by others are used, the verification procedures should be those of the developers. It must be emphasized that the study of gene transfer in soil is a relatively new area of research, and sufficient published data are not available for routine verification and quality assurance. Therefore, verification of gene transfer by novel bacteria by several techniques is required.

Experimental design and statistical analyses

The experimental design and the statistical analysis to be applied to the data must be determined before the start of each study. The assistance of a statistician is helpful at this stage. The number of replicates to be used should be sufficient to detect a P of less than 0.05. This number of replicates is determined in preliminary studies, with the application of appropriate statistical theory and formulas. At the end of each experiment, the data must be statistically analyzed (e.g., means ± standard errors of the means, Student's t-test, ANOVA, regression analyses) and plotted or tabulated (Atlas and Bartha, 1981). As mentioned above, data shoud be expressed in standard terms, or if not, the dimensional units should be clearly defined.

Experimental errors are detected by abnormally high coefficients of variation (greater than 50%) between replicates and by unanticipated and unexplainable deviations in time-response curves. Inasmuch as experiments must not only be sufficiently replicated and statistically analyzed, but also repeated, errors will be readily detected. In the event of such errors, replicated experiments should be repeated. Although statistically significant differences may be observed in some experiments, the potential ecological significance of these differences must be further evaluated (e.g., in repeat experiments or in more complex microcosms),

especially if the differences are transitory.

Data analysis and reporting

Data should be recorded in ink on appropriate data sheets, in duplicate, in permanent, bound laboratory notebooks. All entries should be initialed. One copy should reside with the research technician collecting the day-to-day data, and the other should reside with the Project Manager. A sample data sheet is included (see Appendix).

Internal quality control checks

The routine quality controls of a microbiology laboratory should be followed. For example, periodic checks (at least monthly) must be conducted on laboratory water for microbiological quality (less than 10^4 CFU/ml) and conductance (less than 2 μS/cm); the temperature of refrigerators ($\pm$2 C), freezers ($\pm$4 C), incubators ($\pm$2 C), and autoclaves (121 C) should be continually monitored (usually twice on a measurement day); pH and quality checks should be made on media lots; sterility checks on media lots, water, soils, and other autoclaved or radiation-sterilized materials should be made by plating. A log book of internal quality controls should be maintained. These internal quality control checks are routine in basic research laboratories.

Preventative maintenance

All major pieces of laboratory equipment should be monitored regularly and serviced routinely, as individually required, through service contracts with outside contractors, as well as internally.

Corrective action

If any experiments or parts of experiments have been inappropriately designed, conducted, or analyzed, the entire experiment must be repeated. It must be emphasized, however, that gene transfer among bacteria in soil is essentially an unexplored area and that the development of appropriate experimental design, conduct, and analyses is an intrinsic part of the research.

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APPENDIX

Media Composition (all media prepared with distilled water)

1. Luria Broth (LB) (Maniatis *et al.*, 1982)

tryptone	10 g
yeast extract	5 g
glucose	1 g
sodium chloride	5 g
dH_2O	1 L

2. Luria Broth with calcium and magnesium (LCB) (Zeph *et al.*, 1988)

   LB containing:

   10 m*M* $MgSO_4 \cdot 7H_2O$

   2 m*M* anhydrous $CaCl_2$

3. Luria Agar (LA) (Maniatis *et al.*, 1982)

   LB containing:

   Bacto-agar 15 g/L

4. Luria Top Agar (Maniatis *et al.*, 1982)

   LB containing:

   Bacto-agar 7.5 g/L

5. Minimal Salt Agar (MSA) (Germida and Khachatourians, 1988).

glucose (or lactose)	10 g
vitamin B_{12}	0.1 g
tetracycline	0.1 g
streptomycin	0.2 g
eosin	0.4 g
methylene blue	0.07 g
leucine	0.02 g
threonine	0.08 g
Bacto-agar	15 g
dH_2O	1 L

6. M9 Minimal Agar containing $HgCl_2$ (M9M) (Zeph et al., 1988)

Na_2HPO_4	6 g
KH_2PO_4	3 g
NaCl	0.5 g
NH_4Cl	1 g
Bacto Agar	15 g
dH_2O	1 L

after autoclaving, filter-sterilized solutions of the following are added to yield the indicated final concentrations: 0.2% glucose, 2 m$\underline{M}$ $MgSO_4 \cdot 7H_2O$, 0.1 m$\underline{M}$ $CaCl_2$, and 30 $\mu\underline{M}$ $HgCl_2$.

7. Minimal Liquid (ML) (Curtiss, 1965)

NH_4Cl	5 g
NH_4NO_3	1 g
Na_2SO_4	2 g
K_2HPO_4	9 g
KH_2PO_4	5 g
$MgSO_4.7H_2O$	0.1 g
Glucose	5 g
dH_2O	1 L

Autoclave separately at 121 C at 15 psi; pH = 6.8.

8. Minimal Agar (MA) (Curtiss, 1965)

salts in ML

Glucose (autoclave separately)	5 g
Bacto-agar	15 g
dH_2O	1 L

pH = 6.8

9. Minimal Mating Medium (3M) (Curtiss et al., 1968)

NH_4Cl	5 g
NH_4NO_3	1 g
Na_2SO_4	2 g
K_2HPO_4	4.9 g
KH_2PO_4	6.3 g
Glucose (autoclave separately)	5 g
dH_2O	1 L

pH = 6.3

10. Soil Extract Agar (SEA) (Allen, 1957)

KH_2PO_4	0.5 g
Glucose	1 g
Soil extract	100 ml
Tap water	900 ml

adjust to pH 7.0

Preparation of soil extract:

1.0 kg of garden soil is heated with 1000 ml tap water in the autoclave (121 C, 15 psi) for 30 min. A small amount of $CaCO_3$ is added, and the soil suspension is filtered several times through a double paper filter (Whatman No. 1) until no turbidity is evident. This solution is bottled in 100 ml quantities and autoclaved before storage.

Antimicrobial Agents Commonly Used in Selective Media *

Agent	Abbrev	Special instructions	Initial diluent	Conc (mg/ml)	Approx stability at 4 C (days)	Conc (µg/ml)	ml to add to 1 L
ampicillin	Ap	Filter[a]	dH_2O[c]	25	7	200	8
carbenicillin	Cb	Filter	dH_2O	50	14	100	2
cephalothin	Ceph	Filter	0.1 M PB[d]	12.5	5	25	2
chloramphenicol	Cm	Filter	EtOH	25	30	25	1
chlortetracycline	CTc	Use foil[b]	50% EtOH[e]	10	5	15	1.5
cycloheximide	Cy	Use foil	dH_2O	12.5	5	25	2
gentamicin	Gn	Filter	dH_2O	12.5	30	25	2
kanamycin	Kn	Filter	dH_2O	25	30	50	2
mercuric chloride	Hg	Filter	dH_2O	40	30	40	1
nafcillin	Nf	Filter	dH_2O	1	5	2	2
nalidixic acid	Nx	Filter	0.1 M NaOH	16	30	32	2
rifampicin	Rf	Use foil	MeOH[f]	25	5	100	4
streptomycin	Sm	Filter	dH_2O	12.5	30	25	2
tetracycline	Tc	Use foil	50% EtOH	10	5	15	1.5
tobramycin	Tb	Filter	dH_2O	5	30	10	2
trimethoprim	Tp	Filter	15% 0.06 M HCL	2	30	50	25

All concentrated stock solutions are to be labeled with name of agent, concentration, date, and analyst's initials.
Filter sterilized
Store in dark covered with foil
Distilled water
Phosphate buffer, pH 6
Ethanol
Methanol

courtesy of A. Porteous

Experiment No.: _____

Incubation time: _____

Amendment: _____

Page _____ of _____ pages

Date: _____

	Tube					Tube							
Dilution	Plate	Plate	Plate	S	$\bar{x}$	Plate	Plate	Plate	S	$\bar{x}$	S	N	$\bar{x}$
10^{-}													
10^{-}													
10^{-}													
10^{-}													
10^{-}													
10^{-}													
S													
$\bar{x}$													

Comments:

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S													
$\bar{x}$													

Comments: