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DEVELOPMENT OF GENE PROBE METHODOLOGY FOR DETECTION OF SPECIFIC BACTERIA IN ENVIRONMENTAL SAMPLES

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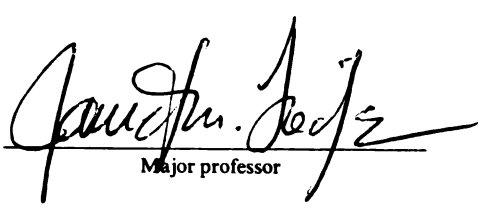

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DEVELOPMENT OF GENE PROBE METHODOLOGY FOR DETECTION OF SPECIFIC BACTERIA IN ENVIRONMENTAL SAMPLES

by

Janet Knutson Jansson

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ABSTRACT

DEVELOPMENT OF GENE PROBE METHODOLOGY FOR DETECTION OF SPECIFIC BACTERIA IN ENVIRONMENTAL SAMPLES

by

Janet Knutson Jansson

A novel method based on DNA-DNA hybridization was developed for detection of specific microorganisms in environmental samples. This methodology involved extraction and purification of total bacterial DNA from soil. Isolated DNA was analyzed by Southern or slot/dot blot analysis using radioactively labeled gene probes. Single stranded ^{32}P-labeled gene probes were used, allowing detection of approximately 10^3 gene copies/g soil.

I used this methodology to detect strains of pseudomonads in soil that were engineered by inserting the *npt*II gene, encoding kanamycin resistance, into their chromosomes. This gene was then used as a specific probe for the recombinant strains. The two strains were distinguished from each other in the same soil sample by Southern blot analysis based on differences in restriction fragment sizes of DNA containing the *npt*II gene. A genomic rearrangement (deletion recombination) of the engineered DNA insert was detected in soil inoculated with one of the recombinant strains.

To assay the fate of engineered microorganisms in soil, a versatile soil core system was developed. Selective plating and gene probes were used to determine the effect of different environmental parameters such as roots, competition, and soil water on survival and movement of the recombinant strains. Roots increased the movement of

bacteria recovered in leachate, but had no effect in unleached cores. The Pseudomonas strains were equally effective colonizing wheat roots but they had different requirements since the presence of one did not impede the colonization of the other.

Gene probe methodology was also used to detect a unique aryl-dechlorinating microorganism, strain DCB-1, in anaerobic enrichments since it cannot be enumerated by conventional methods. Whole chromosomal probes of DCB-1 and a more specific 2.35 kb probe were used to estimate the proportion of DCB-1 in sludge and sediment communities and to determine the relatedness of DCB-1 to organisms in other dehalogenating enrichments. DNA-DNA hybridization also suggested that among the organisms examined, DCB-1 appears to be most closely related to certain sulfate-reducing bacteria. These examples demonstrate that gene probe methodology is a useful tool for detecting specific genes or specific microorganisms in soil and other complex environments.

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CHAPTER 1

INTRODUCTION

General

Microorganisms are crucial members of ecosystems and their activities are important on a global scale. Microorganisms have long been recognized for their role in carbon and nutrient cycling. More recently, microorganisms were discovered that either promoted or inhibited plant growth. In addition, some microorganisms produce antibiotics, or toxins and others show promise as biological control agents of pests. Bacteria have recently been discovered that promote formation of ice nuclei on crops resulting in frost damage. Furthermore, some microorganisms can degrade toxic chemicals that would otherwise persist in the environment. These properties and others make microorganisms useful in industrial and agronomic applications.

Genetic engineering technology has enabled some useful properties of microorganisms to be manipulated resulting in improved microbial products. For example, the gene encoding ice nuclei formation was deleted from a *Pseudomonas syringae* (Lindow, 1985). This organism could colonize plant leaves and prevent later colonization by pseudomonads containing the deleterious gene. Another example is a biological pesticide produced by insertion of a *Bacillus thuringensis* gene encoding toxin production into *Pseudomonas fluorescens* (Watrud et

al, 1985). The engineered strain colonized roots and produced the toxin in an environment where the target insect pest was a problem.

These are just two examples of microbial products that are being developed for use in the environment. In the United States NIH, NSF, USDA and EPA, in a coordinated framework, have oversight over the approval of any environmental testing of genetically engineered microorganisms (Federal Register, June 26, 1986). The responsible agency is determined by the source of research funding. All commercially funded research is under the jurisdiction of EPA as is all research, regardless of funding, on pest control agents since EPA administers FIFRA. Thus, EPA has had the major responsibility to develop criteria and evaluate proposed environmental release experiments. To improve their ability to efficiently and reliably assess risk, EPA has placed a priority on development of methodology for monitoring genetically engineered microorganisms in the environment.

Methodology

Several types of methodology have been developed for tracking specific microroganisms in environmental samples. The most common method, selective plating, depends on a unique trait of the strain of interest allowing only that strain to grow on the selective medium. The most common selective trait used is the resistance to antibiotics. Antibiotic resistant markers can be obtained by selection of spontaneous mutants, or they may be engineered with genes that encode antibiotic resistance. This is accomplished by introduction of plasmid genes, transposons, or by homologous recombination of resistance genes into the chromosome. Other selective markers rely on

unique properties of a gene uncommon to the indigenous bacterial population, such as the ability to use lactose (*lac*) or to degrade unique compounds.

Another method used to trace specific microorganisms in the environment is direct, microscopic counting of organisms that bind a fluorescent antibody. This methodology has been used to trace microorganisms that are no longer viable or no longer culturable on selective media (Rollins and Colwell, 1986; Xu et al, 1982). Although this method is tedious and not very sensitive, it can be specific and is sometimes preferential to selective plating methodology.

Recently, methodology has been developed to trace specific bacteria in environmental samples by use of gene probes (Barkay et al, 1985; Sayler et al, 1985). Gene probes can be developed to be specific for particular microorganisms. For bacteria engineered with a foreign gene, such as the ability to degrade a toxic compound, the foreign gene can act as the probe. This methodology has been used to screen different microbial colonies on solid media for identification of a specific organism or gene. The DNA in each of the colonies is analyzed by colony hybridization where the DNA is bound to a filter and probed with the novel gene of interest. Only those colonies containing like DNA should hybridize to the probe and give a positive signal. This methodology has been very useful, although it has the same drawback as selective plating in that microorganisms must be culturable to be detected.

In Appendix A, Holben and I describe another detection method that we developed for tracing recombinant bacteria in soil. Total soil bacterial DNA is isolated from environmental samples and the

presence of a specific organism is detected using a gene probe. More than one organism can be detected in the same sample using different gene probes or relying on different sizes of DNA fragments that can be distinguished from each other. This methodology overcomes the requirement for growth. Gene probe analysis of DNA isolated form soil can be very specific, and plasmid DNA as well as chromosomal genes can be detected. Although this methodology is not as sensitive as selective plating (Fig. 1), use of highly specific and sensitive probes can increase the detection capability. It is important to recognize that when using any detection method the inability to detect a recombinant strain does not necessarily mean that the organism has "died out" in soil. Instead, the organisms may have "died back" to a level in soil that is below the detection limit of the particular methodology used (Fig. 1).

Fate of microorganisms in the environment

The methods outlined above allow the fate of organisms to be determined after their release to the environment. There are several possible fates to consider. Alexander (1985) has outlined a tier of fates that determine the potential risk of a recombinant microorganism in the environment. First, does a microorganisms survive? If it survives does it multiply? If it multiplies does it disperse, and so on. Another factor of concern, particularly with respect to engineered bacteria, is the possiblity of transfer of the engineered gene to other species. It is at least theoretically possible that a gene harmless in one species may spread to another species where it may cause harm. Also, uncontrolled spread of a particular gene may increase the concentration of a gene to a hazardous level.

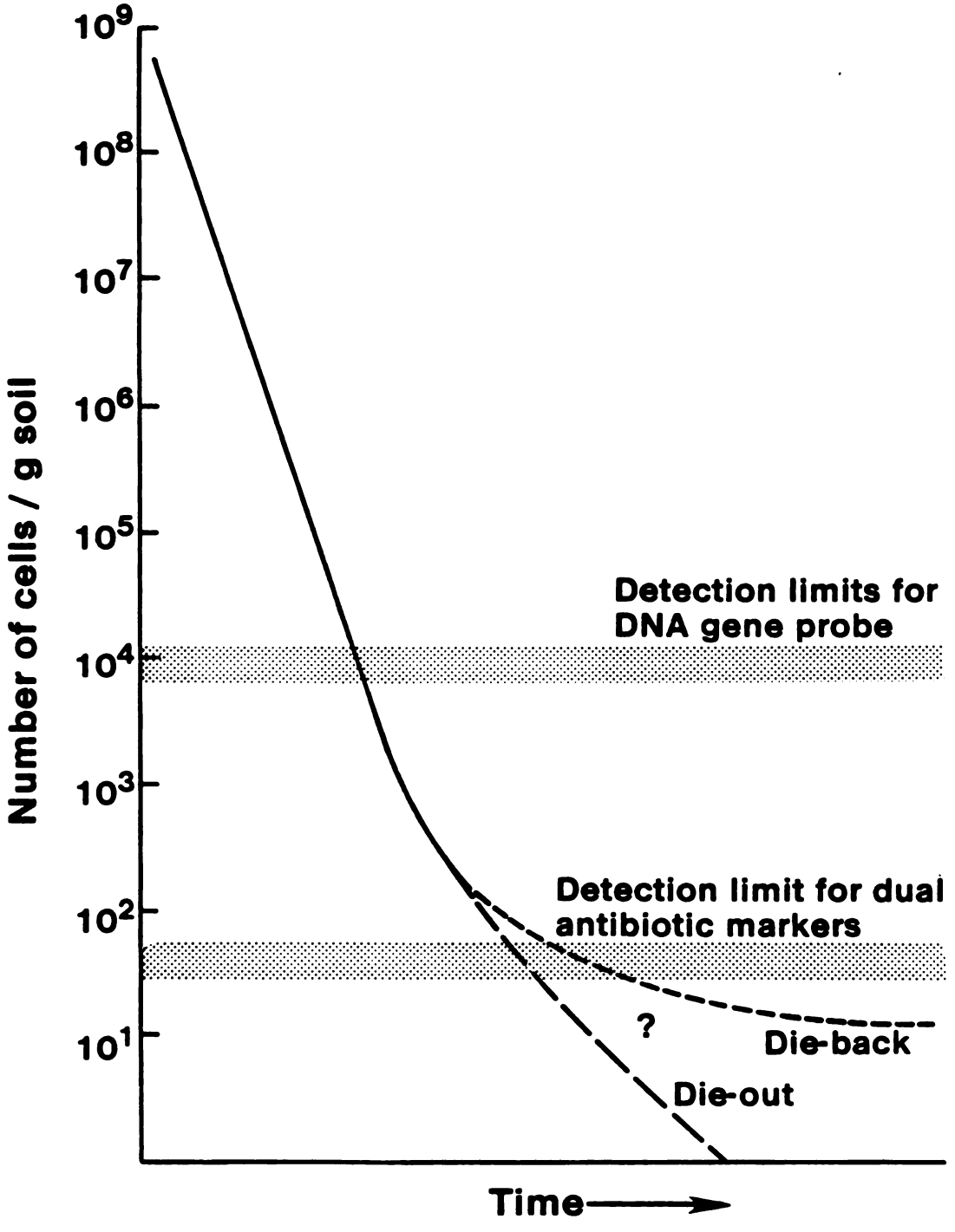

Fig. 1. Theoretical detection limits of gene probe and selective plating methodologies.

Conjugation, transformation, and transduction are the best known means by which DNA can be transferred from one organism to another, and some of these processes have been observed to occur in soil (Graham and Istock, 1981; Weinberg and Stotzky, 1972).

Survival

Survival of a microorganism introduced into the environment is contingent on a variety of factors. Some microorganisms foreign to a particular environment are unable to successfully compete with the indigenous population for nutrients and space. Walter et al. (1987) found that *Escherichia coli* or a plasmid-containing pseudomonad survived poorly in soil extracts that were not filtered, compared to soil extracts in which the indigenous microflora was eliminated by filtration. Weller (1983) found that introduced bacteria occuppied sites in the rhizosphere normally available to native bacteria. However, if the root was already colonized by the native microflora, the introduced strain was unable to displace them. In contrast, space was not a competitive factor for growth of pseudomonads in soil previously colonized by *Arthrobacter* sp. (Labeda et al., 1976).

The initial inoculum density may also influence the subsequent survival of introduced microorganisms. If microorganisms are added to soil at a high concentration they may be subject to predation (Mallory et al, 1983). Alternatively, Dupler and Baker (1984) found that a greater initial population density of *Pseudomonas putida* ultimately resulted in a higher stable population.

Other environmental factors that contribute to an organisms' survivability include moisture, temperature, soil type, and roots. For example, pseudomonads were particularly subject to water stress

(Labeda et al., 1976; Dupler and Baker, 1974) whereas *Arthrobacter* appeared to be very tolerant to dry conditions (Labeda et al., 1976).

Temperature can affect survival of bacteria in soil and on roots. Rouatt et al. (1963) postulated that the highest population of root-colonizing microorganisms was obtained at the temperature optimum for the plant. For example, rhizosphere pseudomonads increased in number on wheat roots as the temperature decreased to the optimum temperature for growth of winter wheat.

Soil type is another factor that can influence survival of an introduced microorganism. The wheat root population of an aggressive root colonizer was found to be dependent on soil type (Fredrickson and Elliot, 1985). An engineered strain of *Pseudomonas fluorescens* survived better in silt loam than loamy sand (van Elsas, 1986). The better survival observed in finer textured soils could be due to increased protection against harsh soil conditions. In addition, Mahler and Wollum (1981) found that both soil texture and water affected the survival of *Rhizobium* sp. and that numbers were particularly depressed under extremes of soil texture (e.g. sand or clay loam).

Roots are another factor that contribute to the survivability of some microorganisms. A direct rhizosphere effect was observed for *E. chloacea* and survival of this strain was poor in the absence of wheat roots (Dijkstra et al., 1987). However, van Elsas et al., (1986) observed no difference in survival of a recombinant pseudomonad in soil with or without roots.

Therefore, the survival patterns of introduced bacteria can vary between different organisms and incubation conditions. Many

microorganisms inoculated into soil decrease in numbers to a lower value which remains relatively constant. This was observed for *B. subtilis* (van Elsas et al., 1986), pseudomonads in soil extracts (Walter et al., 1987) and pseudomonads in two cropped or uncropped soils (van Elsas, 1986, and Weller, 1983). Alternatively, Madsen and Alexander (1982) and Dupler and Baker (1984) observed growth of *Pseudomonas putida* in soil. Conversely, *Rhizobium* (Madsen and Alexander, 1982) and *Arthrobacter* (Labeda et al., 1976) remained at fairly constant population densities in the soil.

The nature of the genetic alteration may also influence survival in the environment. However, plasmids were not found to have a significant effect on survival of pseudomonads in filtered or non-filtered soil extract (Walter et al., 1987). Devanas and Stotzky (1986) had similar results with a plasmid-carrying strain in soil except that the recombinant strain was less tolerant of starvation conditions.

Dispersion

If microorganisms survive and multiply in the environment, the next question is whether there is dispersal from the site of inoculation. The most effective means of dispersal in soil is that caused by water percolation (Bitton et al., 1974; Hambdi, 1971; Wong and Griffin, 1976; Griffin and Quail, 1968; Madsen and Alexander, 1982; Chao et al., 1986; Parke et al., 1986). In the absence of percolating water, microorganisms are seldom found lower than the upper few cm of soil (Madsen and Alexander, 1982; Dijkstra et al., 1987; Bitten et al., 1974). A critical volume of water is necessary for appreciable movement of bacteria (Griffin and Quail, 1968). If

the soil is much drier than field capacity, microbial movement will be limited by the sieving action of the soil (Bitten et al., 1974). Griffin and Quail (1968) suggested that the continuity of water-filled pores of a critical size was the main determinant of bacterial movement. This may explain why bacteria are retained to a greater extent in heavier textured soils (Bitten et al., 1974).

In addition, roots and worms can create channels for transport of bacteria by water (Madsen and Alexander, 1982). Roots alone were found to be ineffective in dispersal (Parke et al., 1986; Madsen and Alexander, 1982). Alternatively, worms were found responsible for transport of *Pseudomonas putida* and *Rhizobium japonicum* below surface layers (Madsen and Alexander, 1982). In a field study, bacterial numbers of a genetically engineered strain decreased progressively with increasing depth and this movement was thought to be due to water percolation (van Elsas et al., 1986).

Movement has also been observed to occur horizontally in soil. *E. Chloracea* inoculated into soil increased in numbers exponentially towards the roots (Dijkstra et al., 1987).

It would be beneficial to have laboratory microcosms to determine the effect of several different environmental parameters on the survival and fate of recombinant microorganisms in soil. I describe the development of a soil core microcosm (Chapter 3) that is useful for simultaneous measurement of survival and dispersal of recombinant organisms in soil.

Adaptation and evolution

The fate of a recombinant microorganism in the environment may in part be dependent on selective forces leading to adaptation or changes in the gene pool of the population due to microevolution.

Selective forces can lead to an increased frequency of a particular gene such as *mer* in mercury-contaminated sites (Barkay and Olson, 1986) or genes encoding ability to degrade a toxic compound, such as naphthalene, in sites contaminated with that chemical (Sayler et al., 1985). In the absence of selection the genes encoding specific traits may gradually be lost from the population. However, Jain et al, (1987) found that plasmid-borne catabolic genes were successfully maintained in groundwater aquifer material regardless of the selective pressure. In a chemostat study, Lundquist and Levin (1986) found that two naturally occurring plasmids increased in frequency and the bacteria carrying them achieved dominance in the absence of selection.

Genes can also be exchanged between members of the microbial population. Dominance of a final most-common phenotype of *Bacillus* in soil was found to be a result of gene transformation (Graham and Istock, 1981). In addition, Weinberg and Stotzky (1982) observed conjugation between strains of *E. coli* in soil leading to changes in the introduced populations. Microevolutionary changes may also result from genome rearrangements within a given organism. Insertion sequences have been shown to promote genome rearrangements in *Pseudomonas cepacia* and this is thought to contribute to the adaptability of this strain (Barsomian and Lessie, 1986). This ability to rearrange genes can be very important in assessing the

stability of a recombinant microorganism. In fact, *Pseudomonas aeruginosa* strains previously scored as having stable gene insertions showed "delayed marker loss" at a frequency of 34-46 out of 100. Deletional-recombinants may display alterred phenotypic traits. Jaoua et al. (1986) observed that recombinants containing the plasmid RP4 integrated into the chromosome have a slower growth rate than cells which deleted all or part of the plasmid. These examples illustrate a possible evolutionary advantage of recombination and horizontal gene transfer. In the presence of selective pressure these forces could produce a rapid adaptive change in the genetic structure of the population.

The use of gene probe methodology in detection of genome rearrangements of a recombinant pseudomonad in soil is described in Chapter 2.

Monitoring bacteria in complex samples by dot/slot blot analysis.

In addition to recombinant microorganisms, some important natural isolates are difficult to monitor by conventional methods. This is the case for strict anaerobic bacteria such as *Bacteroides* spp. in human feces (Salyers et al., 1983), *Vibrio vulnificans* in oyster meat (Morris et al., 1987) and the unique dechlorinating microorganism, DCB-1, in anaerobic enrichments (see Chapter 4). Many of these organisms are hard to isolate and identify and are non-culturable on selective media. Therefore, the methodology of gene probes developed for tracing recombinant microorganisms in soil may be equally adaptable to monitoring these strains.

Slot/dot blot analyses of crude samples, or of DNA isolated from environmental samples offers many advantages. To begin with, many

samples can be analyzed at one time. Secondly, there is no requirement for prior cultivation of the bacterium. In addition, gene probe methodology can be both qualitative and quantitative.

There are some difficulties encountered when using crude samples for DNA hybridization. One problem is that the sensitivity is low. Kuritza et al. (1986) determined that the lower limit of detection of *Bacteroides* species in feces was 1×10^9 bacteria per gram by liquid scintillation counting. Pure cultures of *Bacteroides* added as crude lysates had a detection limit of 10^7 cells if detected by autoradiography (Salyers et al., 1983). As is demonstrated in Appendix A the sensitivity is greatly improved if purified DNA and radioactive probes of higher specific activity are used, allowing detection of 10(4) bacteria per gram of soil. Chapter 4 describes the use of slot blot analyses of purified DNA to detect and estimate the densities of the dechlorinating microorganism, DCB-1, in different anaerobic enrichments.

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CHAPTER 2

DETECTION OF TWO ENGINEERED PSEUDOMONADS AND A SPONTANEOUS DELETION RECOMBINANT DERIVATIVE IN SOIL USING GENE PROBES

INTRODUCTION

Until recently, the fate in soil of an engineered DNA sequence was difficult to assess. With developments in DNA isolation protocols, gene probe methodologies can now be employed to monitor the presence of a particular DNA sequence in soil. Most methods using gene probes for detection of specific organisms in environmental samples rely on the isolation of colonies on solid media which are probed by DNA colony hybridization techniques (Barkay et al., 1985; Sayler et al., 1985). However, many microorganisms become dehabilitated in soil (Devanas et al., 1986) and aqueous samples (Xu et al., 1982; Colwell et al., 1985) so that they are no longer culturable on synthetic media. Recently, we developed a procedure for the detection of specific microorganisms in soil that overcomes this growth requirement (Appendix A). Using this technique, we isolated total bacterial DNA from soil. The purified DNA was then analyzed by Southern blot or slot blot hybridization for the presence of a specific DNA sequence, and hence a specific organism to which that sequence is unique, in the complex mixture of DNA isolated from the soil bacterial community.

In some studies, bacteria engineered with plasmid or transposon markers have been traced in soil (Drahos et al., 1986; Devanas et

al., 1986; van Elsas et al., 1986). However, recombinant microorganisms released into the environment should not contain foreign genes inserted in this manner because of the potential for horizontal gene transfer (Rawlings and Woods, 1985; Gealt et al., 1985; Genthner et al., 1988). Instead, foreign genes should be stably inserted into the chromosomes of recombinant bacteria. For example, we were able to detect a specific gene, *npt*II, engineered into the chromosome of *Bradyrhizobium japonicum* after its addition to soil (Appendix A).

DNA probe methodology has also been used to monitor the distribution of particular bacterial genes in various environments. For example, Barkay and Olson (1986) found a positive correlation between mercury resistance genes (*mer*) and mercury concentration in environmental samples. Similarly, an increase in naphthalene catabolic genes was observed in samples contaminated with this compound (Sayler et al., 1985).

In this study we constructed recombinant strains of *Pseudomonas* as model microorganisms to trace in soil. Pseudomonads are an environmentally and economically important group of organisms. Several are phytopathogens while others seem to promote plant growth. Pseudomonads are also perhaps the most metabolically versatile genus of microorganisms known. For the reasons stated above, pseudomonads are potentially of great importance in current and future applied biotechnology and in fact have been proposed for environmental release as hosts for pesticidal and fungicidal agents (Obukowicz et al., 1986).

In this study we used *Pseudomonas cepacia* strain PCO1224 and *Pseudomonas* sp. B8 as model organisms. *Pseudomonas cepacia* strains are being used as "benchmark" organisms by the Environmental Protection Agency. *Pseudomonas* sp. B8 is an aggressive root colonizing bacterium that inhibits root growth of winter wheat (Fredrickson and Elliot, 1985). These strains were engineered to contain a chromosomal insertion of the neomycin phosphotransferase II gene, *npt*II, which encodes resistance to the antibiotic kanamycin. This gene has previously been shown to be essentially non-existant in the indigenous bacterial community of the soils used in this study. (Appendix A).

In this study, we employed DNA probe methodology to specifically detect two different *Pseudomonas* species of following their addition to soil. In addition, we found evidence of a spontaneous deletion recombination event in the engineered *Pseudomonas cepacia* strain in soil.

MATERIALS AND METHODS

Bacterial strains and plasmids

The bacterial strains, strain abbreviations used in the remainder of the text, and plasmids used in this study are listed in Table 1.

Media and Growth Conditions

Pseudomonas strains were routinely grown in Kings B (KB) liquid medium (King et al., 1954). *Pseudomonas cepacia* cultures were grown at 37°C, and *Pseudomonas* sp. B8 at 25°C. When incubated together the cultures were grown at 25°C. Antibiotics were added as necessary at the following concentrations: to liquid medium, nalidixic acid, 200 ug/ml; kanamycin, 200 ug/ml; rifampicin, 100 ug/ml; and to solid

Table 1. Bacterial strains and plasmids

Strain/plasmid Identification	Abbreviation	Relevant Phenotype	Source or Reference
Pseudomonas cepacia PCO1224	(PC)	Nal^r	R. Olsen
Pseudomonas cepacia PCO1224-1	(PC-1)	Nal^r, Km^r	This study
Pseudomonas cepacia PCO1224-2	(PC-2)	Nal^r, Km^r	This study
Pseudomonas sp. B8	(B8)	Rif^r	L. Elliot
Pseudomonas sp. B8-1	(B8-1)	Rif^r, Km^r	This study
Eshcherichia coli ED8654		Rec^+	Borck et al. (1976)
Eshcherichia coli N100		Rec^-, pro^-	M. Rosenberg
pKC7		Km^r	Rao & Rogers (1978)
pRL161		Km^r	Elhai & Wolk (1988)
pRL425		Ap^r, Cam^r Ery^r	J. Elhai, Michigan State Univ.
pRK2013		Km^r, Tra^+	Figurski & Helinski (1979)
pJJ3		Ap^r	This study
pJJ5		Ap^r, Km^r	This study
pBH1		Ap^r	This study
pJJ6		Ap^r, Km^r	This study

Abbreviations for antibiotic resistance markers are as follows: Nal, nalidixic acid; Km, Kanamycin; Rif, Rifampicin; Cam, Chloramphenicol; Ery, Erythromycin.

medium, nalidixic acid, 400 ug/ml; kanamycin, 500 ug/ml; rifampicin, 200 ug/ml; cycloheximide, 200 ug/ml. Minimal medium (Roehl and Phibbs, 1982) was also used where stated.

E. coli strains were grown and cultivated in Luria broth (LB) or medium solidified with 1.5% agar. Antibiotics, when used, were added at the following concentrations: for liquid medium, kanamycin, 25 ug/ml; ampicillin, 20 ug/ml and for solid medium: kanamycin, 50 ug/ml; ampicillin, 40 ug/ml; chloramphenicol, 20 ug/ml.

Soil inoculation and sampling

The soil used was Capac loam described previously (Appendix A). Bacterial cultures were grown in KB medium containing appropriate antibiotics to a OD (640) of approximately 1 and harvested by centrifugation (10 min at 8000 x *g*). The cells were washed in 0.01 M sodium phosphate buffer (pH 7.0) and resuspended in 1/2 the original volume in the same buffer. Where indicated, cells were starved by incubation in 0.01 M sodium phosphate buffer at room temperature for periods up to 9 hours.

Bacterial suspensions were inoculated into soil by spraying uniformly over the soil surface using a 10 ml syringe with an 18 gauge needle, after which the soil was mixed thoroughly. The soil was moistened to field capacity (approximately 23% moisture) using the cell suspensions, or phosphate buffer alone for controls. Inoculated soils and uninoculated controls were incubated in plastic bags in the dark at 25°C. At appropriate time intervals, 50 g samples of soil were taken for DNA extraction and 10 g samples were taken for enumeration by plate counting, and for dry weight

determination. Bacterial populations are expressed as colony forming units (CFU) per gram dry weight of soil.

Isolation of bacterial DNA from soil

Bacterial DNA was isolated from 50 g soil samples by homogenization and differential centrifugation, followed by cell lysis and cesium chloride density gradient centrifugation essentially as previously described (Appendix A). But, the procedure was modified as follows to reduce the amount of time necessary for the extraction process. 1.) Instead of three rounds of blending and centrifugation, the soil was subjected to two rounds. 2.) Soil bacterial suspensions were washed once in TE buffer. 3.) The lysozyme incubation time was decreased to 45 min, and the pronase incubation time was decreased to 45 min, and the pronase incubation time to 30 min. 4.) After lysis, cell lysates were immediately prepared into cesium chloride -ethidium bromide density gradients rather than being stored on ice overnight.

DNA isolation from liquid culture

Bacterial genomic DNA was isolated from liquid culture by a modification of the procedure by Marmur (1962). Bacterial cultures (10 ml) were brought to 0.1% Sarkosyl and shaken gently by hand for 2 min. The cultures were then spun at 8000 xg for 10 min in Falcon 2059 polypropylene test tubes (Bekton Dickinson). The cell pellets were resuspended in 2 ml of 20% sucrose to which lysozyme (Sigma) was added (0.2 ml of 10 mg/ml solution in water), after which the mixture was incubated at room temperature for 3 min. Sodium EDTA (40 11 of 250 mM) was added followed by the addition of 520 ul of 10% Sarkosyl. The mixture was then incubated at room temperature for 45

min. Pronase (Sigma) (50 ul of a 10 mg/ml soltution preincubated 30 min at 37°C) was added to the mixture followed by an additional 30 min incubation at 37°C. The lysate was combined with an equal volume of TE saturated phenol, mixed by gentle inversion for 5 min, then 2 ml of chloroform was added. If the cell lysate/phenol mixture was overly viscous, additional TE buffer was added (usually 2 ml). The cell lysate mixture was separated into aqueous and organic phases by centrifugation at 8000 x *g* for 10 min. The aqueous phase was again extracted with phenol/chloroform and then twice with chloroform alone. Where desirable, DNA was precipitated by the addition of two volumes of isopropanol and incubated at -20° overnight.

Small scale isolation of plasmid DNA was performed by the method of Birnboim and Doly (1979) for *E. coli*, strains and by the method of Kado and Liu (1981) for *Pseudomonas* strains.

Large scale plasmid purification was performed by the method of Clewell and Helinski (1969).

Strain construction

The strategy used for the construction of the recombinant *Pseudomonas* strains is given in Fig. 1. Genomic DNA from each pseudomonad was digested with EcoRI and single fragments were randomly cloned into the EcoRI site of the mobilizable plasmid pRL425 using standard recombinant DNA techniques (Maniatus et al., 1982). Recombinant plasmids were used to transform *E. coli* strain N100. Transformants were selected by their ability to survive on ampicillin containing medium (ampicillin is conferred by plasmid pRL425) then screened for insertional inactivation of the chloramphenicol resistance gene of plasmid pRL425 which is bordered by EcoRI sites,

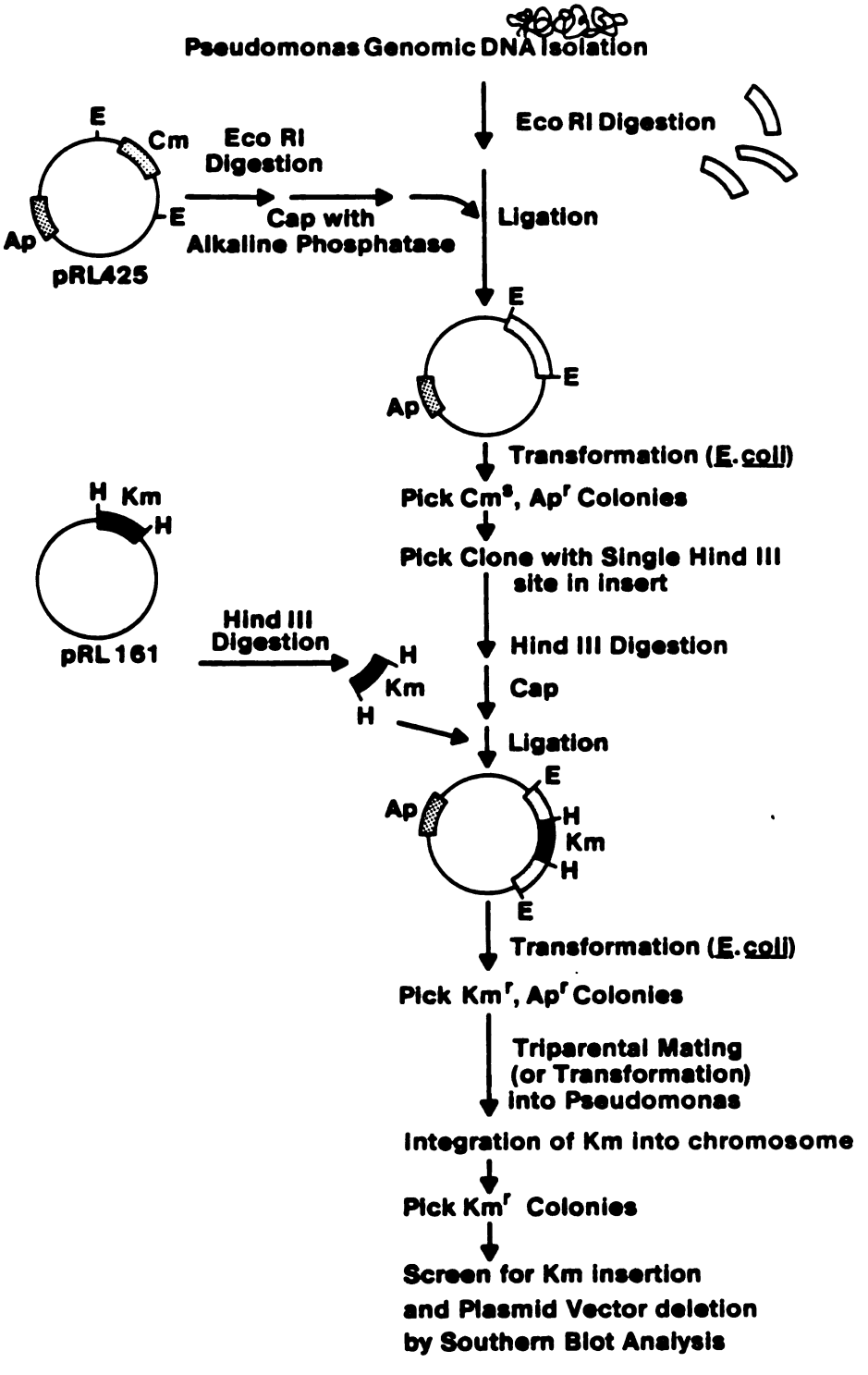

Fig. 1. Procedure for insertion of the kanamycin resistance gene (Km) into chromosomes of pseudomonads by homologous recombination.

by the loss of chloramphenicol resistance. Insertion of *Pseudomonas* DNA into pRL425 was verified by analysis of plasmid DNA from Amp^r, Cam^s transformants on agarose gels. Plasmids were selected which contained one HindIII restriction site centrally located in the cloned *Pseudomonas* DNA fragment. The recombinant plasmids pJJ3 (containing *Pseudomonas* sp. B8 DNA), and pBH1 (containing *P. cepacia* DNA) were selected for use in further strain construction steps. The *npt*II gene was isolated as a HindIII fragment from pRL161 by electroelution from a preparative agarose gel and ligated into HindIII digested pJJ3 and pBH1 that had been treated with calf intestinal alkaline phosphatase (Boehringer Manheim), according to the manufacturers recommendations, to prevent recircularization of the plasmid. The ligation mixtures were used to transform *E. Coli* N100. Transformants were selected on LB containing ampicillin and kanamycin. The structure of clones which had the *npt*II gene inserted into the HindIII site was confirmed by analysis of restriction fragment patterns of HindIII digested plasmid DNA. The structures of the chosen plasmids (pJJ5 for *Pseudomonas sp.* B8 and pJJ6 for *P. cepacia*) are given in Fig. 2.

Triparental mating

Triparental mating was used to introduce *pJJ5 and pJJ6 into Pseudomonas* sp. B8 and *P. cepacia*, respectively to allow for homologous recombination into the chromosome of each organism. *E. coli* strains ED8654, containing pRK2013, and N100, containing pJJ5 or pJJ6, were grown separately in LB containing kanamycin and aliquots were taken so that O.D. 600 x vol (x) = 0.1. The *E. Coli* cultures were centrifuged briefly together in a microfuge tube and washed once

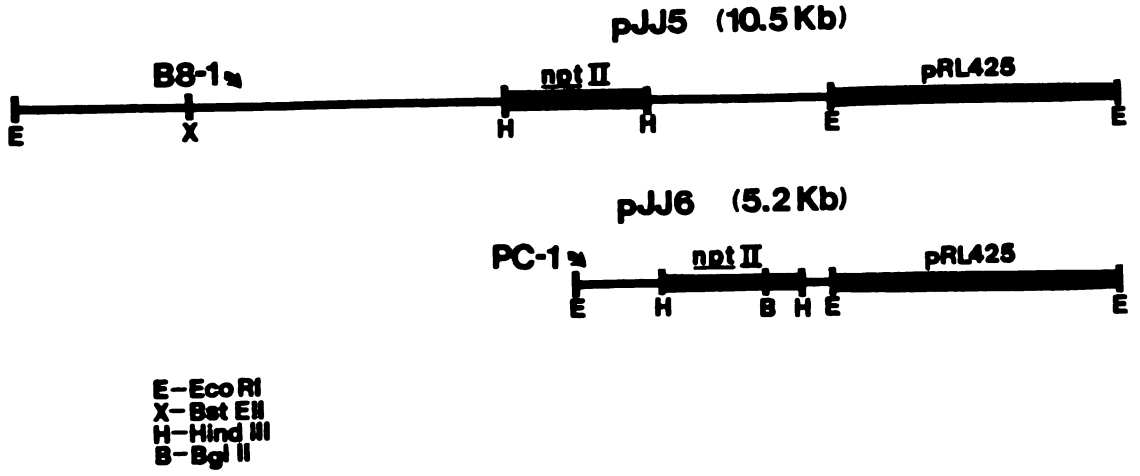

Fig. 2. **Restriction map of plasmids pJJ5 and pJJ6.** Narrow solid lines indicate cloned *Pseudomonas* DNA; wide solid lines indicate the *nptII* gene and vector sequences as designated.

with 5% LB. The pseudomonad recipient culture was then added to the same microfuge tube mixture at a vol (x) = O.D. 600 x vol (x) = 0.176 and collected by centrifugation. The bacterial cell mixture was resuspended in 1 ml 5% LB and added by vacuum filtration to a Millipore filter (0.45 um pore size) that had been previously washed with sterile distilled water. The filters were placed cell side up on LB agar and incubated overnight at 37°C for *P. cepacia*, or 25°C for *Pseudomonas* sp. B8. The filters were then transferred with sterile forceps to LB agar containing nalidixic acid and kanamycin for *P. cepacia*, or rifampicin and kanamycin for *Pseudomonas* sp. B8. and incubated at 37°C or 25°C, respectively. The filters were then lifted and placed in a sterile test tube containing 0.05 M sodium phosphate buffer (pH 7.0) and vortexed to release the bacterial cells. The cell suspension was diluted and plated on the appropriate selective agar medium. Transconjugants were purified by two sequential platings on selective agar. The integration of plasmid DNA into the chromosomes of the transconjugants was verified by Southern blot analysis of genomic DNA.

DNA Hybridization

The single-stranded, ^{32}P-labeled, DNA probe for the *npt*II gene was prepared using the M13 phage-based system described previously (Appendix A). Labeling of whole plasmids by nick translation was performed using a nick translation kit (Bethesda Research Laboratories) according to the manufacturers' directions. Southern transfer of DNA, prehybridization and hybridization conditions were as described (Southern, 1975). Colony hybridization was performed as described (Grunstein and Hogness, 1975).

RESULTS

Pseudomonas cepacia PC01224 and *Pseudomonas* sp. B8 were engineered to contain a chromosomal insertion of the *npt*II gene which encodes resistance to the antibiotic kanamycin, resulting in recombinant strains PC-1 and B8-1 respectively. The strategy for insertion relied on homologous recombination between plasmids carrying cloned fragments of *Pseudomonas* chromosomal DNA containing the *npt*II gene insertion, and the homologous chromosomal sequences. Recombinants were detected by resistance to kanamycin. It was not possible to efficiently screen for the absence of the plasmid vector, pRL425, by antibiotic resistance markers, because the antibiotic selection on this plasmid vector is ampicillin resistance and both pseudomonas strains were naturally resistant to ampicillin. Therefore, to screen for the presence of pRL425 sequences, the recombinants were analyzed by Southern blot analysis of isolated genomic DNA using ^{32}P-labeled pRL425 and pKC7 (which contains the *npt*II gene but has no homology to pRL425) as probes. In each case pRL425 sequences were found to be present in the chromosome of the recombinants, indicating that the vector had not been eliminated.

Since the ColE1 origin of replication of pRL425 cannot replicate in pseudomonads, the entire recombinant vectors (pJJ5 or pJJ6) must have been inserted into chromosomes of the *Pseudomonas* strains by a single cross-over event (refer to Fig. 3). To verify this hypothesis, the genomic DNA of each of the transconjugants was digested with a restriction enzyme that cuts only once in the recombinant vector, (BglII for pJJ6 and BstEII for pJJ5) (see Fig. 2). The DNA fragments were size -fractionated on a 1% agarose gel, transferred to a

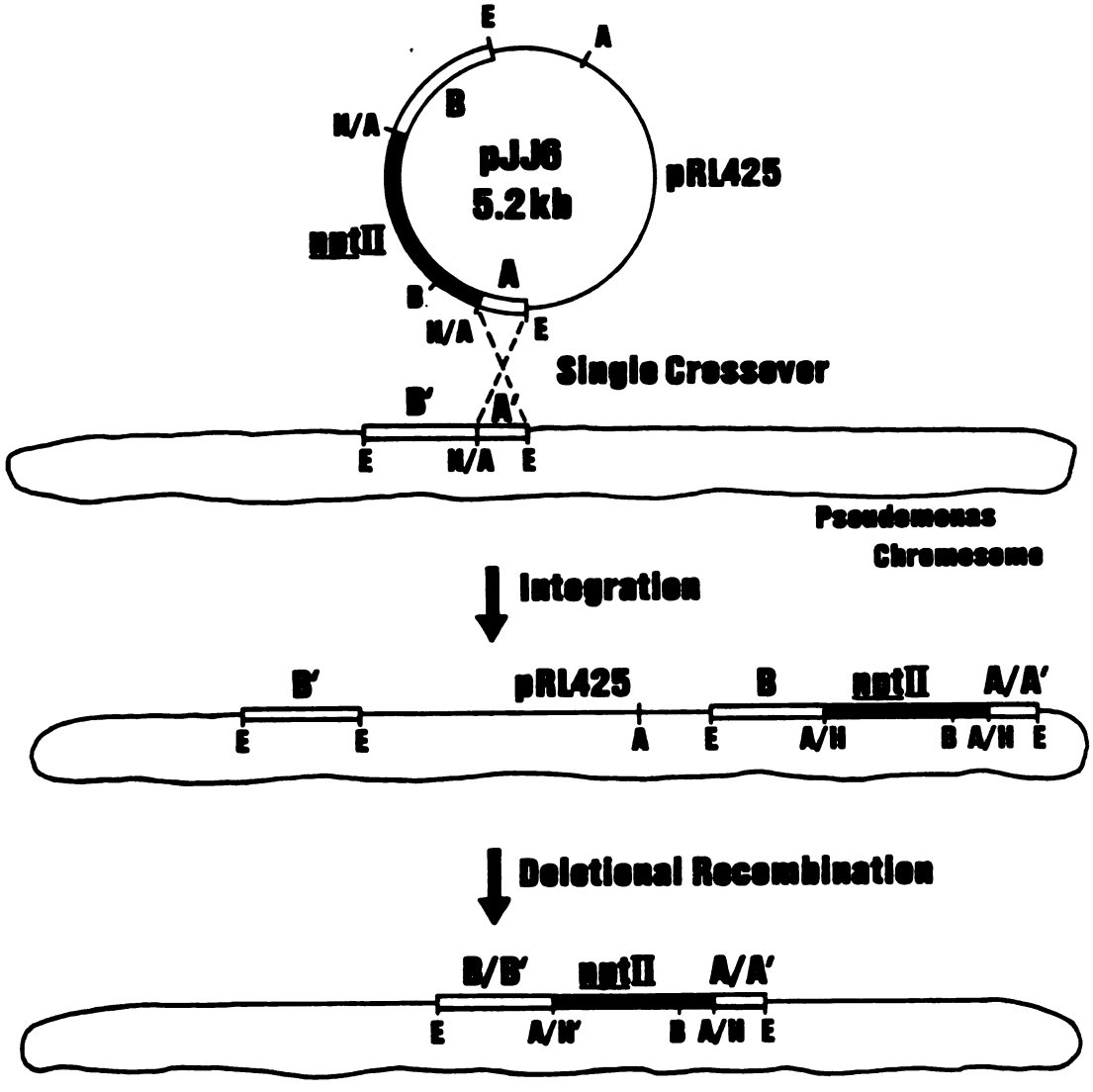

Fig. 3. **Graphic representation of integration of pJJ6 into the *Pseudomonas cepacia* chromosome, and subsequent deletion recombination of the integrated pRL425 sequences.** Restriction sites designated by small capital letters are: E, *Eco*RI; A, *Alu*I; H, *Hin*dIII; B, *Bgl*II. Large capital letters A, B, A′ and B′ represent *P. cepacia* chromosomal fragments. *nptII* = neomycin phosphotransferase II gene insertion.

cellulose nitrate filter, then probed with ^{32}P-labeled pJJ5 or pJJ6. In each case two bands were observed (data not shown) indicating that the entire plasmid had been integrated into the chromosomes of each of the pseudomonads. We tried to detect spontaneous conversion of the single crossover structure to a double crossover structure (see Fig. 3) by incubation in non-selective medium and screening by colony hybridization for spontaneous deletion of the integrated vector sequenc using pRL425 as a probe, but were unsuccessful after screening 600 individual colonies.

Strains PC-1 and B8-1 were inoculated singly or together into non-sterile soil samples. After 1 day of incubation, the soil bacterial DNA was extracted, digested with ClaI, size fractionated by electrophoresis and then transferred to cellulose nitrate and hybridized with the ^{32}P-labeled probe for the *npt*II gene (Fig. 4). A number of restriction enxymes were screened to find one that did not cut in either pJJ5 or pJJ6 to allow each of the two *Pseudomonas* species to be detected as a single band of a discrete size for each organism. The restriction enzyme, ClaI, was found to suitable and this enzyme was subsequently used to digest DNA isolated both from soil and from liquid cultures. DNA from soil inoculated with either strain individually when probed as described, generated a band of identical size when compared to the pseudomonad genomic DNA control (Fig. 4, lanes A, B, C and F, G, H). The two pseudomonads could easily be distinguished from each other in the same soil sample based on the difference in size of the ClaI restriction fragments containing the *npt*II gene insertions (Fig. 4, lanes D and E).

Fig. 4. **Autoradiogram from Southern blot of *Cla*I digested total bacterial DNA isolated from soil samples that were inoculated with PC-1 and B8-1.** Lanes A and H contain genomic DNA isolated from liquid culture of B8-1 and PC-1, respectively. Lanes B through G contain DNA isolated from soil inoculated with: B8-1 alone, (B and C); PC-1 alone, (F and G); or B8-1 + PC-1, (D and E). The lower band in lanes D through F represents a deletion recombination derivative of PC-1.

An unanticipated result was the appearance of an anomalous band when DNA isolated from soil that was inoculated with PC-1 either singly or with B8-1 was digested with ClaI and probed with the *npt*II gene probe (Fig. 4 lanes D, E, F, G). Comparison to DNA size standards of known molecular weight indicated that this band was of the size predicted for a double-crossover recombination event resulting in integration of only the cloned *Pseudomonas* DNA fragment containing the *npt*II gene (refer to Fig. 3). Genomic DNA isolated from liquid pure culture of this organism did not contain this anomalous band indicating that the deletion recombination event occurred in soil.

To determine whether the deletion recombination event occurred in liquid culture before addition to soil or only after incubation in soil, colony hybridization was used to screen for colonies that did not contain pRL425 vector sequences. Colonies plated from the original inoculum were compared to colonies isolated from soil, and from cultures incubated at the same time in minimal medium at 4, 25, or 40 °C (Table 2). After 1 day of incubation, the soil and minimal medium cultures were plated onto KB selective medium containing kanamycin and nalidixic acid. Colonies were picked, inoculated onto KB selective agar plates in an ordered array, and analyzed by colony hybridization for the presence of pRL425 vector sequences by probing with ^{32}P-labeled pRL425 (Fig. 5). The recombination event was only detected in colonies isolated from soil, and in cultures stored at 4 or 40#C, but not those stored at room temperature. (Table 2).

Three colonies which did not hybridize to the pRL425 probe were further analyzed by restriction digest analysis using Southern blots.

Table 2. Frequency of detection of deletional recombinants after incubation of PC-1 in soil or liquid cultures.

Incubation Time (Days)	Condition	Incubation Temperature °C	Frequency %*
0	Inoculum	37	0
1	Soil	25	1
1	Minimal media	4	1
1	"	25	0
1	"	40	1.5

*Frequency is expressed as the percent of total colony isolates on KB selective medium that do not hybridize to the pRL425 probe by DNA colony hybridization. The data presented represent averages from four replicates (100 colonies/replicate) for soil and original inoculum, and two replicates for minimal media cultures. In one instance a frequency of 4% was observed after incubation in soil.

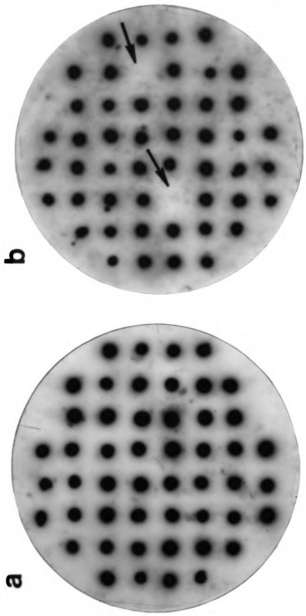

Fig. 5. **Autoradiogram from colony hybridization analysis using [^{32}P]-labeled pRL425 as a probe.** (a), colonies isolated from original inoculum; (b), colonies isolated from soil. Arrows indicate colonies that did not hybridize to pRL425, presumably due to its loss by deletion.

Data from the analysis of DNA from putative deletional recombination derivative colonies isolated from soil is given in Fig. 6a. The three isolates did not hybridize to the pRL425 vector compared to a control with integrated pRL425. The same samples when probed with the ^{32}P-labeled *npt*II gene probe resulted in a single band of the size predicted for precise deletion of the pRL425 vector (Fig. 6b lanes A, C, D).

PC-1 genomic DNA was digested with EcoRI and probed with various ^{32}P-labeled probes on a Southern blot to distinguish the remaining chromosomal fragment that did not participate in the initial crossover event (Fig. 7). The band in lane (a), 2.7 kb, corresponds to pRl425 DNA and in lane (b), 2.3 kb, to the B + *nptII* + A/A' DNA segment (Fig. 3). The broad band in lane (c) consists of the closely positioned 2.7 and 2.3 kb bands seen in lanes (a) and (b) respectively. The arrow indicates the additional (1.0) chromosomal fragment that did not participate in the original crossover event, corresponding to the B' + A'/A DNA fragment.

Since frequencies less than 10^{-2} can not be easily detected by the colony hybridization method because it is not practical to screen large numbers of colonies, bacterial DNA was isolated from soil and from liquid cultures and analyzed by Southern blot analysis. In most cases the deletion was only observed in DNA isolated from soil. However, in one instance the fragment representing the deletion recombination was observed in liquid cultures incubated at room temperature, and in that particular experiment it was also observed in the original inoculum (not shown.

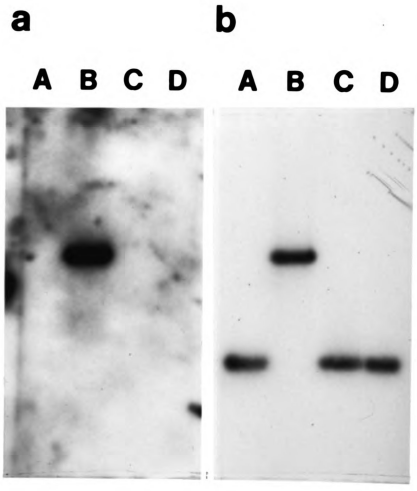

Fig. 6. **Autoradiogram from Southern blot after hybridization with [^{32}P]-labeled probes to *Cla*I digested DNA isolated from putative deletion recombinants.** Duplicate filters were probed with either pRL425 (a) or the *nptII* gene, (b). Lanes A, C and D contain DNA from colonies that did not hybridize to pRL425 by colony hybridization. Lane B is a control with DNA from a colony that did hybridize to pRL425.

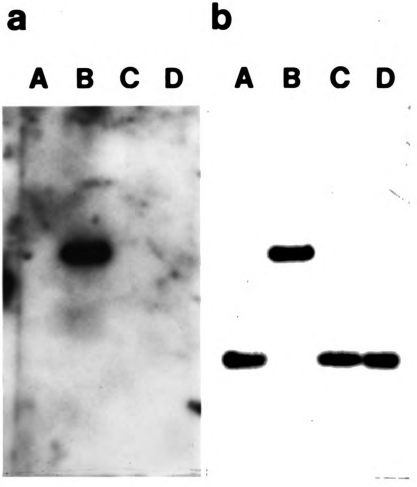

Fig. 7. **Autoradiogram obtained after Southern transfer of *Eco*RI digested PC-1 DNA and hybridization with various probes.** PC-1 genomic DNA (6ug) was digested with *Eco*RI and size fractionated by agarose gel electrophoresis. The Southern blot was probed with pRL425, (a); single-stranded *nptII*, (b); or pBH1, (c).

Results from additional experiments indicated that the freshness of the soil, growth stage of the culture, period of starvation, or presence or absence of nalidixic acid in the growth medium did not affect the frequency of recombination at the level of detection possible with colony hybridization. An experiment to determine whether the proportion of recombinants increased with an increased incubation time in soil was determined by direct comparison of DNA isolated after various times of incubation. The relative proportion of the single crossover band to recombinant band remained constant (data not shown). There was, however, a decrease in overall hybridization intensity for samples incubated 2 weeks compared to 1 week.

DISCUSSION

This study demonstrates the ability of gene probes to detect genomic rearrangements in organisms inoculated into soil. In addition, gene probes were shown to be able to detect and distinguish between two different *Pseudomonas* species present in a single soil sample by the detection of discrete bands of a predicted size in the commplex mixture of DNA isolated from non-sterile soil. We used both Southern blot and colony hybridization methodologies to detect deletion recombinant derivatives of PC-1 in soil. Southern blots were advantageous in that they were capable of detecting recombinants in soil at frequencies lower than could be practically determined by colony hybridization. Colony hybridization was more useful, however, for determining frequency values, since a numerical value was difficult to obtain directly from an autoradiogram resulting from a Southern blot.

Soil is thought to stress many bacteria after priour culturing in liquid media (Devanas et al., 1986). In this study, the number of deletion recombinants were similar in soil and in minimal medium liquid cultures incubated at 4 or 40° C. These extreme temperature conditions are far from the optimum for growth of PC-1, and thus may also be resulting in stress-induced recombination events. We have shown that these pseudomonads do not grow in soil under the conditions used in this study (Chapter 3). Instead, there is a steady decline in numbers over several weeks incubation until an equilibrium number (approximately 10^6 cells per gram dry weight of soil) is reached. This study may represent a case where conditions of stress caused genome rearrangements resulting in deletion of inessential DNA. The *npt*II gene is also inessential in soil but is not flanked by homologous regions as is the pRL425 DNA (refer to Fig. 3).

In one experiment Southern blot analysis showed that the same deletion had occurred in DNA from both the original inoculum and a saturated liquid culture incubated at room temperature. We do not believe that the delection recombination event always occurred before introduction of the strain to soil. For example in Fig. 4 the genomic DNA band is of greater intensity than one of the bands for DNA isolated from soil, however, the lower band is only observed in the soil DNA. It is however, possible that prolonged incubation of saturated cultures, and/or stationary or log-death phase, similarly stress bacteria as indicated in the experiment where the deletion was observed in the original inoculum and in cells stored at room temperature. This particular experiment was at a later date, so it is possible that the strains were becoming more unstable with increased

subculture and storage. Godfrey (1980) found that clones initially scores as stable could become unstable after subculture. This phenomenon has been described as delayed marker loss.

It is not clear why the whole-plasmid insertion was less stable in PC-1 than in B8-1. Lloyd et al (1980) have found that nalidixic acid mutants have a higher frequency of deletion recombination due to the nature of the mutation affecting DNA gyrase. This may explain in part why the deletion was only observed in the nalidixic acid resistant pseudomonad, and not in strain B8-1. It has been postulated (Kiel et al, 1987) that larger regions of homology improve the potential for recombination. However, in strain B8-1 the flanking regions of homology were larger.

The principle of detecting genomic rearrangements by restriction fragment length polymorphism applies whether the rearrangement is within the genome of an individual organism or rearrangement resulting from gene transfer between organisms. Thus the method should be equally useful in assessing horizontal gene transfer in nature.

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CHAPTER 3

THE FATE OF RECOMBINANT PSEUDOMONADS IN MODIFIED SOIL CORE MICROCOSMS (VERSACORES)

INTRODUCTION

Microorganisms have been genetically engineered to act as biological pesticides (Watrud et al., 1985) frost retardants (Lindow, 1985), or other agronomically useful products. Before these organisms can be field tested they must be proven safe for release into the environment. To achieve this objective, effective laboratory test methods must be developed that can assess likely behavior in the environment in order to determine the risk of releasing genetically engineered microorganisms. In addition, laboratory studies can provide information based on topics central to this issue such as survival and competitiveness of microorganisms in soil, and the effect of different parameters on organism fate such as roots, particle composition, water activity and leaching.

Past studies on fate of introduced organisms have usually been done using microorganisms that are spontaneously resistant to antibiotics (Parke et al., 1986; Madsen and Alexander, 1982; Dupler and Baker, 1984; Chao et al., 1986). While this approach has provided much useful information, especially on Rhizobium ecology, it is often limited by a high background of antibiotic resistant organisms in the indigenous soil population (Li and Alexander, 1986) and because the antibiotic resistance trait may not be ecologically

neutral. Furthermore, there are questions unique to the issue of environmental use of genetically engineered organisms that need to be answered in a test microcosm such as the fate of the engineered gene. The engineered strains that have been studied in soil have been labeled with a plasmid or transposon that carries drug resistant markers (Van Elsas, 1986) or the lactose utilization marker (Drahos et al., 1986). These engineered strains have only been monitored in soil by selective plating procedures. Often bacteria become dehabilitated after incubation in soil and are unable to grow on selective media (Devanas et al., 1986). This may result in an underestimation in the survival of the trait or organism in nature.

We have developed an alternative method for detection of genetically engineered microorganisms in soil (Appendix A). This methodology relies on extraction of total soil bacterial DNA. The isolated DNA can then be analyzed using gene probes specific for the added microorganisms. This methodology allowed us to detect engineered *Bradyrhizobium* (Appendix A) and *Pseudomonas* strains (Chapter 2) in soil. The organisms were engineered by insertion of a specific gene *nptII*, which confers kanamycin resistance, into the chromosomes. This gene was found to have a low background hybridization in the indigenous soil bacterial community. The recombinant organisms could then be traced in soil using the *nptII* gene as a specific probe.

The soil core represents the most convenient, realistic microcosm of the soil microorganisms' environment, and thus the most logical approach for establishing a method to assess the fate and behavior of genetically engineered microorganisms. The core maintains the

natural nutrient supply and the physical relationships, including competition, among indigenous organisms. It can also support plants, is easily used in rainfall and leaching studies, and is convenient for use in the laboratory. Thus, the objective of this study was to refine and test a soil core system for studying the fate of introduced genetically engineered microorganisms. This core was designed to allow several modes of organism introduction as well as analysis; because of this versatility we have termed the core Versacore.

Pseudomonas species were chosen as the engineered organisms used to evaluate the Versacore design because of their importance in agronomic and biodegradation applications. *Pseudomonas cepacia* PCO1224, and PCO1215 (R388::*Tn*904) are being considered by EPA as potential benchmark strains for evaluating and standardizing assessment methodology. *Pseudomonas* sp. B8 is an aggressive root-colonizing, toxin-producing microorganism that inhibits growth of winter wheat (Fredrickson and Elliot, 1985). Engineered derivatives of *Pseudomonas cepacia* PCO1224 and *Pseudomonas* sp. B8 in addition to the plasmid containing strain, *Pseudomonas cepacia* PCO1215 (R388::*Tn*904) were used as model organisms in this study. The Versacore was used to study survival and dissemination of recombinant pseudomonads from the point of inoculation to different depths of the core in the presence and absence of plants.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The three bacterial strains used in this study were genetically engineered *Pseudomonas* species. Two strains; *Pseudomonas cepacia*

PCO1224 (obtained from Ron Olsen, University of Michigan) and *Pseudomonas* sp. B8 (obtained from Lloyd Elliot, Washington State University) were engineered by insertion into their chromosomes of the *npt*II gene which confers kanamycin resistance (Chapter 2). The resulting recombinants, *P. cepacia* PCO1224-1 (PC-1) and *Pseudomonas* sp. B8-1 (B8-1), were resistant to high concentrations of kanamycin (500 μg/ml).

The pseudomonads were cultivated on Kings B (King et al., 1954) liquid or solid media. *P. cepacia* PCO1224 and *Pseudomonas* sp. B8 are naturally resistant to nalidixic acid or rifampicin, respectively. Antibiotics, when used for solid media were at the following concentrations: nalidixic acid, 400 μg/ml; rifampicin, 200 μg/ml; kanamycin, 500 μg/ml. *Pseudomonas* sp. B8 and the recombinant derivative B8-1 were cultivated at 25°C, whereas *Pseudomonas cepacia* PCO1224 and the recombinant derivative PC-1 were grown at 37°C. When the two strains were added together or inoculated into soil the incubation was at 25°C. The two recombinant strains could be distinguished from each other based on their colony color: PC-1 formed yellow colonies whereas B8-1 formed white colonies on Kings B. Furthermore, the strains could be distinguished on different agar media based on the differences in their antibiotic resistances.

An additional strain, *Pseudomonas cepacia* PCO1215 (R388::*Tn*904) (obtained from Ron Olsen, University of Michigan) harbors the engineered plasmid R388::*Tn*904 containing trimethoprim, and streptomycin resistance determinants. This strain was selectively isolated on solid minimal medium containing trimethoprim (200 μg/ml) streptomycin (2000 μg/ml), and proline (50 μg/ml). Cycloheximide

(200 μg/ml) was added to all solid media used for selection of the recombinant strains from the soil population to reduce fungal background.

Versacore microcosm design

The Versacore microcosm design is diagramatically represented in Fig. 1. Versacores were constructed from clear cast acrylic tubing (AIN Plastics, Southfield, MI) with an outer diameter of 9.5 cm and a wall thickness of 0.48 cm. The cylinders were cut into 2.5 cm sections. The sections were reassembled to the desired height by taping them together with waterproof tape. For the experiments used in this study, three sections were taped together. A wire mesh (1.5 mm opening) was glued between the bottom of the third section and an additional fourth section using methylene chloride as the cementing agent.

Capac loam soil (fine-loamy, mixed, mesic Aeric Ochraqualf) was sieved through a 2 mm sieve and 550 g was added to the core using a wide mouth funnel, starting from the edges and filling in towards the center. The soil was packed by tapping the core on the bench top as the soil was added to the core to achieve a bulk density of approximately 1.3 g per cm^3.

The cores were brought to field capacity (approximately 23% moisture) by setting in a pan of distilled water overnight. By that time the water had migrated to the surface. The cores were removed from the water, allowed to drain, and then covered at both ends with parafilm.

Cores were planted with five wheat seeds or three corn seeds per core. The corn was pretreated with Captan and Methoxychlor. The

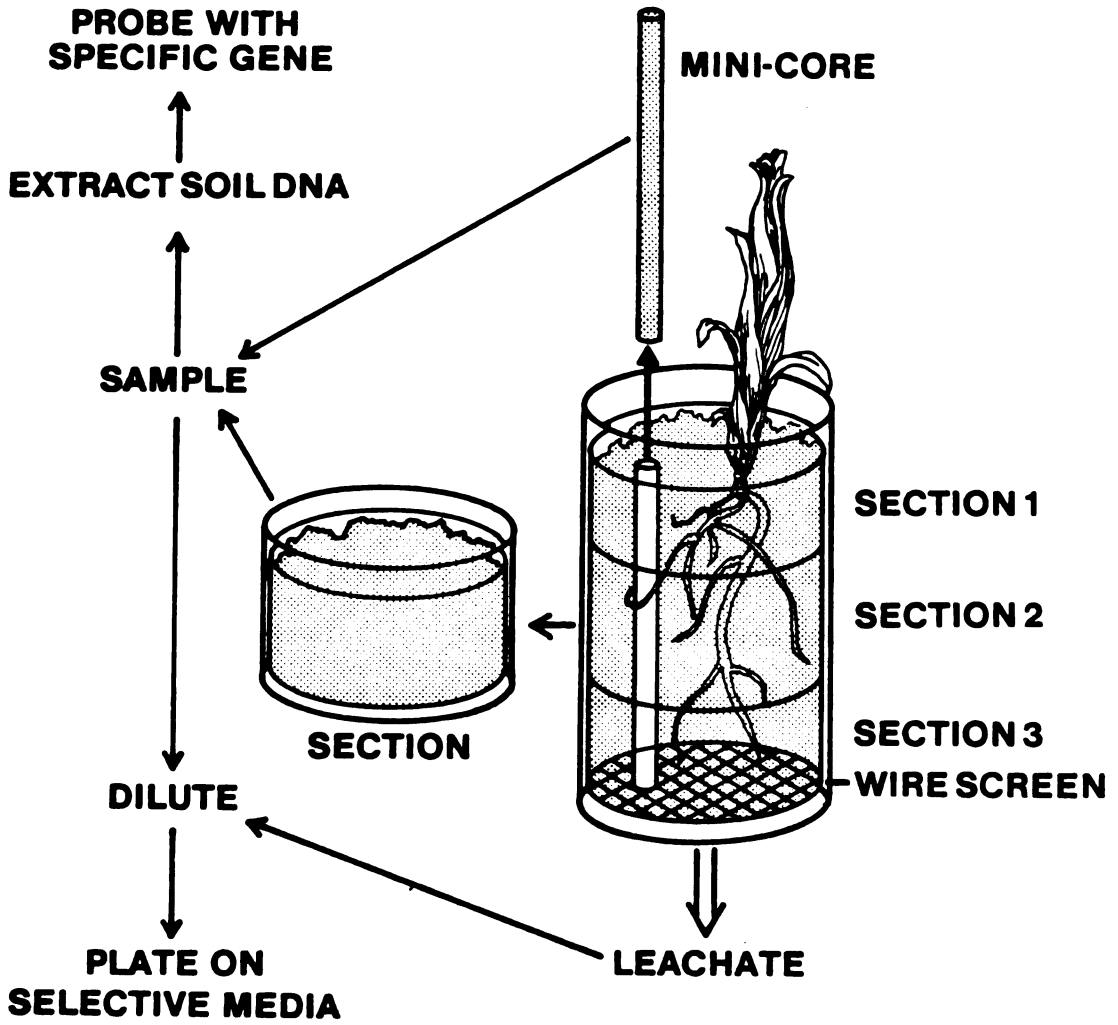

Fig. 1. Versacore design.

wheat seeds were rinsed in 1.5% sodium hypochlorite, followed by a wash in filter sterilized distilled water. The seeds were added to the soil at the top of the cores using flame sterilized forceps.

The cores were incubated under constant temperature and humidity conditions (25°C, 52% humidity) in an incubator room. Constant light was provided by fluorescent lights (intensity equal to 26 uE/s^2). After plant germination, parafilm was removed from the top of the cores, and the moisture content of the soil was maintained by weighing the cores at daily intervals and adding water to account for the amount lost.

Bacterial inoculation

Cells were grown in liquid media to an O.D. (640 nm) of approximately 1.0, harvested by centrifugation, and washed in 10 mM sodium phosphate buffer (pH 7.0). The cells were then starved for 2 to 6 h in the phosphate buffer. If necessary, the suspension was centrifuged 10 min at 8,000 x *g* and resuspended to the desired concentration. In most experiments, the cell suspension was added to the top of the core using a 10 ml syringe with an 18 gauge needle. Care was taken to distribute the inoculum slowly and evenly over the surface. In some experiments the inoculum was thoroughly mixed throughout the soil before the soil was packed into the core. In these experiments the inoculum was again added using a 10 ml syringe and the soil was continually mixed as the inoculum was added.

For the pre-emptive colonization experiments, one *Pseudomonas* strain was mixed throughout the soil, the soil was packed into cores, and the cores were planted with wheat. After 1 week incubation, the other strain was added to the surface of the cores and the cores were

incubated an additional 9 days and sampled every 3 days with mini-cores. As controls, both pseudomonads were also added together either to the top or throughout the soil. Additional controls included cores to which each organism was added singly to the top or singly mixed throughout the soil. Phosphate buffer was added to controls not receiving a microbial inoculum.

Mini-Core sampling

Cores were periodically sampled by taking mini-cores throughout the length of the core using a flame sterilized core borer (9 mm diameter). The holes left in the Versacore were filled with sterile inverted pasteur pipets that fit firmly in the holes. Two different samples were taken per core at each sampling time and combined in a weighing dish. One gram of the sample was used to enumerate the bacteria added. The sample was added to 9.5 ml sodium pyrophosphate (0.1%), sodium chloride (0.9%), Tween 80 (0.1%) and vortexed vigorously for 1 min. The sample was then serially diluted (1:10) in 9 ml of 10 mM sodium phosphate buffer (pH 7.0). The remainder of the sample was used for measurement of soil moisture content. All counts on agar media were converted to log CFU per gram (dry weight) of soil.

Sampling by leaching

For experiments where the cores were sampled by leaching, the cores were set into individual holes of a wooden platform. A funnel was placed underneath the core and into the mouth of a preweighed 250 ml centrifuge bottle. The cores were leached with 200 ml non-sterile tap water (equivalent to one inch rainfall), using a 50 ml syringe taking care to prevent the water from draining down the sides of the

cylinder. The leachate was collected and the bottle reweighed to determine the amount of water that drained through the soil column. The leachate was centrifuged 15 min at 23,000 x *g* to concentrate the cells. The cell pellet was resuspended in 10 ml 50 mM Tris buffer (pH 7.0), and serially diluted (1:10) in Tris buffer. Dilutions were plated onto solid medium and counts were converted to a per ml leachate basis.

Sampling by sectioning

Cores were divided into sections by removal of the waterproof tape. The soil core was cut between sections using a flat spatula prewashed in 10% sodium hypochlorite followed by a rinse with distilled water. The spatula was rewashed between each slice to avoid contamination. Each slice of soil (+/- roots) was mixed on an individual sheet of aluminum foil. A 50 g sample was taken for extraction of total soil bacterial DNA. A 10 g sample of the soil was used for enumeration by selective plating. An additional soil sample was taken for soil dry weight measurement. Cores sampled by sectioning were not leached before dismantling in these experiments to avoid movement of bacteria to lower layers with the large volume of water. Instead, only small amounts of water were added to maintain soil moisture.

Rhizosphere and rhizoplane

The root populations of the recombinant strains in Versacores planted with wheat or corn was determined essentially as described by Fredrickson and Elliot (1985). After sacrificing the cores, the roots were collected and gently shaken in 50 ml buffer consisting of sodium pyrophosphate (1 g/l), sodium chloride (9 g/l) and Tween 80

(0.1%) in Erlenmeyer flasks. The rhizosphere population was determined by shaking the soil in the flask for 1 hour then serially diluting and plating onto selective media. The soil was collected by vacuum filtration and dried to determine the dry weight of the rhizosphere soil. Rhizoplane populations were enumerated by shaking the roots in 50 ml of the buffer described above for 1 hour, then plating dilutions onto selective media. The roots were collected for dry weight determination. Rhizosphere or rhizoplane counts were expressed as Log CFU per g dry weight of soil or per g dry weight of root, respectively.

Soil DNA extraction and hybridization

Soil DNA was extracted by our previously described method (Appendix A). Briefly, a portion of the soil bacterial community was eluted from soil by repeated rounds of blending and differential centrifugation. The bacterial cell pellet was lysed and DNA separated, concentrated, and purified by ultracentrifugation in a CsCl-ethidium bromide density gradient.

The isolated soil DNA was analyzed by slot blot hybridization (Kafatos et al., 1979). A known concentration of DNA was added to nitrocellose through individual wells of a slot blot manifold (Bethesda Research Laboratories, Rockville, MD). The DNA was first denatured with 1/10 volume 3 N NaOH and heated 5 min at 100°C. Then the DNA was neutralized with 1 volume 2 N ammonium acetate on ice, and added to a nitrocellulose filter equilibrated with 1M ammonium acetate using the slot blot aparatus. Following vacuum filtration of the DNA onto the membrane filter, the filter was air dried and baked 2 h at 80°C. Prehybridization was carried out in bags containing

approximately 15 ml prehybridization solution with 50% formamide at 42°C for 12-24 h. After prehybridization, single stranded probe was prepared using a M13 phage vector as we have previously described (Appendix A). Alternatively, if sensitivity was not a critical issue, the *npt*II probe was prepared by nick translation of the plasmid pKC7 which contains the *nptII* gene (Borck et al., 1976). The R388::*Tn*904 plasmid was also labeled by nick translation. Hybridization was then conducted for an additional 12-24 h at 42°C. After hybridization, the nitrocellulose membrane was washed under stringent conditions to remove unbound probe as follows: briefly in 6X SSC + 0.1% SDS at room temperature, 15 min each at room temperature in 2X SSC + 0.1% SDS, 0.5X SSC + 0.1% SDS, and 0.1X SSC + 0.1% SDS respectively, followed by 30 min at 50°C in 0.1X SSC + 1% SDS. The filter was examined for bound sequences by autoradiography using Dupont Cronex 4 Medical X-Ray film.

Southern blot analysis was conducted by standard methodology (Southern, 1975). DNA isolated from soil was digested with the restriction enzyme ClaI. The DNA fragments were separated on a 1% agarose gel and transferred to a nitrocellulose filter. The filter was prehybridized and hybridized as described as above for slot blots, with the addition of 5% dextran sulfate in the hybridization solution.

RESULTS

Survival of genetically engineered pseudomonads in soil cores

The fate of the three engineered strains in the Versacores as determined by plate counts are shown as follows: Fig. 2, *P. cepacia* PCO1215 engineered by plasmid transformation with R388::*Tn*904; Fig.

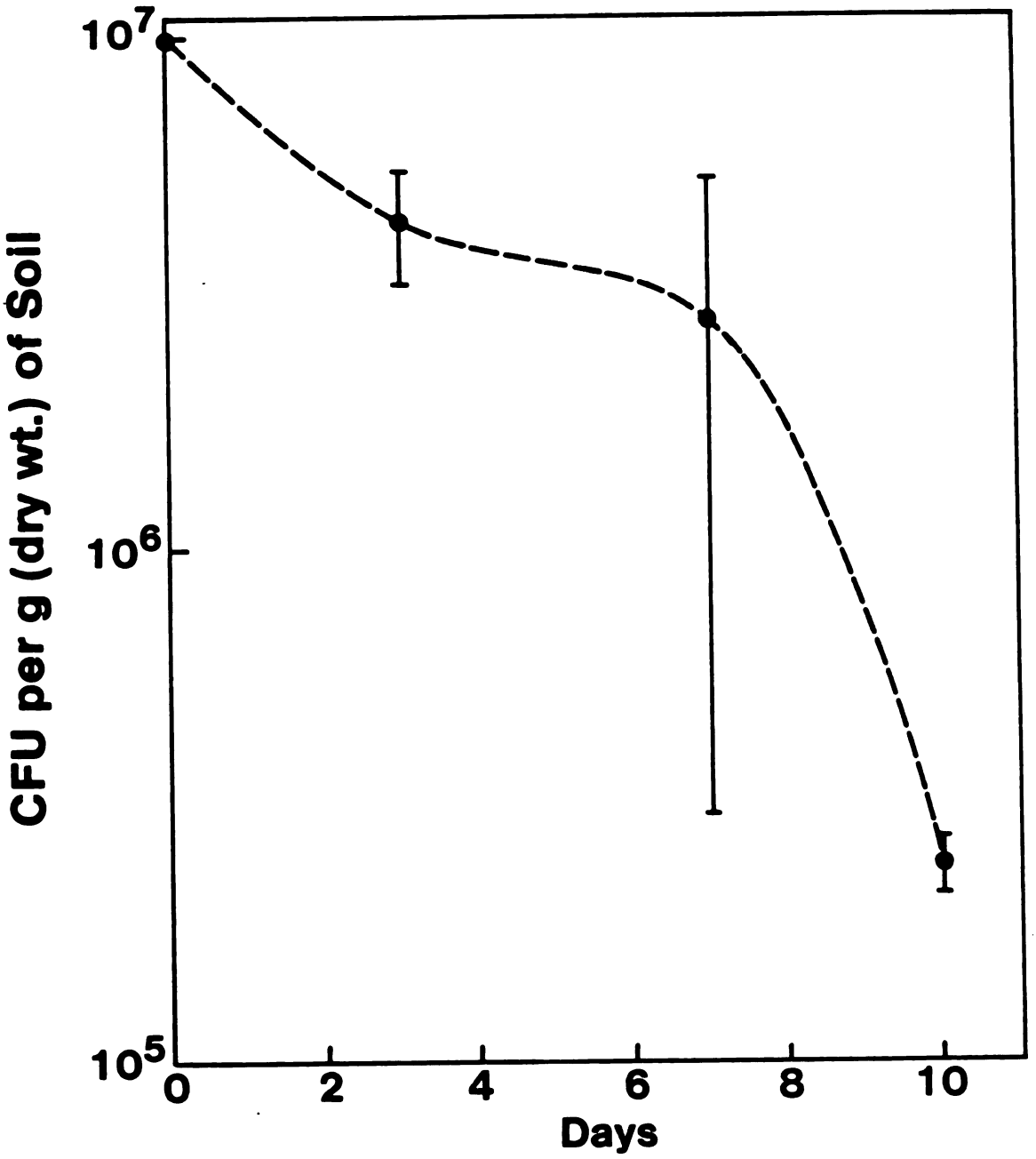

Fig. 2. **Survival of *Pseudomonas cepacia* PCO1215 (R388::*Tn*904) in Versacores planted with corn.** Counts represent the mean of three replicate cores.

3, *P. cepacia* PC-1 (with *npt*II); and Fig. 4, *Pseudomonas* sp. B8-1 (with *npt*II). Minicore sampling showed that all these strains decreased to the range of 10^5 organisms per g soil after 10-14 days, with PC-1 maintaining the highest density perhaps due to its larger inoculum (Fig. 3). The presence of corn roots did not have any significant effect on the soil populations on either PC-1 or B8-1 (Figs 3 and 4).

Movement of genetically engineered pseudomonads in soil cores

Versacores were sampled in a variety of ways to assay movement of engineered pseudomonads in soil. These included leaching of bacteria through the soil profile with water, and examination of bacterial density at different depths by selective plating and gene probe methodologies. Versacores inoculated with B8-1 were leached after 29 days incubation and the leachate plated onto selective medium (Fig. 4 insert). When the cores were leached, the number of cells in the leachate was higher for the cores that were planted, presumably due to the enhanced movement of bacteria along root channels.

In a separate experiment, after 20 days incubation, the cores were not leached but the density of PC-1 cells at different depths in the soil cores was determined by separating the cores into three sections and plating samples from each section on selective medium. The data indicate that PC-1 maintained a high population at the top of the core (the point of addition) and in the middle core section. The numbers of PC-1 in the bottom section was significantly lower, but cells were still detected at this depth. When sampling by sectioning, we did not find a significant difference between planted

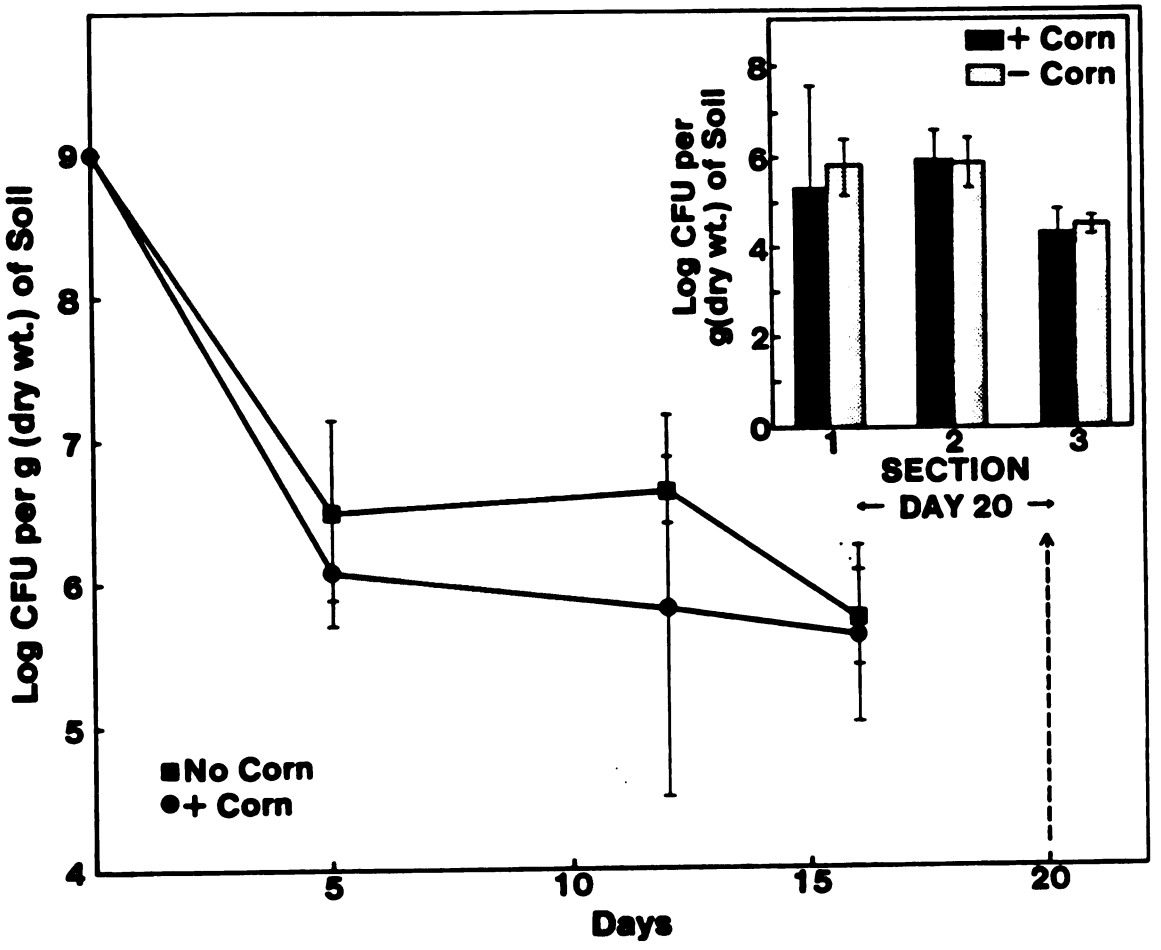

Fig. 3. Survival of *Pseudomonas cepacia* PC01224-1 in Versacores planted or not planted with corn. Minicore samples were taken until day 17 of incubation. After 20 days the cores were divided into three 1 inch sections and cells at each depth were enumerated on selective medium. Counts represent the mean of three replicate cores.

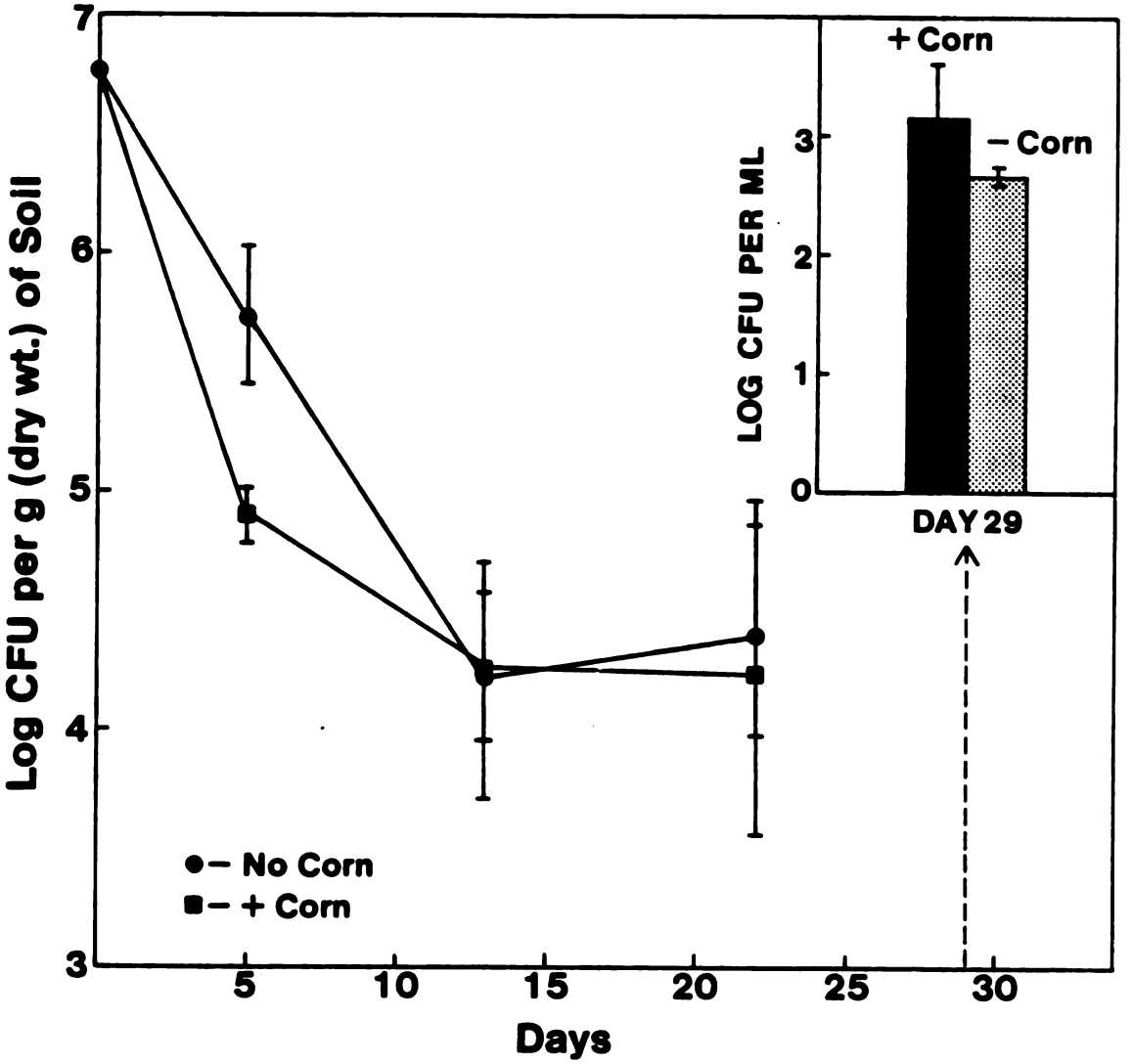

Fig. 4. **Survival of *Pseudomonas* sp. B8-1 in Versacores planted or not planted with corn. Minicore samples were taken until day 22 of incubation. After 29 days the cores were leached with the equivalent of 1 inch rainfall and the leachate was plated onto selective medium. Counts represent the means of three replicate cores.**

and not planted cores, indicating that roots had no apparent effect on movement through this non-leached soil. The amount of water added to the cores to maintain the moisture content were very small compared to the 200 ml used to leach the cores in the previous experiment, which is a likely explanation for the observed differences in bacterial movement when this and the previous experiment are compared.

Gene probes were employed as another method to follow movement of bacteria in soil. The single-stranded *nptII* gene probe was used to detect PC-1 and B8-1. The intensity of hybridization of the probe increased with an increase in the concentration of the cells added to soil (Fig. 5). Versacores inoculated with PC-1 or B8-1 and incubated for 7 days were then dissassembled into the three sections. DNA extracted from each section, was probed with the *nptII* gene (Fig. 6). The highest number of cells were detected in the upper section of soil corresponding with plate count data for the two organisms (Table 1).

Gene probes were also used to trace the plasmid-containing strain *P. cepacia* PC01215 at different depths in the soil core. A slot blot of DNA extracted from soil at the end of the incubation period for the experiment reported in Fig. 2 is shown in Fig. 7. The isolated DNA was probed with the R388::*Tn*904 plasmid. The greatest intensity of hybridization was to DNA isolated from the upper layer of the cores. There was some background hybridization to the control probed with R388::*Tn*904, possibly due to the use of a plasmid probe that might have homologous sequences, such as replication regions, in the indigenous bacterial community. Previous results (Appendix A)

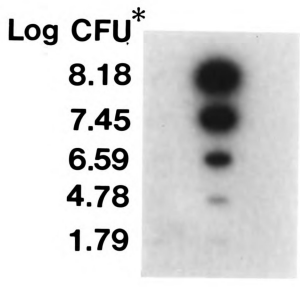

Fig. 5. **Slot blot of DNA isolated from soil samples inoculated with different concentrations of B8-1.** The same amount (10 ug) of DNA was added to each slot. Therefore, differences in hybridization intensity were due to differences in the *nptII* gene concentration in the soil samples. Cell density was verified by selective plating at the time of extraction. *Log CFU was calculated per g dry weight of soil.

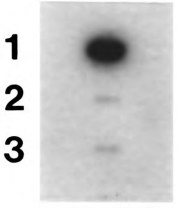

Fig. 6. Slot blot of DNA isolated from different depths of soil in Versacores inoculated with PC-1. The same amount of DNA was added to individual slots on the filter, and the blot was probed with single-stranded *nptII*. Numbers 1, 2 and 3 refer to soil core depths of 2.5, 5.0 and 7.5 cm, respectively. Similar results were obtained for B8-1.

Table 1. Log CFU per g (dry weight) of soil of two recombinant *Pseudomonas* strains, at three depths, after 8 days incubation in Versacores planted with wheat.

Depth (cm)	B8-1	PC-1
2.5	5.93	7.83
5.0	2.55	4.84
7.5	1.14	0.89

Counts represent the mean of three replicate cores.

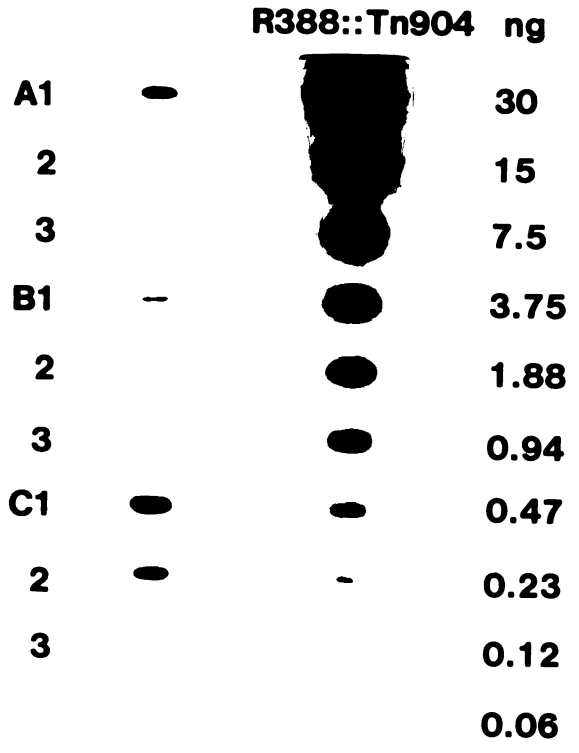

Fig. 7. **Slot blot of DNA isolated from 3 depths of soil cores inoculated with** *Pseudomonas cepacia* **PC01215 (R388::*Tn*904).** The blot was probed with [^{32}P]-labeled R388::*Tn*904. A and C are samples inoculated with the *P. cepacia* strain, and B is an uninoculated control. Numbers 1, 2 and 3 refer to core depths of 2.5, 5.0 and 7.5 cm, respectively. Serial 1:2 dilutions of purified R388::*Tn*904 DNA are given for comparison.

indicated that a non-indigenous gene, *npt*II, had negligible background in the soil community.

Effect of co-inoculation on the fate of two recombinant pseudomonads in soil

The two pseudomonads were added to the same soil core to determine the effect of co-inoculation and movement on survival. Plate counts taken over time are presented in Table 2. The two organisms were distinguished from each other using two different selective media. DNA was extracted at the first two sampling dates to detect the presence of the added organisms by slot blot analysis (Fig. 8). Duplicate samples from 0.5 and 4 days were analyzed using the single-stranded *npt*II gene as a probe. Comparison of intensity indicated that the greatest numbers of the added recombinant pseudomonads were in the uppermost layers of the soil (the point of inoculum). The plate count data did not correlate to the hybridization results at the 2nd sampling date.

PC-1 and B8-1 cannot be distinguished from each other by slot blot analysis when added together since they are engineered with the same gene insertion. Therefore, we cannot tell how much of the hybridization in Fig. 8 was due to each strain. However, the strains can be distinguished from each other in the same soil sample by agarose gel separation of ClaI restriction fragments followed by Southern blot analysis, since the ClaI fragment containing the *npt*II is a different size for the two strains (See Chapter 2).

The two *Pseudomonas* strains were added together to soil to determine the effect of co-inoculation on colonization of wheat roots. *Pseudomonas* sp. B8 is known to be an aggressive root

Table 2. Enumeration of PC-1 and B8-1 added together to soil cores, planted or not planted with wheat.

		Log CFU per g (dry weight) soil at depths of 2.5, 5.0, and 7.5 cm from surface					
		B8-1			PC-1		
Wheat	Time (days)	2.5	5.0	7.5	2.5	5.0	7.5
+	0.5	7.83	<2	<1	8.52	4.92	<1
+	4	6.37	<2	<1	7.92	4.63	<1
+	18	4.42	2.73	1.72	6.05	4.33	0.91
-	18	4.64	<2	3.17	5.91	2.32	0.29

Counts represent the mean of three replicate cores.

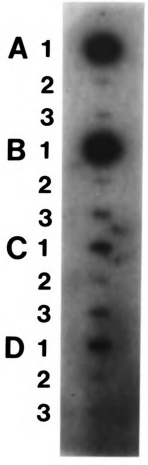

Fig. 8. **Slot blot of DNA isolated from different depths of soil inoculated with both PC-1 and B8-1.** The same amount (10 ug) of DNA was loaded into each slot, and the blot was probed with single-stranded *nptII*. (A) and (B) were isolated from duplicate cores after 0.5 days, and (C) and (D) from duplicate cores after 4 days incubation. Numbers 1, 2 and 3 represent soil core depths of 2.5, 5.0 and 7.5 cm, respectively.

colonizer (Fredrickson and Elliot, 1985). When the engineered strain was compared to the wild type organism, they did not differ in root colonizing ability (Fig. 9).

The known root colonizer, B8-1, was then compared to PC-1 for root colonization ability (Table 3). The two strains were added together or separately to soil planted with wheat. Samples were taken from the rhizosphere, rhizoplane, and leachate after 2 weeks and analyzed by selective plating. Interestingly, the two microorganisms showed similar root colonization ability. In addition there did not appear to be a competitive effect when the two organisms were co-inoculated into the same cores.

The effect of the method of co-inoculation (e.g. top or throughout) on subsequent root colonization of the two strains was determined (Table 4). There was no significant difference in the survival of either strain by these different inoculation methods (see Fig. 11, below). At the end of the experiment, the cores were sacrificed and the rhizosphere, rhizoplane, and soil bacterial populations were enumerated on selective media (Table 4). B8-1 counts were the same for both inoculation methods. PC-1 had higher counts when added to the top than when added throughout the soil and the soil population of PC-1 was higher than that of B8-1.

Effect of pre-emptive colonization on survival

Since the PC-1 and B8-1 strains appeared to be similar in their root colonization ability, we investigated the effect of pre-emptive colonization by one of the strains on establishment of the second, compared to controls with just one organism added. When either strain was allowed to colonize alone for 7 days and then the second

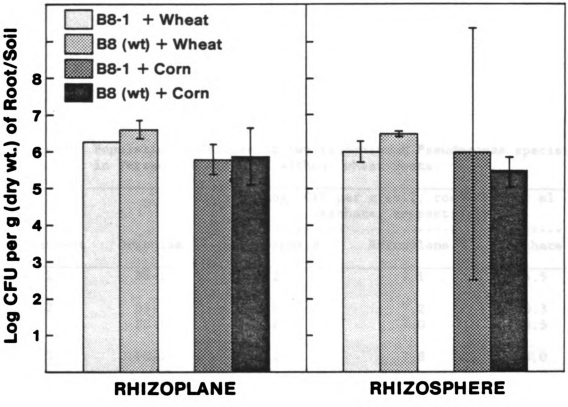

Fig. 9. **Comparison of *Pseudomonas* sp. B8 (wild type [wt]) and *Pseudomonas* sp. B8-1 (engineered derivative) as to root colonizing ability on wheat or corn.** Small cores were packed with 40 g Capac soil and planted with wheat or corn, or left unplanted. Duplicate cores were inoculated with either B8 or B8-1 (10^7 cells per g soil). After 9 days incubation, roots were removed from the cores and the rhizosphere and rhizoplane populations were enumerated on selective media.

Table 3. Population densities of two recombinant *Pseudomonas* species in Versacores with and without wheat roots.

Treatment	Organism	Log CFU per g soil, roots or per ml leachate, respectively		
		Rhizosphere	Rhizoplane	Leachate
1	B8-1	7.2	7.1	5.5
2	B8-1	7.0	7.2	6.3
	PC-1	6.8	7.0	5.5
3	PC-1	7.2	7.8	3.0
4	B8-1	-	-	6.2
	PC-1	-	-	6.8

Treatments 1,2, and 3 were planted with wheat and treatment 4 was not planted. B8-1 was added alone in treatment 1, and PC-1 alone in treatment 3. Both PC-1 and B8-1 were added in treatments 2 and 4. Counts represent the mean of triplicate cores.

Table 4. Rhizosphere, rhizoplane, and bulk soil populations of PC-1 and B8-1 added together to the top or mixed throughout soil in Versacores.*

	Log CFU per g dry weight root or soil**			
	Top		Mixed Throughout	
Sample	PC-1	B8-1	PC-1	B8-1
Rhizosphere	3.7	4.2	2.6	3.6
Rhizoplane	3.9	4.2	1.8	4.0
Soil	5.6	4.3	5.3	4.2

*Incubation time was 3 weeks after addition of strains if added throughout, and 2 weeks if added to the top (see Fig. 10).

**Counts represent the mean of three replicate cores.

strain was added, there was no noticeable interference on colonization of the second strain by the prior establishment to the first when compared to controls (Fig. 10 and Fig. 11).

DISCUSSION

A versatile soil core design was successfully used to monitor the fate and competitiveness of genetically engineered microorganisms introduced into a natural soil community. The design allowed temporal sampling by extraction of mini-cores from the larger core, assessment of movement and leaching of the added organisms and determination of the effect of roots on colonization and movement. Both conventional selective plate counting and gene probe methodologies were conveniently used to assess the fate of singly added organisms or to distinguish between two co-introduced organisms. These methods were used to trace genetically engineered bacteria labeled with either chromosomal DNA insertions, or with autonomously replicating plasmid DNA. In other studies we have shown that this design can also be used to study recombination in soil by adding donor and recipient strains in soil to different sections either by mixing into soil or by injecting into the soil from a side port in the core (Dwyer et al., unpublished data). Thus, this versatile design is both convenient and appropriate for evaluating a number of experimental questions important to assessing the fate of genetically engineered microorganisms in soil.

The two detection methods employed - selective plating and gene probe - have complementary advantages and disadvantages in these studies. Selective plating is easier, more sensitive and offers more capacity for sample number. Gene probe methods, however, detect

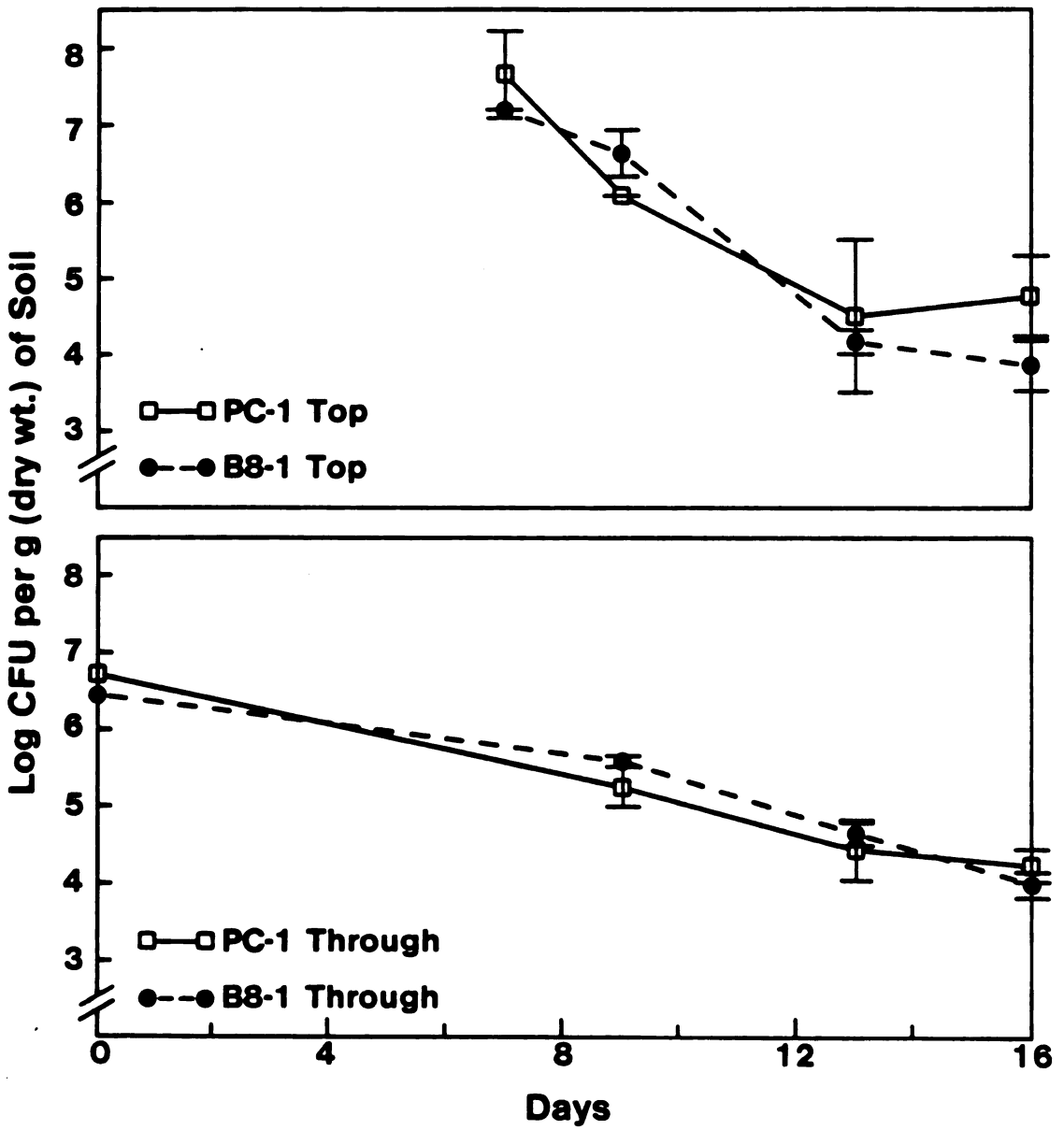

Fig. 10. Survival of PC-1 and B8-1 added together to the top or mixed throughout soil in planted Versacores. Counts represent the mean of three replicate cores.

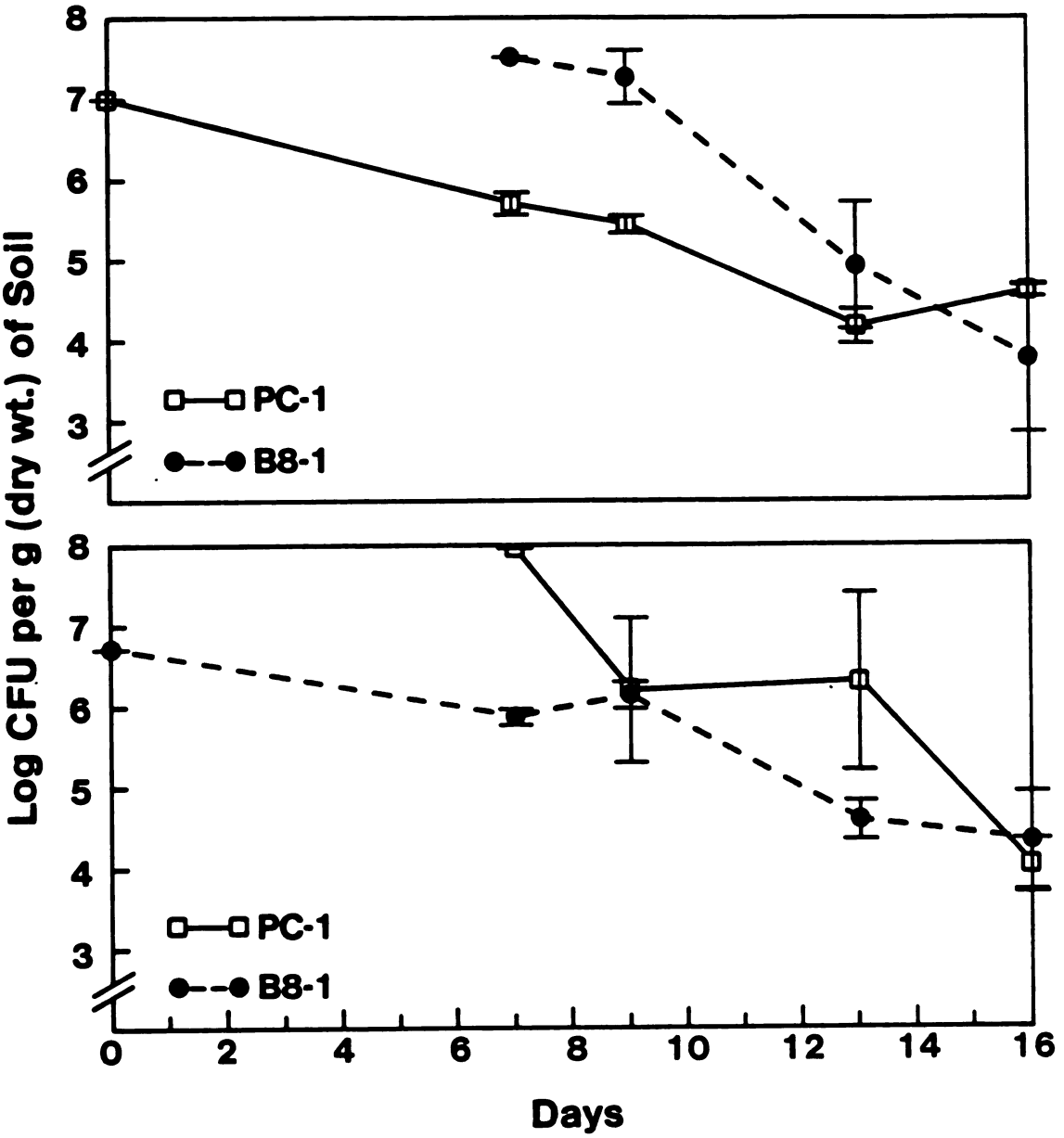

Fig. 11. **Establishment and subsequent colonization and survival of PC-1 and B8-1 added consequtively to soil in Versacores planted with wheat.** At day 0, the strains were added by mixing throughout the soil and the cores were planted. After 7 days incubation the second strain was added to the surface of the soil core. Counts represent the mean of three replicate cores.

organisms that are stressed or otherwise non-culturable on selective media and can also detect rearrangements or exchanges of the target gene (Chapter 2). Slot blots are the method of choice for analysis of a large number of samples due to the ease of preparation. However, if two organisms, such as the two pseudomonads in our study, are engineered with the same DNA insertion they cannot be distinguished from each other in the same soil sample by slot blot analysis but they can by Southern blot analysis. The later, however, is more time consuming and is slightly less sensitive. On the other hand, it usually reduces any non-specific background binding that can occur in slot blots (Appendix A).

Using these detection methods, Versacores were sampled in a variety of ways to determine the fate of the recombinant pseudomonads in soil. We did not find an effect of roots on survival or movement except when the cores were leached (Fig. 4). In this case greater numbers were obtained in the leachate of cores planted with corn. This could be due to movement along root channels. These findings are similar to those obtained by Madsen and Alexander (1982) who found that roots did not affect movement of *Pseudomonas putida* or *Rhizobium japonicum* unless sufficient water was added to leach the cores. Van Elsas et al. (1986) confirmed in a field study that survival and movement of *P. fluorescens* in soil was not influenced by roots in cropped and uncropped columns.

Most studies concerned with movement of bacteria in the soil profile have found that the bacteria remain in the upper layer of soil in the absence of saturated water flow. Van Elsas et al. (1986) found the highest numbers in the upper layers, but numbers were

detected at all depths to 25 cm. Chao et al. (1986) found that in non-sterile soil pseudomonads could not be detected in the rhizosphere greater than 3 cm below the planted seed. Madsen and Alexander (1982) also found that movement of *R. japonicum* or *P. putida* was not detected below 2.7 cm in absence of worm, plant, or percolating water. In this study we found the highest numbers consistently at the upper level of the cores. In addition, we could detect some bacteria at all depths after inoculation. Since we continually watered the cores to maintain field capacity, this could have contributed to the movement. The high numbers at lower depths obtained in cores sampled with minicores may have resulted from movement along the core plug. This suggests that if movement is to be studied in Versacores, the same cores should not be sampled with vertical mini-cores.

We did not find any competitive affect when the two recombinant strains were co-inoculated into the same soil samples. Therefore, the two organisms must not compete for the same sites or nutrients. Both species declined in number after inoculation in non-sterile soil. This could be due to predation or competition from the indigenous soil population. Chao et al. (1986) found that the biotic status of the soil markedly affected the colonization of microbes on roots. Bacteria competed more strongly in the rhizosphere for similar resources such as nutrients and space with other bacteria than with fungi. The known aggressive root-colonizing strain *Pseudomonas* sp. B8 did not establish rhizosphere populations as high as previously reported (Fredrickson and Elliot, 1985). This could be due to the root zone temperature, or to the length of incubation.

Fredrickson et al. (1987) found that B8 achieved and maintained higher populations at lower temperatures as a result of increased competitive ability.

The more rapid population decline of both pseudomonads after the second inoculation in the pre-emptive colonization experiment was likely due to desiccation of the soil surface at that time period. The top of the cores dried out (it reached 5% moisture during this sampling period). This interpretation is consistent with the reported sensitivity of pseudomonads to soil drying (Lebeda et al., 1976; Dupler and Baker, 1974).

While repacked cores are necessary for some experiments, this disruption of soil structure does change the microbes' micro-environment, most notably by increasing the supply of nutrients and destroying natural channels. For studies of bacterial movement where inoculation is at the top of the core, intact soil columns such as described by Van Elsas et al. (1986) provide a better estimate of movement under field conditions.

In conclusion, Versacores, were useful for tracking recombinant *Pseudomonas* strains and determining the effect of different environmental parameters on survival and dissemination. These factors are important for assessing the relative risk of releasing genetically engineered organisms into the environment.

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CHAPTER 4

USE OF DNA-DNA HYBRIDIZATION TO DETERMINE THE RELATIONSHIP OF THE DECHLORINATING ORGANISM, DCB-1 TO MEMBERS OF ANAEROBIC ENRICHMENTS AND PURE CULTURES

INTRODUCTION

The dechlorinating anaerobic microorganism, DCB-1, appears to be unique based on its morphological and physiological properties. DCB-1 was originally isolated from sewage sludged enriched for degradation of 3-chlorobenzoate (Shelton and Tiedje, 1984). DCB-1 was found to be responsible for removal of the chlorine substituent from the aromatic ring of this compound resulting in stoichiometric conversion to benzoate and chloride (Shelton and Tiedje, 1984, Dolfing and Tiedje, 1986). The benzoate could then be further degraded by other microorganisms in the enrichment. Three microorganisms were isolated from this enrichment, including DCB-1, that together were able to completely degrade 3-chlorobenzoate (Dolfing and Tiedje, 1986).

DCB-1 is important because it is the only anaerobic microorganism isolated in pure culture that is capable of aromatic reductive dehalogenation reactions. This type of reaction has great potential for the detoxification of serious environmental pollutants, e.g. PCBs and dioxins. Dechlorination of several classes of chloromaromatic compounds is known to occur in the environment, (Tiedje et al., 1987, Brown et al., 1987), but nothing is known about the organisms in the habitat that are responsible for the dechlorinations. To determine if

the microorganisms(s) were related to, or similar to DCB-1, new methodology had to be developed since DCB-1 cannot be selectively grown on media.

Gene probe methodology provides an alternative mechanism for detection and quantitation of indigenous populations that overcomes the growth requirement. Gene probes have been successfully used to detect other obligate anaerobes in complex samples such as feces (Kuritza and Salyers, 1985). Gene probes also offer the advantages of increased sampling ease, and the capability to assay several different samples at once. In this study we used a specific DNA probe, as well as the whole chromosome as a probe, to detect and estimate the density of DCB-1 cells in various anaerobic enrichments.

There is some evidence that DCB-1 is a sulfidogen. This is based on the ability of DCB-1 to produce sulfide from thiosulfite, the stimulatory effect of oxysulfur compounds on growth, and the presence of a c-type cytochrome and desulfoviridin (Linkfield, 1985). Therefore, in addition to using DNA probes to enumerate DCB-1 in anaerobic enrichments, we used the same methodology to assay the relatedness of DCB-1 to various known species, especially sulfate reducing microorganisms.

MATERIALS AND METHODS

Enrichment cultures and bacterial strains

Cultures enriched for the degradation of various halogenated compounds are listed in Table 1. Cultures were incubated anaerobically in sealed Erlenmeyer flasks at 37° C for sludge enrichments or 22° C for sediment enrichments. DNA isolated from the

Table 1. Enrichment cultures.

Source of Inoculum	Substrate	Source or Reference
Sludge	3-chlorobenzoate	Shelton and Tiedje, 1984
Sludge	3-chlorobenzoate	T. Linkfield, Battelle Lab. Columbus, Ohio
Sludge	3-chlorobenzoate	S. Schoberth, Institut fur Biotech., Julich, Germany
Sludge	3-bromobenzoate	This laboratory*
Sediment	3-bromobenzoate	T. Linkfield, 1985
Sludge	3-chlorophenol	M. Mikesell, Michigan State Univ.
Sludge	Phenol	Krumme, 1986

*Developed from the 3-chlorobenzoate sludge enrichment of Shelton and Tiedje (1984).

cricket gut microflora was supplied by Sheridan Kidd-Haack (Michigan State University) for use as a control.

In addition, the reconstructed consortium of which DCB-1 is an obligate member was probed to quantitate DCB-1. The consortium also contained a methanogen, *Methanospirillum* strain (PM-1) and a benzoate oxidizer (BZ-1) (Dolfing and Tiedje, 1986). DNA from these additional members of the consortium was isolated separately to determine the background hybridization, if any, due to them.

Organisms possibly related to DCB-1 were probed with DCB-1 DNA to determine their relatedness to this organism. The organisms examined and their sources are listed in Table 2.

Media and growth conditions

Basal medium (BM) (Shelton and Tiedje, 1984) was used for growth of most anaerobic cultures with various supplements. BM contained mineral salts, trace elements, vitamins, clarified rumen fluid (10-20%), sodium sulfide (0.24 g/l), sodium bicarbonate (1.2 g/l), and resazurin as a redox indicator. DCB-1 was grown in BM with pyruvate (2 g/l) and sometimes chlorobenzoate (400 uM). *Desulfovibrio* spp. and *D. multivorans* were grown in BM containing 20 mM sodium sulfate and 0.2% pyruvate. Sodium propionate (1.5 g/l) was used instead of pyruvate for *D. propionicus*. *Wolinella* was grown in BM with the addition of sodium formate (2 g/), sodium fumarate (3 g/l) and hemin (5 mg/l). *Clostridium* was grown in BM with yeast extract (0.03 g/l), 0.1% peptone, and 0.1% glucose. *Bacteroides* was grown in BM with 0.1% glucose and 1% peptone. *D. orientis* was grown in medium #1249 (ATCC) containing sodium lactate (3.5 g/l), sodium citrate (5 g/l), yeast extract (1 g/l), $CaSO_4$ (1 g/l), NH_4Cl (1 g/l), and K_2HPO_4 (0.5 g/l).

Table 2. Bacterial Strains.

Bacterial Strain	Source or Reference
Bdellovibrio bacteriovorus	ATCC 25631
Myxococcus xanthus	ATCC 25232
Desulfobulbus propionicus	DSM 2032
Desulfovibrio desulfuricans DG2	Dwyer and Tiedje, 1986
Desulfovibrio desulfuricans P3	Dwyer, this laboratory
Desulfovibrio desulfuricans DDS	Dwyer, this laboratory
Bacteroides sp.	Dwyer, this laboratory
Desulfococcus multivorans	Widell and Pfennig, 1984
Desulfotamaculum orientis	ATCC 19365
Wolinella succinogenes	DSM 1740
Clostridium bifermentens	Microbiology Dept, Michigan State Univ.

After autoclaving, 0.1% ferrous ammonium sulfate was added to 0.1%. The anaerobic cultures were incubated with a headspace consisting of 20% CO_2 and 80% N_2, except for *W. succinogenes* which had a gas mixture of 10% CO_2: 80% N_2: 10% H_2.

Aerobic bacteria were grown in the following media. *Bdellovibrio* medium contained peptone (10 g/l), and yeast extract (3 g/l). *Myxococcus* medium contained casitone (20 g/l), $MgSO_4$ (1 g/l) in a 0.01 M postassium phosphate buffered (pH 7.2) solution. All cultures were incubated at 37° C, except for *Bdellovibrio* and *Myxococcus* which were incubated at 25° C.

Chemicals and enzymes

All restriction enzymes, T4 ligase, purified phenol, and salmon sperm were purchased from Bethesda Research Laboratories (Gaithersburg, Maryland) , calf intestinal alkaline phosphatase from Boehringer Mannheim Biochemicals (Indianapolis, Indiana), and ^{32}P-labeled deoxycytidine from Amersham Corporation (Arlington Heights, Illinois). All antibiotics and other chemicals were purchased from Sigma Chemical Company (St. Louis, Missouri).

DNA isolation

Genomic DNA was isolated from the anaerobic enrichments and pure cultures using a modification of the procedure by Marmur (1961). Cells were grown in anaerobic media to the maximum O.D. for the culture in 150 ml serum bottles. To harvest the cells, NaCl was added to a final concentration of 1M, and the cultures were centrifuged 10 min at 8000 x *g*. The cells were washed once with T_8ES (3 mM Tris, pH 8.0; 5 mM EDTA; 50 mM NaCl). The cells were pelleted in a preweighed centrifuge tube. T_8ES was added (1 ml/ g cells) and the cells were

resuspended. This suspension was frozen at -70° C at least 12 h and could be stored at this stage until later use.

To isolate DNA the cells were thawed and lysed. T_8ES was added (5 ml/ g cells) followed by lysozyme (3 mg/ml in T_8ES) to 1/10 volume. The cells were put on ice 15 min and 1/15 volume proteinase K was added (10 mg/ml in T_8ES, predigested 30 min at 37° C). Sarkosyl was aded (1/9 volume), and mixed well, and incubated 3 h at 37° C.

At this stage lysis was complete, and the lysate was purified by extraction with phenol saturated with T_8ES. The resulting organic phase was back extracted once with T_8ES. The combined aqueous phases were then repeatedly extracted with phenol until a clear interface was observed between the top and bottom layers. The aqueous phase was then extracted four times with diethyl ether until both layers were clear. The DNA was dialyzed against three changes of 2 liters of 6 mM Tris, pH 7.4; 0.1 mM EDTA; 10 mM NaCl.

The DNA concentration was measured spectrophotometrically at a wavelentgth of 260 nm, and if necessary the DNA was concentrated through Centricon filters (Amicon), or precipitated. In some cases the DNA concentration was confirmed based on ethidium bromide fluorescence compared to a series of standards (Maniatus et al., 1982). DNA was frozen at -20° C for long term storage. In later experiments, to avoid degradation from repeated thawing and freezing, DNA was stored at 4° C with a drop of chloroform.

Construction of DNA probe specific for DCB-1

DCB-1 genomic DNA was isolated and digested to completion with the restriction enzyme BglII. The DNA was ligated with pKC7 (Rao and Rogers, 1978) (digested with BglII and treated with alkaline

phosphatase) at a molar ratio of 3:1 insert: vector using T4 ligase. The ligation mixture was used to transform competent cells of *E. coli* HB101 and the cells were plated onto Luria agar plates containing ampicillin (50 ug/ml). Colonies which formed were picked and replica plated onto either kanamycin or ampicillin. Colonies were isolated which did not grow on kanamycin due to insertional inactivation of the resistance gene by a DCB-1 DNA fragment. Plasmid DNA was isolated from these clones using the mini-prep procedure of Birnboim and Doly (1979). The DNA was idgested with BglII to excise the inserts and the fragments resolved on an agarose gel.

Clones were tested for specificity by colony hybridization using methods previously described (Grunstein and Hogness, 1975). Clones with inserts of DCB-1 DNA were plated onto duplicate agar plates. DCB-1 genomic DNA was used to probe one of the plates as a positive control. The replicate plate was probed with DNA isolated from the bromobenzoate sediment enrichment (Linkfield, 1985). This enrichment had been shown to hybridize to whole genomic DCB-1 DNA, but no DCB-1 like cells were observed (see results). One clone, designated pJJ7 was isolated that showed a hybridization signal to DCB-1 DNA, but a very weak signal to bromobenzoate sediment DNA. Thus, pJJ7 was used in subsequent experiments as a specific probe for DCB-1. pJJ7 had a DCB-1 DNA insert of 2.35 kb, resulting in a total plasmid size of 8.15 kb (Fig. 1).

DNA hybridization

Slot blot analysis of DNA (Kafatos et al, 1979) was conducted as follows. A known concentration of DNA was denatured by heating at 100 C for 5 min in 0.3 N NaOH. The denatured DNA was neutralized by

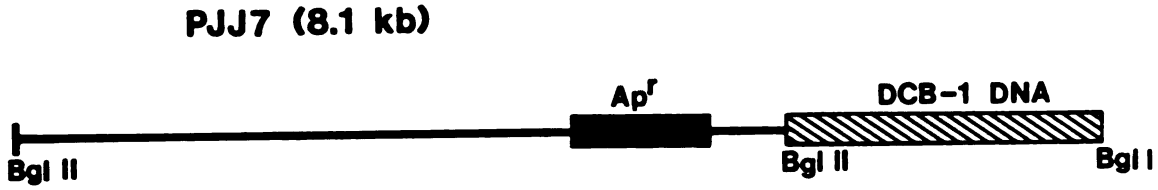

Fig. 1. **Restriction map of plasmid pJJ7.** Size was estimated by agarose gel electrophoresis of restriction fragments that were compared to *Hin*dIII digested phage lambda DNA standards. kb = kilobase pairs.

addition of 1 volume 2 N ammonium acetate on ice. The DNA was serially diluted 1:2 in 1 N ammonium acetate and filtered onto nitrocellulose (Amersham) using a slot-blot manifold with 24 wells (Bethesda Research Laboratory). Whatman 3 MM paper was wet with 1 N ammonium acetate and placed on the slot blot apparatus underneath the nitrocellulose which was also prewet with ammonium acetate. DNA samples in the wells were drawn onto the nitrocellulose by vacuum filtration. The nitrocellulose filter was air dried and baked 2 h at 80 °C to fix the DNA to the filter. The filter was prehybridized in a solution containing 50% formamide and salmon sperm DNA for 12 to 24 h. Then approximately 0.5 ug 32P-labeled DCB-1 DNA was added.

The radioactive probes were prepared by nick translation using the commercial kit supplied by Bethesda Research Laboratories. The probe was denatured by heating at 100 °C for 5 min and added to the bag.

When the cloned DNA fragment was used as a probe for slot blots, pJJ7 was first digested with BglII. The DNA was run on an agarose gel (1%) to separate the fragments, and the DCB-1 specific fragment was excised. The DNA fragment was electroeluted into a dialysis membrane containing 100 ul standard electrophoresis buffer. The DNA in solution was taken up into an Eppendorf tube and extracted once with phenol followed by one extraction with diethyl ether and precipitation to remove contaminating agarose. The isolated fragment was nick translated and used as a probe as described above.

Alternatively, for Southern blots there was no need to separate the specific fragment from the plasmid vector. Southern blot analysis of BglII digested genomic DNA was conducted using standard methodology

(Southern, 1975). Therefore, the whole pJJ7 plasmid was nick translated and used as a probe.

Stringency during hybridization was controlled by the formamide concentration and the incubation temperature. For high stringency, hybridization and prehybridization was conducted with 50% formamide at 42 °C and for lower stringency with 30% formamide at 37 °C. The salt concentration and temperature were adjusted during subsequent washing steps depending on the desired stringency. For high stringency the SSC concentration and washing times were as follows: briefly in 2X SSC + 0.1% SDS at room temperature, followed by three washes for 15 min each in 2X SSC + 0.1% SDS, 0.5X SSC + 0.1% SDS, and 0.1X SSC + 0.1% SDS respectively, all at room temperature, and finally for 30 min in 0.1X SSC + 1% SDS at 50 °C. For lower stringency, the filters were washed briefly in 6X SSC + 0.1% SDS at room temperature, followed by three washes for 15 min each in 4X SSC + 0.1% SDS at room temperature, and finally for 30 min in 2X SSC + 0.1% SDS at 42 °C.

The amount of probe hybridized was assayed by autoradiography using Dupont Cronex 4 Medical X-Ray film. Exposure times varied from 24 h to 1 week, depending on intensity of the signals. The amount of DNA that hybridized to samples on the nitrocellulose was quantitated relative to hybridization of DCB-1 DNA to itself. Serial 1:2 dilutions of known concentrations of DCB-1 DNA were added to each filter as positive controls. Serial dilutions of sample DNA were examined for the concentration at which hybridization decreased linearly with a decrease in DNA. This was necessary due to the known problem of saturation of nitrocellulose at high DNA concentrations (Thomas, 1980). The DNA at a suitable concentration was examined

visually and compared to the DCB-1 DNA hybridization intensity. The known concentration of DCB-1 DNA was taken to be the equivalent amount of DCB-1-like hybridizing DNA for slots with the same intensity of hybridization. The percentage of DCB-1 hybridizing DNA in the sample was then calculated from the ratio of ug DCB-1 DNA to ug sample DNA at a given intensity. Concentrations were interpolated for sample DNA between the intensity levels of two concentrations of DCB-1 DNA.

RESULTS

Slot blot hybridization of DCB-1 DNA to the dechlorinating consortium

To estimate the density of DCB-1 in the defined consortium we used whole genomic DNA isolated from DCB-1, or a specific DNA fragment as molecular probes. The hybridization of DCB-1 DNA to the DNA from the other two consortium members, the methanogen and benzoate degrader, was examined to determine the amount of background hybridization. The amount of hybridization to the methanogen alone was also determined. Since the benzoate-degrader is an obligate syntroph and must be grown in co-culture with a methanogen, the amount of DNA hybridization attributed to the benzoate degrader was obtained by subtraction.

By slot blot analysis, approximately 13% of the anaerobic consortium consisted of DCB-1 (Table 3). There was no observed hybridization of the methanogen DNA to the dechlorinator. There was some hybridization for the benzoate degrader, but this was negligible. Therefore, this type of methodology could be used to assay population dynamics in the defined consortium.

Slot blot hybridization of DCB-1 DNA to various anaerobic enrichments.

DCB-1 genomic DNA was used as a probe for the presence of DCB-1 or a similar organism in various anaerobic cultures enriched for the degradation of haloaromatic compounds (Fig. 2, and Table 4). Negative controls were anaerobic microbial communities not expected to have organisms similar to DCB-1, i.e. cricket gut and phenol enrichment. Negligible hybridization was observed in both of these

Table 3. Relative hybridization intensity of DCB-1 DNA to the members of the defined 3-chlorobenzoate-degrading consortium.

	% Relative hybridization (Probe/Stringency)		
Sample	DCB-1/ High	DCB-1/ Low	pJJ7/ Low
DCB-1	100	100	100
Consortium	13	>10	13
Benzoate degrader + Methanogen	<1	<1	1
Methanogen	0	n.d.	n.d.

n.d. = not determined

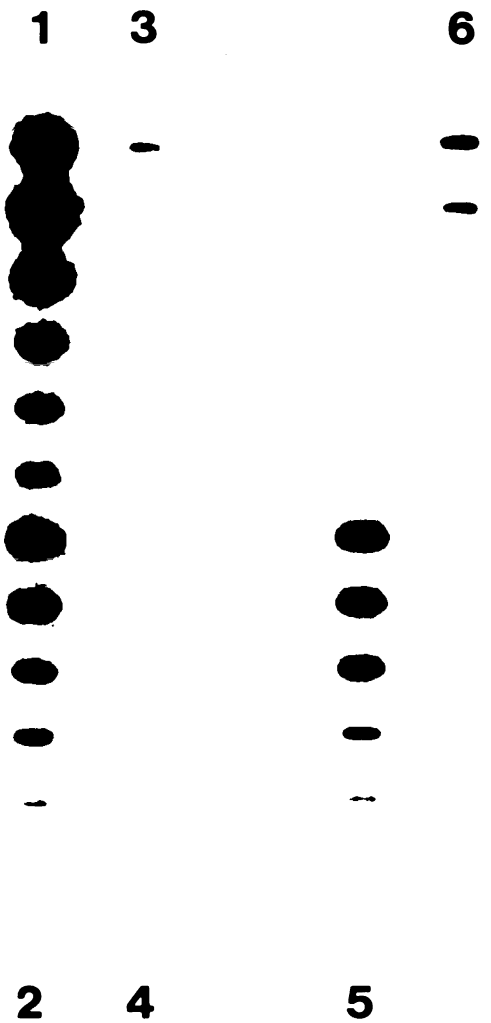

Fig. 2. Slot blot of serial 1:2 dilutions of DNA isolated from anaerobic enrichments. Sources of DNA and initial concentrations are as follows: DCB-1 (1 ug), 1; consortium (2 ug), 2; benzoate degrader + methanogen (5 ug), 3; methanogen (5 ug), 4; 3-bromobenzoate sediment enrichment (5 ug), 5; 3-chlorophenol sludge enrichment (5 ug), 6.

Table 4. Hybridization of DCB-1 DNA to DNA from various anaerobic enrichments.

DNA source	% Relative hybridization (Probe/Stringency)		
	DCB-1/ High	DCB-1/ Low	pJJ7/ Low
DCB-1	100	100	100
3-Chlorobenzoate Sludge (original)	10	>10	13
3-Chlorobenzoate Sludge (Schoberth)	4	3	5
3-Chlorobenzoate Sludge (Battelle)	<1	1	2
3-Bromobenzoate Sludge	n.d.	>10	13
3-Bromobenzoate Sediment	3	2	4
3-Chlorophenol Sludge	<1	n.d.	n.d.
Phenol Sludge	<1	n.d.	n.d.
Cricket Gut	n.d.	<1	<1

n.d. = not determined.

controls (Table 4) thereby demonstrating the specificity of this methodology.

There were varying degrees of hybridization detected for the different enrichments examined (Table 4). The highest degree of hybridization was to the original 3-chlorobenzoate enrichment where DCB-1 is known to be present, and to the bromobenzoate sludge enrichment. The 3-chlorobenzoate enrichment obtained from Schoberth also showed some relation, followed by the 3-bromobenzoate sediment enrichment. The other 3-chlorobenzoate enrichment (Linkfield, Battelle Laboratories) was weakly related to DCB-1, and the 3-chlorophenol enrichment was not related at all.

Southern blot analysis of DCB-1 in various enrichments.

Southern blot analysis was used to determine whether the hybridization observed by slot blot analysis was due to the presence of DCB-1. The only DNA that hybridized to the DCB-1 specific fragment was from samples where DCB-1 was known to be present (i.e. the reconstructed consortium, and the original 3-chlorobenzoate sludge enrichment) (Fig. 3). To confirm that the plasmid vector did not participate in the hybridization reaction, a duplicate filter was probed with the pKC7 vector alone (i.e. no DCB-1 DNA fragment). THe absence of hybridization when the vector alone was used as a probe indicated that the bands obsrved were due to hybridization of the DCB-1 probe to DCB-1-specific DNA sequences.

Slot blot hybridization of DCB-1 to various known cultures.

Since DCB-1 is thought to be a sulfidogen, DNA-DNA hybridization was done to examine the relatedness of DCB-1 DNA to several known sulfate reducers as well as to other possibly related strains (Table

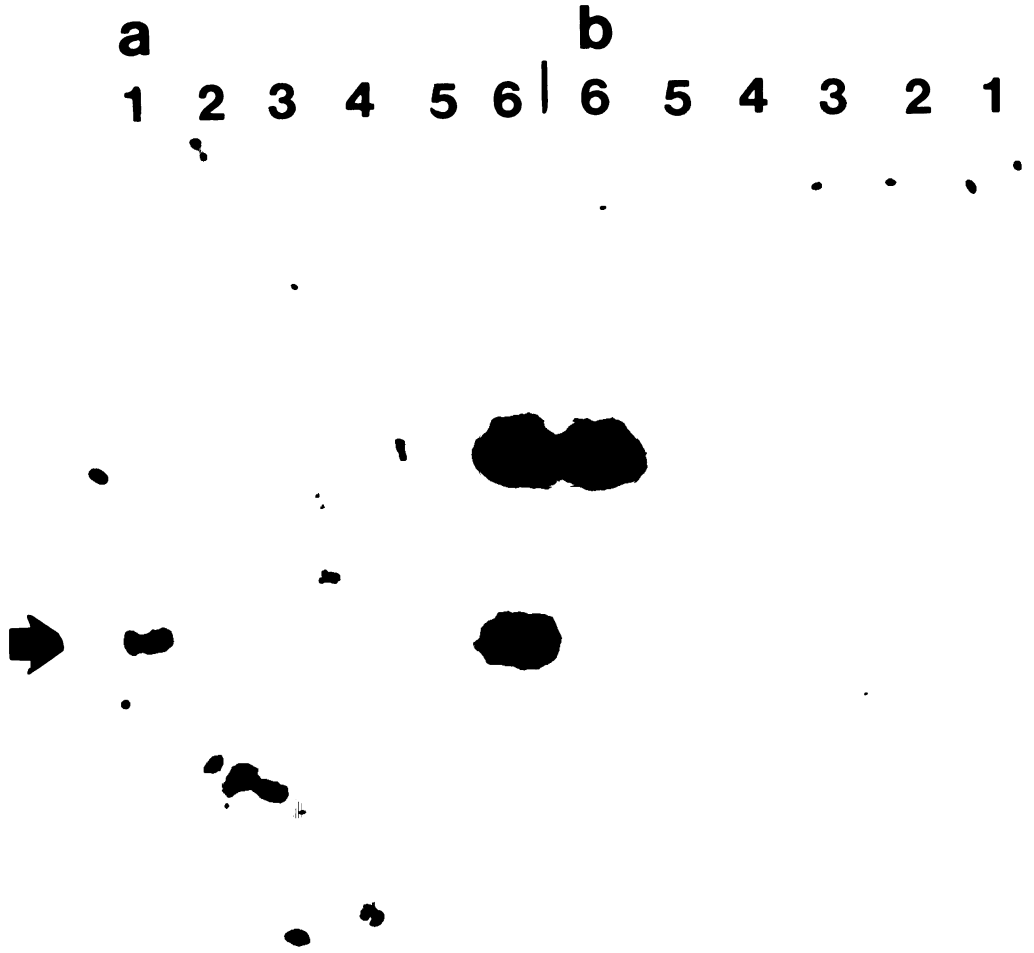

Fig. 3. Southern blot of DNA isolated from various anaerobic cultures and digested with *Bgl*II. Sources of DNA were as follows: 3-chloro-benzoate sludge enrichment (Linkfield), 1; 3-bromobenzoate sediment enrichment, 2; phenol sludge enrichment, 3; reconstructed consortium, 4; DCB-1, 5; pJJ7, 6. Samples on the left (a) were probed with the [^{32}P]-labeled pJJ7 and samples on the right (b) with [^{32}P]-labeled pKC7. Arrow indicates presence of the DCB-1-specific DNA fragment. Upper band corresponds to the pKC7 vector.

5). These results indicate that the cultures which exhibited the highest degree of relatedness to the DCB-1 genomic or specific probes were the three strains of *Desulfovibrio*. In addition, there was some hybridization above background to *Desulfotomaculum orientis* and to *Desulfomomas propionicus*. The DCB-1 fragment but not the total genomic probe hybridized to DNA isolated from *Bdellovibrio*.

Wolinella, Clostridium, and *Bacteroides* spp. were chosen as representatives of other classes of strict anaerobes, none of which showed any relatedness to DCB-1 DNA.

DISCUSSION

The results of this study indicated that DNA hybridization can be utilized as a method to detect DCB-1 in anaerobic enrichments. We isolated purified DNA from the different cultures, which enabled us to measure the exact concentration of DNA used in the hybridization experiments. In previous work using DNA probes in anaerobic communities, dot blots of crude cell lysates, standardized based on the number of cells in the sample, was used (Salyers et al, 1983). Although using a crude lysate method is simple, it does not allow for standardization of very dissimilar samples such as those used in this study. Also, it is not possible to standardize by optical density the number of cells present in crude enrichments. Furthermore, Kuritza and Salyer (1985) found that non-DNA components interfered with hybridization if greater that 10^8 bacteria were added to the filter.

We used slot blot hybridization instead of solution hybridization to measure the DNA relatedness between DCB-1 and different pure cultures. Solution hybridization is the method of

Table 5. DNA hybridization to pure cultures

Strain	% Relative hybridization (Probe/Stringency)		
	DCB-1/ High	DCB-1/ Low	pJJ7/ Low
DCB-1	100	100	100
Bdellovibrio bacteriovorus	n.d.	<1	4
Myxococcus xanthus	n.d.	<1	<1
Desulfomonas propionicus	n.d.	2	3
Desulfovibrio desulfuricans			
DG2	3	n.d.	n.d.
DDS	3	3	4
P3	5	n.d.	n.d.
Bacteroides sp.	1	n.d.	n.d.
Desulfococcus multivorans	<1	<1	<1
Desulfotomaculum orientis	n.d.	2	2
Wolinella succinogenes	n.d.	<1	1
Clostridium sp.	<1	n.d.	n.d.
Lambda	<1	n.d.	n.d.

n.d. = not determined.

choice for examining DNA-DNA relatedness between species because the kinetics of reassociation in solution are well known (Britten and Davidson, 1985). By contrast, the kinetics of reassociation of DNA bound to a filter have not been well studied, although it is thought to resemble pseudo first order kinetics (Anderson and Young, 1985). Solution hybridization was not an attractive option because relatively high amounts of DNA are needed. In some enrichments, and pure cultures, the cell yield was so low that the amount of DNA was insufficient for solution hybridization. In addition, slot blot analysis has the advantage of the ease of sampling and the ability to assay multiple samples at one time. Furthermore, visual determination of the degree of hybridization intensity is known to be accurate (greater than 2 fold over a 100-fold range taking into account both the intensity and diameter of the spots) (Anderson and Young, 1985).

Quantitation of DCB-1 in the reconstructed consortium by slot blot analysis resulted in a lower percentage of DCB-1 cells than was obtained by microscopic counting. Dolfing and Tiedje (1987) determined that 31% of the cells in the consortium were DCB-1 compared to 13% in this study. We do not know the reason for this disparity, but it may have been due to a difference in the number of DCB-1 cells in the two different cultures examined.

The 3-chlorobenzoate and 3-bromobenzoate sludge enrichments showed comparable amounts of hybridization in slot blot analyses using low stringency conditions (Table 4). The two enrichments were obtained from the same sludge source (Linkfield, 1985). Microscopic examination of the 3-bromobenzoate sludge enrichment revealed that

DCB-1-like cells were common; DCB-1 cells are easy to distinguish based on their large and characteristic morphology (Shelton and Tiedje, 1984). The presence of DCB-1 in the other enrichments in Table 4 was less likely. However, there does appear to be an increase in hybridization over that of negative controls for the other chlorobenzoate enrichments and for the bromobenzoate sediment enrichment which may indicate the presence of DCB-1 related organisms. DCB-1-like cells have never been microscopically observed in the bromobenzoate sediment enrichment (Linkfield, personal communication) or the 3-chlorobenzoate enrichment from Germany (Schoberth, personal communication)

We expected to see a significant difference in the amount of hybridization obtained at the different stringency levels, or between the different probes used. However, the total genomic DNA appeared to be as specific for DCB-1 in these studies as the cloned fragment (Table 3). This may reflect the uniqueness of this organism, or the limited species compositon of anaerobic enrichments.

Southern blot analysis under high stringency conditions was used to identify the DCB-1 organisms in the various anaerobic enrichments (Fig. 3). The results suggest that the hybridization observed for the bromobenzoate sediment enrichment in the slot blot experiment was not due to DCB-1 but to related organisms. Alternatively, the concentration of DCB-1 in that enrichment could have been too low to be detected on a Southern blot. We have previously found (Holben et al, 1988) that slot blots were more sensitive than Southern blots for detection of bacteria in a mixed community. This is likely due to

the capacity for loading more DNA in a concentrated area by slot blot analysis.

Slot blot DNA hybridization revealed that of the different anaerobic strains tested, DCB-1 appears to be most closely related to sulfate reducing bacteria, although the degree of relatedness by this methodology was low. The DNA hybridization results nonetheless support the physiological information which also suggests that DCB-1 is a sulfidogen. DCB-1 is most likely related to the gram (-), sulfate-reducing, non-spore forming bacteria. The percent G+C of DCB-1 DNA was determined to be 49% (J. Johnson, personal communication). This value falls within the range reported to be common for sulfidogens (36-67%) (Widdel and Pfennig, 198).

There was some hybridization above background to *Bdellovibrio* using the cloned DCB-1 fragment as a probe. This may reflect some nucleic acid relatedness, such as is known based on rRNA homology studies of sulfate reducers and this species (Fowler et al., 1986). However, *Myxococcus*, which is also related to sulfate reducers on the basis of rRNA homology showed no degree of hybridization to DCB-1.

In this study, a randomly cloned fragment of DCB-1 DNA was used as a probe for DCB-1 instead of the gene for the specific dechlorinase activity which would have been prefered. I did some initial experiments to look for the presence of the dechlorinase gene on plasmid DNA. However, using five different plasmid DNA isolation procedures, I was unable to detect any plasmids in this organism. Attempts to clone the dechlorinase gene have so far been unsuccessful.

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APPENDICES

APPENDIX A

DNA PROBE METHOD FOR THE DETECTION OF SPECIFIC MICROORGANISMS IN THE SOIL BACTERIAL COMMUNITY.

WILLIAM E. HOLBEN,[1,2] JANET K. JANSSON,[1] BARRY K. CHELM,[2,3] AND JAMES M. TIEDJE[1,3]*

Department of Crop and Soil Sciences[1], Plant Research Laboratory[2] and Department of Microbiology and Public Health[3], Michigan State University, East Lansing, Michigan, 48824.

ABSTRACT

We have developed a protocol which yields purified bacterial DNA from the soil bacterial community. The bacteria were first dispersed and separated from soil particles in the presence of polyvinylpolypyrrolidone which removes humic acid contaminants by the formation of an insoluble complex. The soil bacteria were then collected by centrifugation and lysed using a comprehensive protocol designed to maximize the disruption of the various types of bacteria present. Total bacterial DNA was purified from the cell lysate and remaining soil contaminants using equilibrium density gradients. The isolated DNA was essentially pure as determined by UV spectral analysis, was at least 48 kb long, and was not subject to degradation, which indicated that there were no contaminating nucleases present. The isolated DNA was readily digested by exogenously added restriction endonucleases and successfully analyzed by slot blot and Southern blot hybridizations. Using single-stranded, [^{32}P]-labelled DNA probes we could detect and quantitate the presence of a specific microbial population in the natural soil community based on the presence of a DNA sequence unique to that organism. The sensitivity of our

methodology was sufficient to detect *Bradyrhizobium japonicum* at densities as low as 4.3 x 10^4/g dry weight of soil which corresponds to ~0.02 pg of hybridizable DNA.

INTRODUCTION

Methods for the detection of specific microorganisms in environmental samples have traditionally used fluorescent antibody and selective plating techniques each of which is useful but limited in some aspects. The prospect for the intentional or accidental release of genetically engineered microorganisms into the environment has served to highlight the need for further methodologies for monitoring the fate of particular microorganisms in the environment, especially for those that become non-culturable (8, 22) or for the detection of gene transfer to new populations. The methods of molecular biology that allow detection of particular DNA sequences can be used to detect particular genes, and hence organisms containing these genes, in the environment. If the total DNA of soil microorganisms could be recovered directly from soil, then the fate of particular genes or organisms could be monitored directly without the requirement for successful culture of the recovered organism.

Previously used protocols for the isolation of bacterial DNA from soil involved trichloroacetic acid treatment and ethanol/ether extraction of soil bacterial fractions filtered through diatomaceous earth (24), or purification of DNA from detergent and high salt in cell lysates by hydroxyapatite column chromatography (J. Goksoyr, personal communication). These methods were tedious and allowed processing of only a few samples per week and have not been demonstrated to yield DNA of sufficient purity for use in restriction digestion and hybridization reactions.

In order to adapt DNA probe methodology for use in soils, the following features of a protocol needed to be improved or developed.

First, a procedure was needed which would allow processing of more samples simultaneously and in a shorter period of time in order to analyze the number of treatments and replicates needed for ecological studies. Secondly, the isolated DNA had to be of sufficient purity and size for use in experiments involving digestion with restriction endonucleases, transfer to cellulose nitrate membranes, and hybridization to DNA probes. Humic acids and other contaminants, if not removed, could reduce the efficiency of digestion by restriction endonucleases and the specificity of hybridization. Third, it was also necessary to develop probes that were both sensitive and specific enough to detect the presence of a particular sequence of low frequency in the complex mixture of DNA isolated from the soil bacterial community. The standard method of labelling probes by nick translation (16) did not appear to be sensitive or specific enough to be a candidate method for probing natural populations.

The objective of this work was to develop a protocol for the use of sequence-specific DNA probes to detect specific genes and microorganisms in soil. We successfully used both slot blot and Southern transfer to fix DNA to cellulose nitrate filters which were subsequently used in hybridization experiments to detect *Bradyrhizobium* strains in soil. Both a naturally occurring sequence, the *rbcL* gene, and a sequence engineered into *Bradyrhizobium*, the *nptII* gene, were used as probes.

MATERIALS AND METHODS

Bacterial strains and phages. *Bradyrhizobium japonicum* strain BJ110 is a stable, small-colony derivative of *B. japonicum* strain USDA 3I1b110 (18). *B. japonicum* strain CRM52 is a

ribulose-1,5-bisphosphate carboxylase-oxygenase deficient (RUBISCO$^-$), phosphoribulokinase deficient (PRK$^-$) derivative of *B. japonicum* strain BJ110 obtained by gene-replacement with the *nptII* (kanamycin^r) gene (18). *Escherichia coli* strain JM103 and the M13 mp phage vectors are described elsewhere (7).

Culture media. *Bradyrhizobium japonicum* strains BJ110 and CRM52 were cultured at 30°C on YEMN medium which contains 0.04% yeast extract, 1.0% mannitol, 3 mM K_2HPO_4, 0.8 mM $MgSO_4$, 1.1 mM NaCl and 10mM KNO_3. All other strains were cultured at the appropriate temperature (30°C or 37°C) in Luria broth (LB) which contains per liter: 10 g Bacto-tryptone, 5 g yeast extract and 5 g NaCl. Enumeration of *B. japonicum* strain CRM52 in soil was accomplished by plating appropriate dilutions of soil suspensions onto YEM (same as YEMN except containing no KNO_3) agar containing 30 ug/ml chloramphenicol (to which *B. japonicum* strains BJ110 and CRM52 are naturally resistant) and 50 ug/ml kanamycin since the *nptII* gene confers kanamycin resistance and also containing 300 ug/ml cycloheximide (to control fungal growth). The plates were incubated for 7 to 10 days at 30°C prior to counting.

Inoculation and sampling of bacteria in soil. Bacterial cultures *of B. japonicum* strains BJ110 and CRM52 were grown to mid-log phase (OD_{600} = 0.5) in YEMN broth. The cultures were harvested by centrifugation (23,000 x *g* for 20 min at 4°C), washed once with an equal volume of sodium phosphate buffer (15 mM, pH 7.0), and again collected by centrifugation. The cell pellets were resuspended in 1/20th volume of sodium phosphate buffer and starved for 48 h by incubation at room temperature. Appropriate dilutions of the

bacterial suspensions were made with 15 mM sodium phosphate buffer such that, regardless of cell density, 50 ml of total inoculum was added to 10 g of vermiculite (W.R. Grace and Co., Cambridge, MA) and thoroughly mixed. The vermiculite/inoculum mixture was then thoroughly mixed with 700 g (dry weight) of Capac loam soil (a fine-loamy, mixed, mesic, aeric ochraqualf) which had been sifted through a 2-mm sieve. The inoculated soil was transferred to plastic pots with slotted bottoms and hydrated to approximately 80% of field capacity by standing in 5-6 cm of sterile, distilled water until moisture visibly reached the soil surface. At appropriate time intervals, "mini-core" samples were taken from the soil pots using 28 mm diameter x 170 mm lengths of copper pipe with the resulting hole being filled by a polypropylene test tube of the same dimensions. This non-destructive sampling method allowed multiple samples to be taken from a single soil pot. A 10 g subsample of the mini-core was used for enumeration of *B. japonicum* by selective plating and a 50 g subsample was used for the isolation of total bacterial DNA from soil.

Isolation of the bacterial fraction from soil. The bacterial fraction was isolated from soil using a modification of the fractionated centrifugation technique described by Goksoyr and co-workers (11) and is depicted schematically in Fig. 1. Soil samples (50 g) were combined with 200 ml of homogenization solution (described below) and 10 g of acid-washed polyvinylpolypyrrolidone (PVPP) blended in a Waring blender for three 1 min intervals with cooling in an ice water bath for 1 min between intervals. The homogenate was transferred into 250-ml centrifuge bottles and the fungal biomass and soil debris pelleted by centrifugation at 1000 x *g* for 15 min at 4°C.

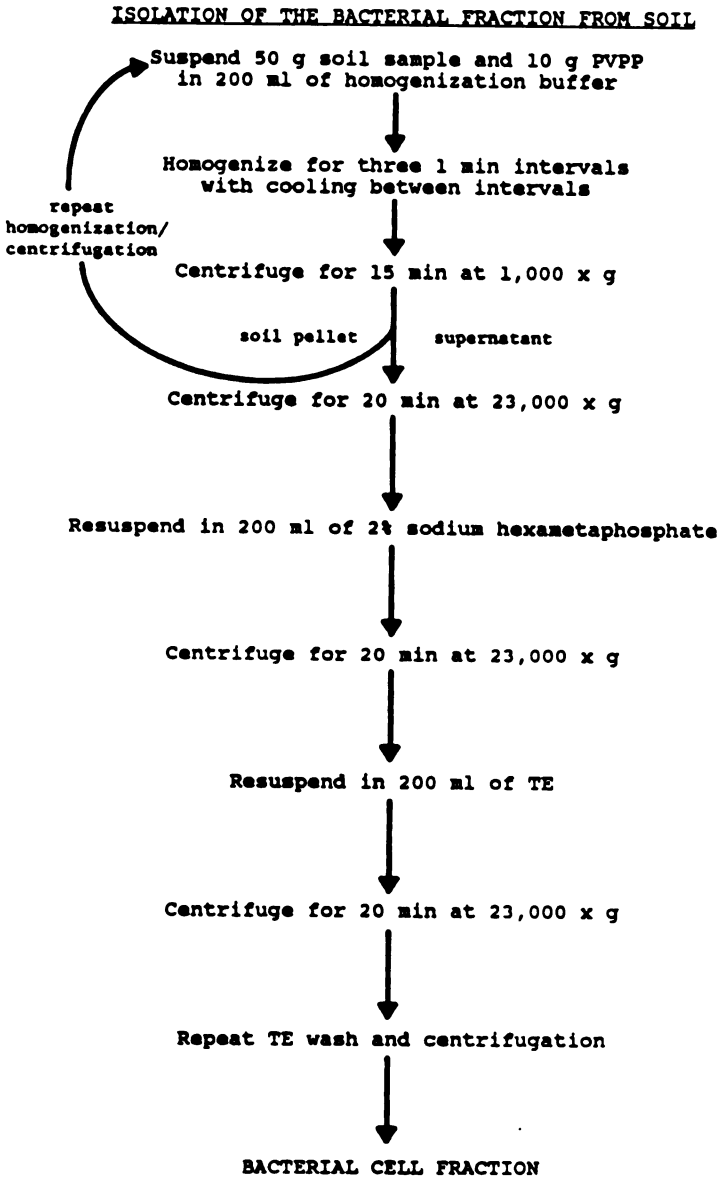

Fig. 1. Scheme for isolating the bacterial fraction from soil by fractionated centrifugation of soil suspensions.

The supernatant (SN1) was transferred to a fresh 250-ml centrifuge bottle and subjected to centrifugation at 23,000 x *g* for 20 min at 4°C to collect the bacterial fraction while the soil pellet (P1) was subjected to further (usually two more) rounds of homogenization and centrifugation. The combined bacterial pellets for all rounds of homogenization were resuspended in 200 ml of 2% w/v sodium hexametaphosphate, pH 8.5 (Pfaltz and Bauer, Inc., Waterbury, CT) then collected by centrifugation at 23,000 x *g* for 20 min at 4°C. The bacterial pellet was then washed twice by resuspending in 200 ml of TE which contains 33 mM Tris (pH 8.0) and 1 mM EDTA. The bacteria were collected by centrifugation and resuspended in 100 ml of TE to yield the bacterial suspension for lysis.

The homogenization solution consisted of Winogradsky's salt solution (20), diluted 1:20 (1.43 mM K_2HPO_4, 1.01 mM $MgSO_4 \cdot 7H_2O$, 2.14 mM NaCl, 4.75 uM $Fe_2[SO_4]_3 \cdot 7H_2O$, 14.8 uM $MnSO_4 \cdot 4H_2O$) to which 0.2 M sodium ascorbate was added just prior to use. The acid-washed PVPP was prepared as described by Evans *et al.* (10). Insoluble PVPP (Sigma Chemical Company, St. Louis, MO) was suspended in 3 M HCl (typically 300 g of PVPP was suspended in 4 l of 3 M HCl) for 12 to 16 h at room temperature. The suspension was then filtered through MIRACLOTH (Chicopee Mills,Inc., Milltown, N.J.) and the PVPP resuspended in 4 l of 20 mM potassium phosphate (pH 7.4) and mixed by stirring for 1 to 2 h. This process was repeated until the suspension reached pH 7.0, after which it was filtered through MIRACLOTH and the PVPP air-dried overnight.

Lysis of the bacterial fraction. The procedure used to lyse the bacterial fraction is outlined in Fig. 2. The bacterial suspension

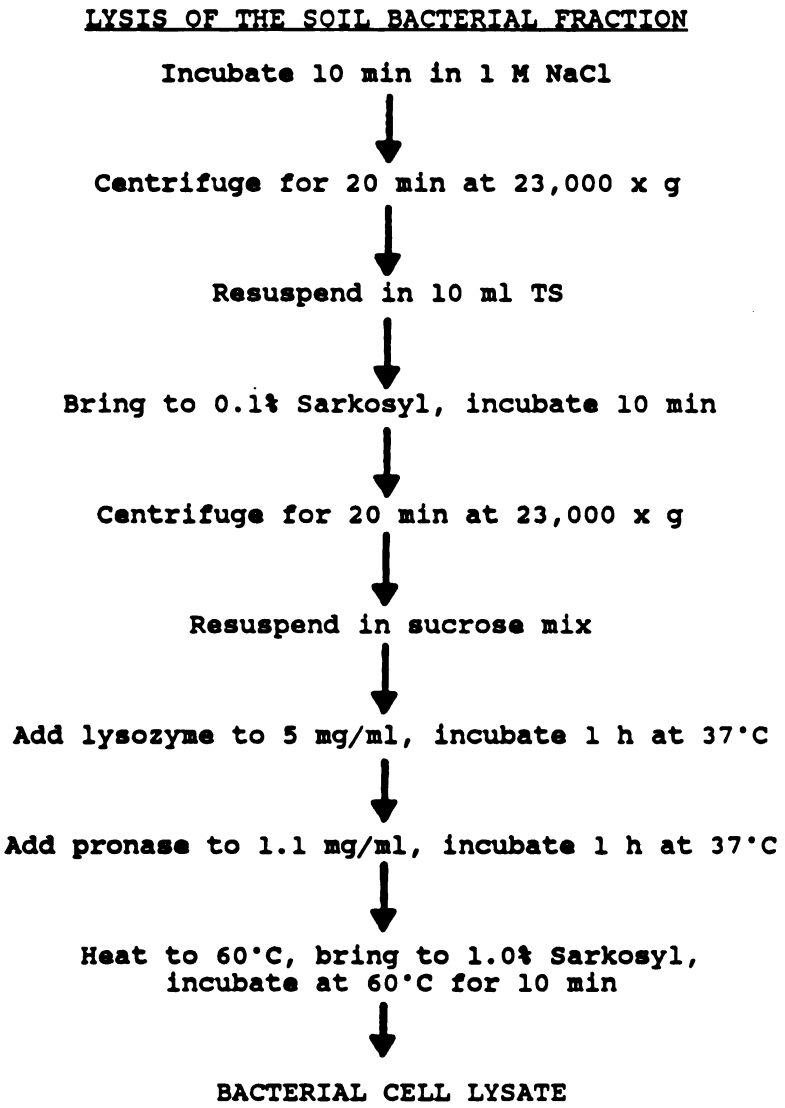

Fig. 2. Scheme for the lysis of the soil bacterial fraction using the comprehensive protocol we describe.

(in 100 ml of TE) was brought to 1 M NaCl by the addition of 25 ml of 5 M NaCl, incubated at room temperature for 10 min, then collected by centrifugation at 23,000 x *g* for 20 min at 4°C. The pellet was resuspended in 10 ml of TS which contains 50 mM Tris (pH 8.0) and 50 mM NaCl, transferred to a 50-ml polycarbonate centrifuge tube and then brought to 0.1% Sarkosyl by the addition of 50 ul of 20% Sarkosyl. This mixture was incubated at room temperature for 10 min, after which the bacteria were again collected by centrifugation. The bacterial pellet was drained well and resuspended in 3.5 ml of Tris/sucrose/EDTA which contains 0.75 M sucrose, 50 mM Tris (pH 8.0), and 10 mM EDTA. Lysozyme was added to a final concentration of 5 mg/ml by the addition of 0.5 ml of a 40 mg/ml solution of lysozyme in TS and the samples were then incubated at 37°C for 60 min. Pronase (0.5 ml of a 10 mg/ml solution in TS of Type XIV pronase from *Streptomyces griseus* [Sigma Chemical Company, St. Louis, MO]) that had been predigested by incubation for 30 min at 37°C was added to the bacterial cell/lysozyme mixture, mixed by vortexing, then incubated at 37°C for 60 min. This mixture was brought to 65°C followed by the addition of 0.25 ml of 20% Sarkosyl, then further incubated for 10 min at 65°C. The lysates were then stored on ice overnight. The lysis protocol described above was used for all bacterial fractions isolated from soil. Other methods of bacterial lysis used for comparison were the method of Doi (9) and extensive sonication (3 min on ice) with a Branson Sonic Power sonifier model S-125.

Cesium chloride-ethidium bromide equilibrium density centrifugation. Bacterial lysates were cleared by centrifugation at 40,000 x *g* for 1 h at 4°C and the supernatant transferred to a clean 50-ml polycarbonate

centrifuge tube. The volume was adjusted to 25 ml using distilled water and 27 g of finely ground cesium chloride (CsCl) was added and mixed by gentle inversion. After the CsCl was fully dissolved, 2.5 ml of 10 mg/ml ethidium bromide (EtBr) was added and the refractive index adjusted to 1.3865 by the addition of distilled water (if > 1.3865) or cesium chloride (if < 1.3865). The DNA was banded by equilibrium density centrifugation at 18°C for 16 to 20 h in a VTi 50 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 45,000 rpm. The DNA band was made visible by illumination with long-wave ultraviolet light, which caused the ethidium bromide-DNA complex to fluoresce, and the DNA removed from the gradient using a 16 gauge needle and syringe. The DNA, still in CsCl-EtBr, was again banded by ultracentrifugation under the same conditions for increased purity. Following the second banding, the EtBr was removed from the DNA by multiple rounds of extraction with isopropanol (stored over a saturated NaCl solution) until the aqueous (lower) phase was colorless, and then extracted once more. The sample was then diluted with two volumes of distilled water followed by the addition of twice the diluted volume of cold (-20°C) 100% ethanol and the DNA precipitated at -20°C overnight. The DNA was collected by centrifugation at 7,000 x *g* for 30 min at 4°C, dried *in vacuo*, then resuspended in 400 ul of distilled water. The DNA solution was transferred to a microfuge tube to which 40 ul of 3 M sodium acetate (pH 5.2) was added and the DNA reprecipitated by the addition of 840 ul of cold ethanol followed by incubation overnight at -20°C. The DNA pellet was collected by centrifugation for 15 min at 4°C in a microfuge, dried *in vacuo*, and resuspended in a small volume (usually 100 ul) of distilled water. The concentrations of the

purified DNA solutions obtained were determined by absorbance at 260 nm (e_{260} = 6,500).

Restriction endonuclease digestion of DNA. All restriction digestions of DNA were performed according to the manufacturer's specifications.

Synthesis of single-stranded DNA probes. Figure 3 provides a graphic representation of the protocol for the synthesis of single-stranded DNA probes. Single-stranded DNA probes specific for the *nptII* gene were synthesized by primer extension using the universal M13 sequencing primer and M13 mp19 phage available from Bethesda Research Laboratories (BRL), Gaithersburg, MD. To maximize the specific activity of the probes all four radiolabeled deoxynucleotides (i.e. [$a^{32}P$]-dATP, [$a^{32}P$]-dTTP, [$a^{32}P$]-dCTP and [$a^{32}P$]-dGTP) were used in the primer extension reaction. The *nptII* gene was cloned as a 1.1-kb *Hin*dIII-*Sma*I fragment into the double-stranded replicative form of M13 mp19 using standard recombinant DNA techniques (7, 16). Recombinant phage were transformed into *E. coli* strain JM103 and single-stranded phage DNA isolated from the mature phage as described elsewhere (7). The universal primer was hybridized to the single-stranded DNA by incubating a mixture of 0.5 ug of DNA, 3.4 pmol of universal primer and 0.2 ul of 10X Klenow buffer (70 mM Tris [pH 7.5], 70 mM $MgCl_2$, 500 mM NaCl) in 2 ul total volume at 95°C for 5 min, then cooling slowly to room temperature. For the primer extension reaction, the above mixture was combined with 0.8 ul of 10X Klenow buffer, 1.7 ul of each of the four [$a^{32}P$]-radiolabeled deoxynucleotides (3000 mCi/mmol, Amersham Corporation, Arlington Heights, IL) and 0.5 units of the Klenow fragment of DNA polymerase I

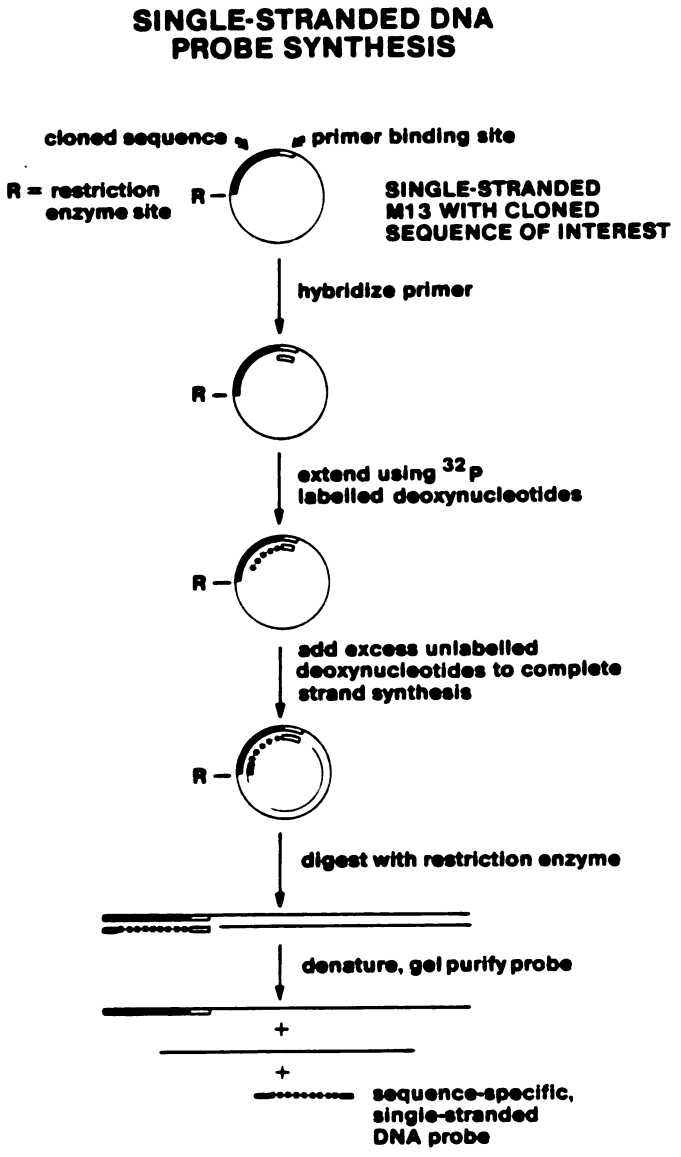

Fig. 3. **Synthesis of [^{32}P]-labeled, single-stranded DNA probe.** Probes were labelled by a primer extension reaction from a single-stranded DNA template isolated from recombinant M13 phage. The * indicate the ^{32}P-labelled region.

in a total volume of 10 ul, then incubated at room temperature for 60 min. The reaction was completed by the addition of 2 ul of an aqueous solution containing each of the four deoxynucleotides (unlabelled) at a concentration of 3 mM (pH 7.0) and incubating at room temperature for an additional 60 min. The DNA (now double-stranded) was digested with the restriction endonuclease *Hin*dIII in 50 ul total volume for 60 min.

The digested sample was combined with 150 ul of single-stranded DNA loading buffer (89 mM Tris [pH 8.0], 89 mM boric acid, 2 mM EDTA, 0.1% w/v xylene cyanole, 0.1% w/v bromophenol blue, 80% formamide), denatured by immersion in boiling water for 10 min, and size fractionated by electrophoretic separation in a 1.5 mm x 22 cm x 26 cm denaturing gel consisting of 4% polyacrylamide and 50% w/v urea in 1X TBE buffer (89 mM Tris-base, 89 mM boric acid, 2 mM EDTA) for 10 h at 400 volts. The labeled probe fragment was localized in the gel by autoradiography (30 sec to 1 min exposure time was generally sufficient), excised, then eluted from the polyacrylamide by being pulverized in 400 ul of elution buffer (0.3 M LiCl, 10 mM Tris [pH 7.5], 0.05% w/v sodium dodecyl sulfate, 0.1 mM EDTA) and incubated at 37°C for at least 6 h. The eluant was filtered thru a QUIK-SEP column (Isolab, Inc., Akron, OH) to yield the purified probe.

DNA hybridization. Cellulose nitrate filters for slot blot analyses were prepared essentially as described by Palva (19). Typically, 1 ug of sample DNA was denatured in 0.3 M NaOH for 5 min at 100°C, chilled on ice, then neutralized by the addition of an equal volume of cold 2 M ammonium acetate. Using a slot blot manifold (Schleicher and Schuell, Keene, NH), the samples were spotted onto a cellulose nitrate

filter that had been prewetted with 1 M ammonium acetate. For Southern blot analyses, DNA was size fractionated by electrophoresis in 0.7% agarose gels and transferred to cellulose nitrate filters as described elsewhere (6, 23). All cellulose nitrate filters were baked *in vacuo* at 80°C for 2 h to fix the DNA samples.

The solutions used for DNA hybridization analyses have been described elsewhere (16). The filters were prehybridized for at least 6 h at 42°C in heat sealed pouches containing 100 ul of prehybridization fluid per cm^2 of filter. Prehybridization fluid contains 5X Denhardt's solution, 5X SSPE, 50% formamide and 200 ug/ml of denatured (by immersion for 10 min in boiling water) salmon sperm DNA. Denhardt's solution (1X) consists of Ficoll, polyvinylpyrollidone (M.W. = 40,000) and bovine serum albumin, each at 200 ug/ml. SSPE (1X) contains 0.18 M NaCl, 10 mM NaH_2PO_4 (pH 7.4) and 1mM EDTA. Hybridization was performed at 42°C for 24 to 30 h using 100 ul of hybridization fluid per cm^2 of filter. Hybridization fluid consists of 5X Denhardt's solution, 5X SSPE, 50% formamide, 10% w/v dextran sulfate and 200 ug/ml denatured salmon sperm DNA. Following hybridization, the filters were washed once for 20 min with agitation at room temperature with each of the following solutions: [2X SSC, 0.1% SDS]; [0.5X SSC, 0.1% SDS]; [0.1X SSC, 0.1% SDS]; then once at 50°C with [0.1X SSC, 1.0% SDS]. SSC (1X) consists of 0.15 M NaCl and 0.015 M sodium citrate (pH 7.0). After washing, filters were exposed to X-ray film (Kodak X-Omat AR) at -70°C with a Quanta III intensifying screen (E. I. DuPont Nemours and Co., Wilmington, DE). Exposure times were based on the anticipated intensity of the radioactive signal (generally 2 to 10 days).

Viable cell counts. Viable cell counts of the soil and separated bacterial fractions were performed by plating appropriate dilutions (in duplicate) onto PTYG medium (5) which contains the following ingredients per liter of distilled water: peptone, 0.25 g; tryptone, 0.25 g; yeast extract, 0.5 g; glucose, 0.5 g; $MgSO_4 \cdot 7H_2O$, 30 mg; $CaCl_2 \cdot 2H_2O$, 3.5 mg; Bacto-Agar (Difco Laboratories), 15.0 g and, in addition, 300 ug/ml cycloheximide. The plates were incubated aerobically for 1 week at 30°C after which the total number of colonies were counted.

Acridine orange direct counts (AODC). Enumeration of bacteria by AODC was performed essentially as described by Ramsay and Bawden (21). The soil pellets obtained after 1, 2, or 3 rounds of homogenization/centrifugation (P1, P2 and P3) were resuspended in 200 ml of homogenization solution then homogenized in a Waring blender for 1 min. These soil suspensions and the bacterial cell suspensions (supernatants SN1, SN2 and SN3) were serially diluted into 50 mM Tris buffer (pH 7.5). Acridine orange was added to a final concentration of 5 ug/ml. After staining, 10 ml samples were filtered onto Nucleopore 0.2 um polycarbonate filters which were previously stained with Irgalan black. Enumerations were made with an Olympus AHBS microscope equipped with a mercury lamp and appropriate filters for epifluorescent microscopy.

RESULTS AND DISCUSSION

Efficiency of recovery of the bacterial fraction from soil. The DNA isolation protocol involves an initial separation of the soil bacterial fraction from fungal mycelia and soil debris. By first isolating the bacterial fraction from soil we insure that the DNA

obtained is of bacterial origin and not of fungal or other origin. We also sought to protect the DNA from direct contact with soil since nucleic acids in soil are readily degraded by nuclease producing microorganisms (13) and because the high binding capacity of soil for phosphate and for DNA is well-known (B.G. Ellis personal communication, 13). It is also likely that methods for the direct extraction of DNA from soil involving alkaline hydrolysis would result in contamination of the DNA by humic and fulvic acids since alkali extraction is the most common method for their recovery from soil (14).

Separation of the bacterial fraction from soil was accomplished using a modification of the fractionated centrifugation technique first described by Faegri *et al.* (11). Fractionated centrifugation involves the homogenization of a soil sample into a buffered salt solution followed by a low speed centrifugation step which pellets the soil debris and fungal mycelia while leaving the bacterial cells in suspension. We used Waring blenders for the homogenization steps as did Faegri *et al.* since the use of blenders has been shown to be superior to mechanical shaking for the release of bacteria from soil (15). This cell suspension was then subjected to high speed centrifugation to collect the bacterial fraction.

The efficiency of recovery of the soil bacterial fraction was determined by comparing the number of bacteria recovered to the number of bacteria remaining in the soil. Since accurate enumeration of bacteria in soil is problematic (1, 11), we employed two widely used techniques for counting bacteria, namely, enumeration of viable counts

by plating on non-selective medium and acridine orange direct counts (AODC). For each method, enumerations of bacteria were performed on the supernatants (SN1, SN2 and SN3 which contain bacteria in suspension) and the pellets (P1, P2 and P3 which contain the soil, fungal biomass and unreleased bacteria) for each of the low-speed centrifugation steps through three rounds of homogenization/ centrifugation. The results obtained using each method expressed as percent recovered are given in Table 1. The efficiencies of recovery were similar whether determined by viable counts or AODC although the actual numbers of cells enumerated by each method differed by two orders of magnitude. The disparity in the number of cells enumerated when the two methods are compared is a well-documented phenomenon (1, 11), and is based on the inability to culture a large percentage of organisms directly from soil. The inability of soil bacteria to efficiently survive transfer from soil directly onto culture medium seems not to affect determinations of the efficiency of extraction made by the viable count method probably because culturable and non-culturable cells are released from soil with the same efficiency.

Approximately 10% of the bacteria present in the soil used were released per round of homogenization/centrifugation (Table 1). Others have shown, based on bacterial cell size distribution, that the fractionated centrifugation technique releases all bacterial types from soil with equal efficiency through up to eight rounds of homogenization/centrifugation (1, 2). Thus, the bacterial fraction released after a single round of homogenization/centrifugation is apparently as representative of the total bacterial population as the bacterial fraction collected after multiple rounds of extraction. The

TABLE 1. Efficiency of recovery of the bacterial fraction from soil.

Sample	numbers of organisms	
	Direct counts	Viable counts
SN1	1.7×10^9	6.2×10^6
SN2	5.0×10^8	6.1×10^6
SN3	1.1×10^9	4.8×10^6
Total recovered [SN1+SN2+SN3]	3.3×10^9	1.7×10^7
Number remaining [P3]	6.3×10^9	3.4×10^7
Recovered (%) $\left[\frac{SN1+SN2+SN3}{SN1+SN2+SN3+P3}\right]$	34.9	33.3

number of rounds of soil extraction is thus determined by the sensitivity needed for the experiment rather than by the need to obtain the majority of the population to insure recovery of all types of bacteria. We have found that three rounds of homogenization/centrifugation were sufficient when the organism of interest was present at levels of 10^4 or more per gram of soil. It is likely that actual recoveries of organisms will depend on the type of soil being tested. It has been shown, for example, that the clay content of soil affects the dispersion of microorganisms by homogenization (1, 15).

Lysis of the bacterial fraction and DNA purification. The isolated bacterial fraction was lysed using a protocol that includes the salient features of several individual lysis protocols designed for use with different types of bacteria. We developed this comprehensive lysis protocol in an effort to insure the maximal disruption of the various types of bacteria present in the natural soil population. Bacterial cell lysates were then subjected to equilibrium density centrifugation on cesium chloride-ethidium bromide gradients to purify the total bacterial DNA from proteins, RNA and other contaminants.

The ability of our protocol to lyse the bacterial fraction was assessed by comparing it to the method of Doi (9) and to extensive sonication. Pure cultures of *Streptomyces coelicolor*, *Escherichia coli* strain ED 8654, *Bradyrhizobium japonicum* strain CRM52, *Bacillus subtilis* strain 168, *Corynebacterium floccumfaciens*, *Agrobacterium tumefaciens* strain A6, *Pseudomonas putida* and *Pseudomonas cepacia* strain PC01224 were grown to mid-log phase under appropriate culture

and temperature conditions. Each culture was divided into three aliquots and each aliquot lysed by one of the methods described above. All three lysis methods resulted in a total clearing of the cell suspensions indicating complete lysis. However, lysis by sonication resulted in DNA of very small size (approximately 500 bp), whereas the Doi method and our own lysis protocol yielded DNA in the 50 to 150 kb range as indicated by agarose gel electrophoresis (some data not shown). However, the Doi method was inferior to our own method of lysis when the purity of DNA isolated from soil was compared. The DNA recovered from soil using the Doi method had a brownish tint, was substantially contaminated as indicated by U.V.spectral analysis and was refractory to digestion by restriction endonucleases (data not shown).

Sensitivity and specificity of the single-stranded DNA probes. Since it was important to be able to detect organisms present at low population densities (by detecting DNA sequences present at very low levels in a complex mixture of DNA), we devised a probe system that would maximize both specificity and sensitivity. We developed a strategy (Fig. 3) by which a sequence-specific, single-stranded DNA probe was synthesized from a single-stranded DNA template. Each of the four deoxynucleotides in the initial reaction were labelled to maximize the amount of radioactive label incorporated. DNA probes synthesized in this fashion offer several advantages over probes synthesized by nick tranlation of double-stranded DNA, namely: i) Very high specific activities are obtained. e.g. nick translation reactions typically yield specific activities of 10^7 to 10^8 cpm/ug of DNA (this includes non-probe vector sequences which are also labelled by this

process), while probes synthesized by the primer extension reaction typically have specific activities of 1 to 2 x 10^{10} cpm/ug of DNA (which is essentially all probe sequence). ii) Background hybridization resulting from non-specific binding, cross-homology to other sequences, or hybridization of labelled vector sequences is eliminated or dramatically reduced since the probe product is a single strand of DNA with complete homology to the sequence of interest (except for about 20 bp of upstream primer). This feature is likely to be important since it has been shown that the genomes of many organisms contain homology to common, broad host range plasmids and that many plasmids have homology to other plasmids (3,12). iii) Concerns of probe sequences rehybridizing to their complement in solution, as can happen when denatured double-stranded probes are employed, are obviated since the probe is single-stranded. iv) The ability to carefully control the specific activity of the synthesized probe DNAs by controlling the reaction conditions makes it possible to synthesize different probes with the same specific activity so that results obtained using various probes can be directly compared.

The results of experiments to determine the sensitivity of the single-stranded probes both in slot blot and in Southern blot analyses are shown in Figure 4. In the slot blot experiment, the single-stranded probe was capable of detecting as little as 0.02 pg of the sequence of interest (Fig. 4A). This assumes that the 1.2 kb fragment containing the sequence of interest comprises about one five-thousandth of the genome of *B. japonicum*. Similarly, in the Southern blot experiment, the single-stranded probe was capable of detecting as little as 0.1 pg of the sequence of interest (Fig. 4B and C). The

Fig. 4. **Single-stranded DNA probe sensitivity.** The sensitivity of [^{32}P]-labeled, single-stranded DNA probe for the *nptII* gene was determined by hybridization to cellulose nitrate filters to which a serial dilution of known amounts of genomic DNA isolated from a pure culture of *B. japonicum* strain CRM52 were fixed. (A) autoradiogram obtained after hybridization of the *nptII* gene probe to undigested *B. japonicum* strain CRM52 genomic DNA fixed to cellulose nitrate. Lanes 1 through 15 contain, respectively, 1.00 ug; 0.50 ug; 0.25 ug; 0.13 ug; 0.062 ug; 0.031 ug; 0.016 ug; 0.0078 ug; 0.0039 ug; 0.0019 ug; 0.00098 ug; 0.00049 ug; 0.00024 ug; 0.00012 ug; 0.000063 ug of total genomic DNA. (B) ethidium bromide stained 0.7% agarose gel containing *B. japonicum* strain CRM52 genomic DNA digested with *Eco*RI. (C) autoradiogram obtained after transfer of the DNA from the agarose gel to cellulose nitrate and hybridization with the *nptII* gene probe. Lanes 1 through 14 of (B) and (C) contain, respectively, 2.00 ug; 1.00 ug; 0.50 ug; 0.13 ug; 0.063 ug; 0.031 ug; 0.016 ug; 0.0078 ug; 0.0039 ug; 0.0019 ug; 0.00098 ug; 0.00049 ug; 0.00024 ug of *Eco*RI digested total genomic DNA.

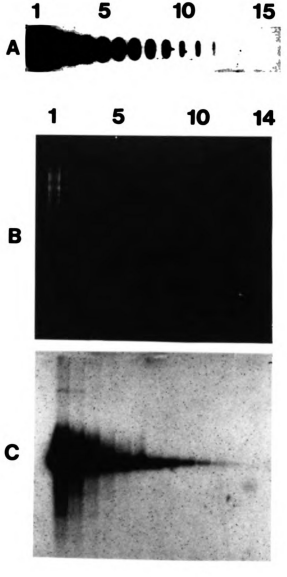

Fig. 4.

difference in sensitivity when the two methods are compared probably results, at least in part, from incomplete transfer of DNA from the agarose gel to the cellulose nitrate filter when Southern transfer is employed.

The 1 ug DNA samples used in the slot blot experiments and the 2 ug DNA samples used in the Southern blot experiments do not exceed the DNA binding capacity of the cellulose nitrate filters (data not shown). An increase in sensitivity could therefore be gained by increasing the amount of DNA in the sample. However, when binding large amounts of DNA to cellulose nitrate filters, control experiments must be performed to show that the DNA binding capacity of the filter has not been exceeded. In more recent experiments we have successfully detected as few as 10^3 organisms per gram of soil. The results from the slot blot and the Southern blot analyses represent a substantial improvement in sensitivity and specificity as compared to DNA probes labeled with either radioactive or biotinylated residues by nick translation (25).

Comparison of DNA isolated from soil to DNA isolated from pure culture. A major consideration in the isolation of bacterial DNA from soil was to purify the DNA from soil contaminants, especially humic acids which make DNA refractory to complete restriction endonuclease digestion and hybridization analyses (our unpublished observations). This was facilitated by including insoluble PVPP and sodium ascorbate in the initial homogenization steps of our protocol. The sodium ascorbate serves as a reducing agent to prevent the oxidation of phenols while phenolic compounds such as humic acids are removed by

the formation of an insoluble complex with PVPP (10). The inclusion of sodium ascorbate and PVPP in the DNA isolation protocol resulted in a two-fold increase in DNA yield and a significant increase in the purity of bacterial DNA obtained (Fig. 5). The ultraviolet (UV) spectral profiles of total genomic DNA isolated from a pure culture and total bacterial DNA isolated from soil using our technique were also compared. The results obtained for each DNA compare quite favorably indicating that DNA isolated from soil using our protocol is of comparable purity to DNA isolated from pure culture (Fig. 5).

The results of experiments designed to compare total bacterial DNA isolated from non-sterile soil inoculated with *B. japonicum* strain CRM52 (hereafter designated soil DNA) to DNA isolated from a pure culture of *B. japonicum* strain CRM52 (hereafter designated culture DNA) are seen in Figure 6. The experiments were designed to assess: i) The average size of the DNA obtained from each source. ii) The presence of contaminating (endogenous) nucleases. iii) The ability of the DNAs to be readily digested to completion by exogenously added restriction endonucleases. iv) The quality of data obtained from DNA:DNA hybridization analyses using radioactively labeled single-stranded probes. Untreated soil DNA was found to be of similar size (~50 kb) when compared with untreated culture DNA (Fig. 6A). Both DNAs are considered to be free of contaminating nucleases since there was no decrease in apparent size when incubated for 4 h under conditions favorable for nuclease digestion (Fig. 6A). When the soil DNA and the culture DNA were incubated with 5 units of *Eco*RI per ug of DNA both appeared to be readily digested to completion (Fig. 6A). The characteristic "ladder" of DNA fragments obtained when *B. japonicum*

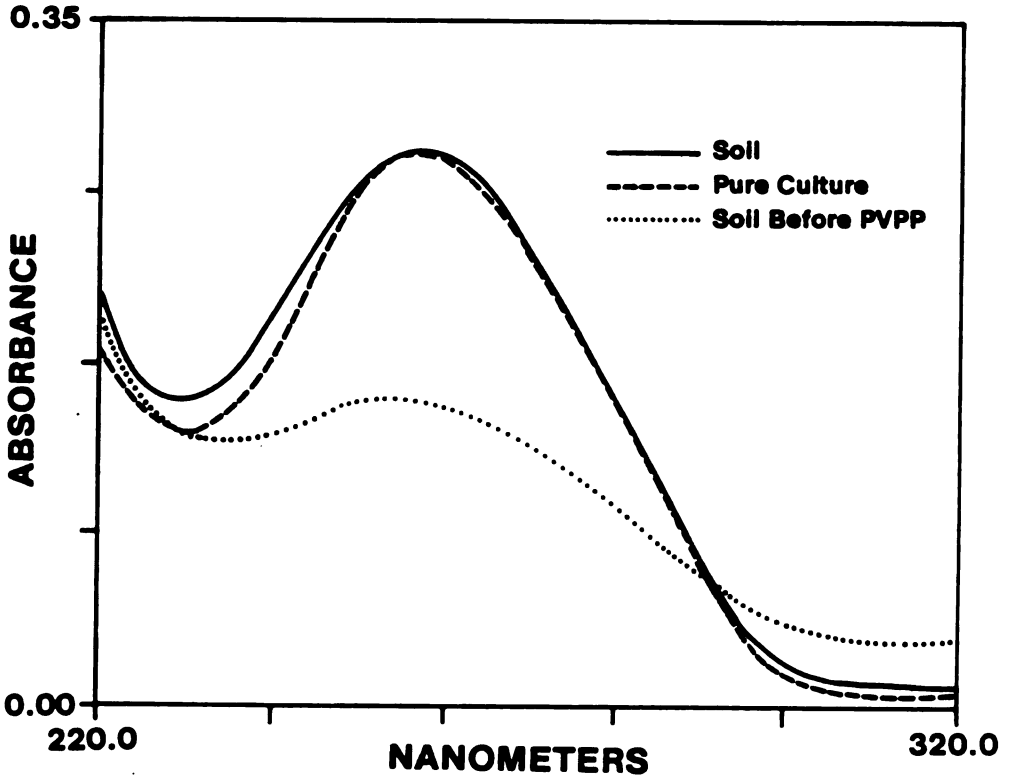

Fig. 5. Ultraviolet absorption spectra of DNA isolated from soil and from pure culture. (___), DNA isolated from the soil bacterial population using our protocol. (···), DNA isolated from the soil bacterial fraction using our protocol but without polyvinyl-polypyrrolidone and sodium ascorbate in the homogenizing solution. These two soil DNA samples represent the DNA obtained from 10 g of Capac soil. (---), DNA isolated from a pure culture of *B. japonicum* strain CRM52 grown in YEMN medium, lysed by the method of Doi, then purified by equilibrium gradient centrifugation.

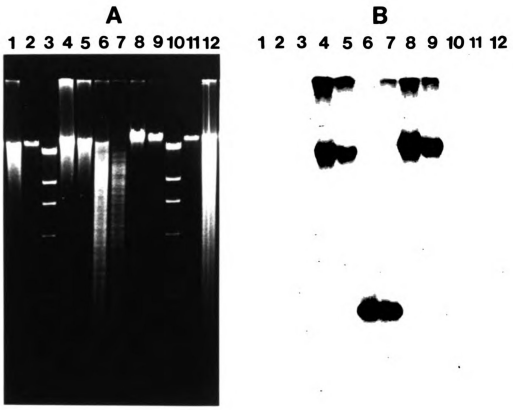

Fig. 6. **Comparison by restriction endonuclease digestion and DNA probe hybridization of DNA isolated from soil and from pure culture.** (A) ethidium bromide stained 0.7% agarose gel. (B) autoradiogram obtained after Southern transfer from the gel to cellulose nitrate and hybridization with the single-stranded *nptII* gene probe. Lanes: 1, *Eco*RI digested DNA isolated from non-sterile soil inoculated with 10^9 *B. japonicum* strain BJ110 per gram dry weight; 2 and 11, linearized DNA from phage lambda (48 kb); 3 and 10, *Hind*III digested DNA from phage lambda (23.1, 9.4, 6.6, 4.4, 2.3, 2.0, 0.56, and 0.12 kb); 4, 5 and 6, respectively, unincubated, incubated (for 4 h at 37 °C in 1X React 3 buffer [BRL]) with no exogeneous nuclease added, and *Eco*RI digested DNA isolated from non-sterile soil inoculated with 10^9 *B. japonicum* strain CRM52 per gram dry weight; 7, 8 and 9, respectively, unincubated, incubated (as above) with no exogeneous nuclease added, and *Eco*RI digested DNA isolated from a pure culture in YEMN medium of *B. japoncum* strain CRM52; 12, *Eco*RI digested DNA isolated from uninoculated non-sterile soil.

DNA is digested with *Eco*RI is apparent in the DNA obtained from pure culture. This ladder is not as apparent in the DNA obtained from the inoculated non-sterile soil since it is a complex mixture of DNA from the soil bacterial community. However, because *B. japonicum* cells added at 10^9 per g soil comprised approximately half of all the bacteria in the soil sample, elements of the ladder can still be seen. Overall, the DNA isolated from soil performed essentially the same as that obtained from pure culture as indicated by both the ethidium bromide stained agarose gel (Fig. 6A) and the autoradiogram obtained after hybridization with the probe for the *nptII* gene (Fig. 6B). In particular, lanes 6 and 7 of Fig. 6B demonstrate that we are capable of specifically detecting the sequence of interest (representing the organism of interest) as a fragment of predicted size in DNA isolated from non-sterile soil. The specificity of the *nptII* gene probe is also demonstrated in Fig. 6B since there was a lack of any detectable hybridization signal to total bacterial DNA isolated from non-sterile soil inoculated with *B. japonicum* BJ110 (the parental strain of CRM52 which does not contain the *nptII* gene) and DNA from uninoculated non-sterile soil.

Results of slot blot experiments using soil bacterial DNA. The slot blot apparatus allows DNA obtained from soil samples to be fixed onto cellulose nitrate filters in discrete, slot-shaped areas. The filters were subsequently used in hybridization experiments to detect the DNA sequence of interest. The results of an experiment in which the fate of *B. japonicum* CRM52 inoculated into non-sterile soil was monitored for 2 weeks after inoculation are given in Fig. 7. Since each DNA sample applied to the cellulose nitrate filter contained the same

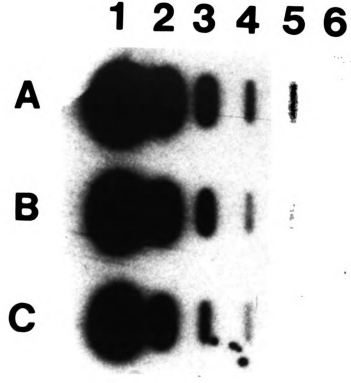

Fig. 7. **Detection of *B. japonicum* strain CRM52 in soil by slot blot analysis.** Each sample applied to the filter contained 1 ug total of undigested DNA isolated from soil inoculated with *B. japonicum* strain CRM52. Capac soil was inoculated with: 4.6×10^8, (1); 4.6×10^7, (2); 5.4×10^6, (3); 3.6×10^5, (4); 4.3×10^4, (5); or, (6), no *B. japonicum* strain CRM52 per gram dry weight at 0 weeks and incubated in covered pots at room temperature for the specified interval. Soil bacterial DNA was isolated at: (A), 0 weeks; (B), 1 week; or (C), 2 weeks after inoculation.

total amount of DNA (1 ug), the intensity of the hybridization signal at each position indicates the number of copies of the *nptII* gene present in the DNA sample. *B. japonicum* strain CRM52 contains one stably integrated copy of the *nptII* gene in its chromosome, therefore, the intensity of hybridization reflects the number of *B. japonicum* CRM52 present in the soil. We were able to detect this strain in soil throughout the range of 10^8 to 10^4 per g dry weight of soil based on the presence of this single-copy sequence. Thus, we were able to detect the microorganism of interest in soil, even though it comprised only a very small fraction (10^{-5}) of the total bacterial population. The results obtained using the DNA probe methodology indicate that the numbers of *B. japonicum* strain CRM52 in the soil did not change significantly during the course of the experiment regardless of the initial inoculum density and are in agreement with results obtained by selective plating (Fig. 7, some data not shown).

There is some low-level background hybridization observed in the uninoculated control (Fig. 7). It is not clear whether this results from hybridization to *nptII* homology in the DNA obtained from indigenous microorganisms or simply to non-specific hybridization. As shown previously, there is no background hybridization apparent when DNAs isolated from soil are analyzed by Southern transfer (Fig 6). The lack of detectable background hybridization in this format probably derives from low levels of background hybridization being dispersed throughout the entire lane when the DNA samples are size fractionated by agarose gel electrophoresis. This is in contrast to the slot blot format where the entire DNA sample is localized to a very small area of the filter which effectively concentrates very low

levels of background hybridization resulting in a detectable signal. If background hybridization obscures the results of slot blot analyses, the Southern blot technique can be employed to reduce or eliminate this problem.

Detection of parental and engineered derivative strains in a single soil sample. A probe was constructed for the *B. japonicum rbcL* gene which encodes the RUBISCO large subunit. The *rbcL* probe was constructed by cloning a 0.95-kb *Eco*RI-*Bam*HI fragment which contains the 3′ two-thirds of the *rbcL* gene (T. Cotter, personal communication) into M13 mp9. This probe flanks the site of the insertion of the *nptII* gene in CRM52 (Fig. 8). When genomic DNA from *B. japonicum* strains BJ110 and CRM52 was digested with a restriction enzyme which cuts the DNA outside of the region used as the probe (in this case *Sma*I), the fragment containing the 3′ end of the *rbcL* gene will be of a different size for each organism (Fig. 8). This forms the basis for the identification of both strains in a single soil sample using a single probe. *B. japonicum* strains BJ110 and CRM52 were readily identified, either singly or together, in the soil sample by virtue of the *rbcL* hybridization signal of the predicted size (Fig. 9). By comparison to known standards, or by scanning densitometry, quantitation of the numbers of organisms should be possible. Note that in this case, the probe used hybridizes to wild-type genomic sequence rather than to an engineered DNA sequence, and that using this probe we were able to detect and differentiate between a wild-type *B. japonicum* strain BJ110 and an engineered derivative of this strain.

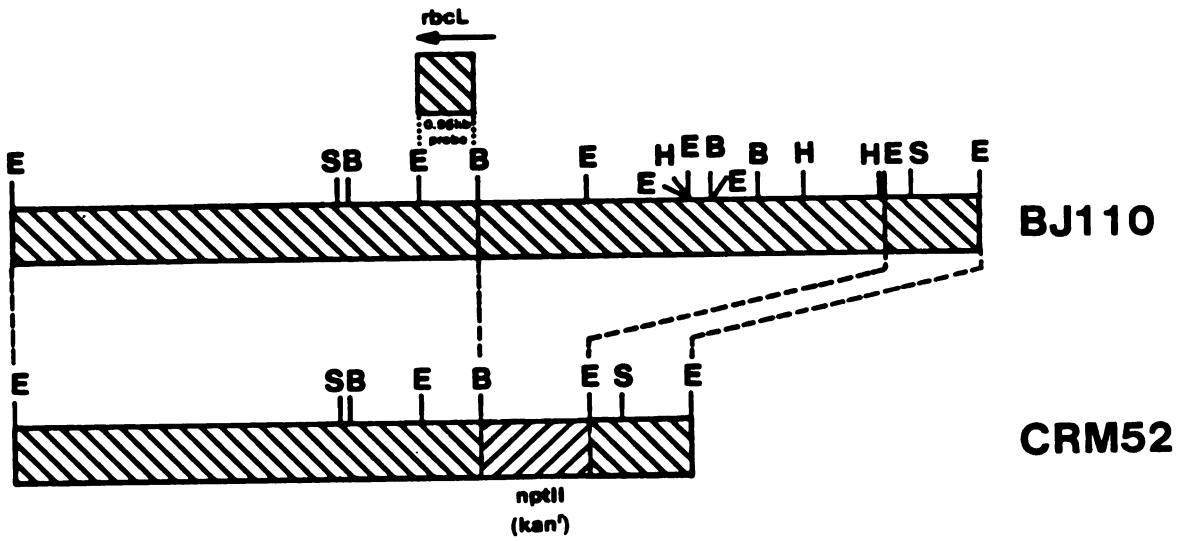

Fig. 8. **Restriction map of the *rbcL* region of the chromosomes of *B. japonicum* strains BJ110 and CRM52.** The location and orientation of the *rbcL* gene are designated by the arrow. *B. japonicum* strain CRM52 was constructed (18) using site-directed mutagenesis by deleting the 8.6 kb *Bam*HI-*Eco*RI fragment of the chromosome and replacing it with a 1.8 kb *Bam*HI-*Eco*RI fragment containing the *nptII* gene but is otherwise isogenic to strain BJ110. Letters indicate restriction sites: E, *Eco*RI; S, *Sma*I; B, *Bam*HI; H, *Hin*dIII.

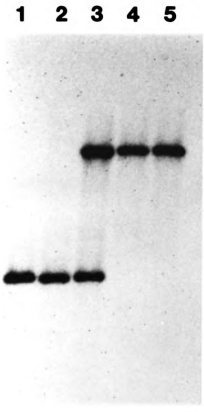

Fig. 9. **Detection of parental and engineered derivative strains in a single soil sample.** Autoradiogram obtained after hybridization of the single-stranded *rbcL* gene probe to DNA fixed to cellulose nitrate by Southern transfer from a 0.7% agarose gel. Lanes contain *Sma*I digested DNA as follows: (1), *B. japonicum* strain CRM52 genomic DNA isolated from pure culture; (2), soil bacterial DNA isolated from Capac soil inoculated with 10^7 *B. japonicum* strain CRM52 per gram dry weight; (3), soil bacterial DNA isolated from Capac soil inoculated with 10^7 *B. japonicum* strain CRM52 and *B. japonicum* strain BJ110 per gram weight; (4), soil bacterial DNA isolated from Capac soil inoculated with 10^7 *B. japonicum* strain BJ110 per gram dry weight; (5), *B. japonicum* strain BJ110 genomic DNA isolated from pure culture.

The same principle, that of restriction fragment length polymorphism, which allows us to detect a parental strain and its engineered derivative in this experiment can also be applied to allow the detection of more than one species (or genus) in a single soil sample as we have done for two different species of Pseudomonads (manuscript in preparation). It should also be possible to detect multiple organisms in soil by combining probes (for wild-type or engineered DNA sequences) unique to each organism for hybridization to total bacterial DNA isolated from soil containing these organisms.

Using our method we detected not just an organism of interest, but a particular gene or DNA sequence of interest in an entire community of soil bacteria. This technique, in conjunction with Southern transfer, has the potential to detect genetic rearrangements as well as horizontal gene transfer to other species. The basis for detecting such phenomena is that if a gene or DNA sequence of interest were involved in a genetic rearrangement within the original organism or incorporated into the DNA of a different organism (e.g. as the result of a horizontal gene transfer event) the DNA flanking the sequence of interest would likely be different. The result of this would be that the DNA sequence of interest would reside on either a larger or smaller DNA fragment, when digested with an appropriate restriction enzyme, than was the case in the original organism. This would be detected as a band of a different size during subsequent hybridization and autoradiography.

Some advantages of the described methodology are: i) The bacterial fraction, and hence the DNA, is isolated *in situ* and therefore

requires no culturing of soil microorganisms. The importance of this distinction is that the majority of microorganisms in environmental samples can not readily be cultured under laboratory conditions (8, 17, 22). In fact, where transfer of genetic information to other organisms is of concern, the inability to culture all types of soil microorganisms would be a major consideration. The isolation of DNA *in situ* also allows direct quantitations and comparisons to be made. ii) A specific gene or DNA sequence particular to a microorganism is detected so that gene expression is not required. This is important because bacteria in the environment often exist under conditions of nutrient limitation and therefore may not be actively expressing a gene product required for detection (4). iii) Multiple organisms can be monitored in a single sample. iv) The potential exists to detect genetic rearrangements and horizontal gene transfer. v) Reporter genes and phenotypes are not required since the probe directly detects the sequence of interest. These methods, therefore, are useful not only with mutant or genetically engineered microorganisms but with natural isolates as well.

The use of this probe methodology in conjunction with Southern transfer is analytically quite powerful, being able to detect multiple organisms, genetic rearrangements and possibly also gene transfer. However, when simply monitoring the presence of an organism in soil using DNA probes, the slot blot approach is more appropriate as it allows for rapid processing of large numbers of DNA samples in a simpler format.

Acknowledgements

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APPENDIX B

THE EFFECT OF FREEZING ON DETECTION OF RECOMBINANT PSEUDOMONADS IN SOIL BY GENE PROBE AND SELECTIVE PLATING TECHNIQUES

The bacterial DNA extraction protocol we developed (Holben et al, 1988) can confortably accomodate the processing of 6 to 8 samples per day per person. We were interested in determining whether it would be possible to freeze additional samples that would be involved in experiments involving multiple treatments and/or replicates until they could be processed for DNA extraction. In a preliminary experiment, identical soil samples inoculated with *Pseudomonas cepacia* PCO1224-1 (PC-1) were processed immediately or were stored in plastic bags and placed in a -20° C freezer for 24 hours prior to processing. The level of DNA hybridization was the same when frozen and non-frozen samples containing PC-1 were compared (Fig. 1). By contrast, the numbers of bacteria detected by selective plating from these samples were lower by two orders of magnitude for the samples that were frozen compared to unfrozen samples.

Since there was a considerable decrease in culturability for samples frozen at -20° C, we tried several other methods to freeze samples to increase stability during freezing and thawing. Glycerol is commonly used to maintain stocks of frozen laboratory cultures to maintain their viability. Therefore, PC-1 was added to soil with or without glycerol (15%) and frozen at -20° C, quick frozen in liquid nitrogen and stored at -70° C, or stored at room temperature. The number of culturable cells on selective medium decreased initially

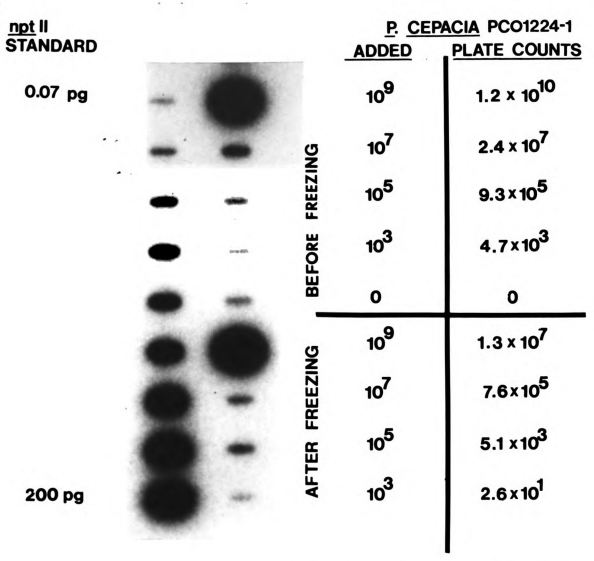

Fig. 1. **Comparison of detection of PC-1 in soil samples, incubated at room temperature or stored at -20 °C for 1 day, by selective plating and gene probe methodologies.** Duplicate soil samples were inoculated with different dilutions of a PC-1 liquid culture to the given cell densities. One replicate was frozen at -20 °C and the other replicate was incubated at room temperature. The autoradiogram was obtained after hybridization with the [^{32}P]-labeled *nptII* gene to a slot blot with each slot containing an equivalent amount (10 ug) of soil bacterial DNA. The same samples were enumerated on KB agar containing kanamycin and nalidixic acid.

and then stabilized, with the -20° C, no glycerol, treatment showing the greatest decrease (Fig. 2). The corresponding slot blot of soil bacterial DNA hybridized to the *npt*II gene probe indicated that at 1 and 7 days of frozen storage there was less hybridization for the -20° C treatments than the -70° C treatments (Fig. 3).

For comparison, we also determined the effect of freezing on *Pseudomonas* strain B8-1 (B8-1). This strain was added to duplicate samples of soil and then frozen at -20° C, or incubated at room temperature. Non inoculated controls were incubated at room temperature. The population of B8-1 was monitored over time in frozen samples using gene probes and selective plating. The number of culturable bacteria decreased in both frozen and non-frozen samples, but the decrease in culturable bacteria was much more dramatic in the frozen samples (Fig. 4). After 7 days of storage, there were no culturable bacteria in the frozen samples. However, the gene was still detectable by hybridization to the gene probe. Furthermore, even in the samples that were not frozen a dramatic decrease in culturable organisms was seen from day 7 to 9 (possibly due to soil drying). However, the amount of hybridizable DNA remained fairly constant. Controls to which no organisms were added but the DNA extracted at the same time exhibited no background hybridization. B8-1 appeared to be less resistant to freezing than PC-1 since after 7 days incubation there was no counts on selective media for these samples whereas some PC-1 cells could still be cultures on selective medium (Fig. 3). However, the results from these two experiments cannot be directly compared due to possible

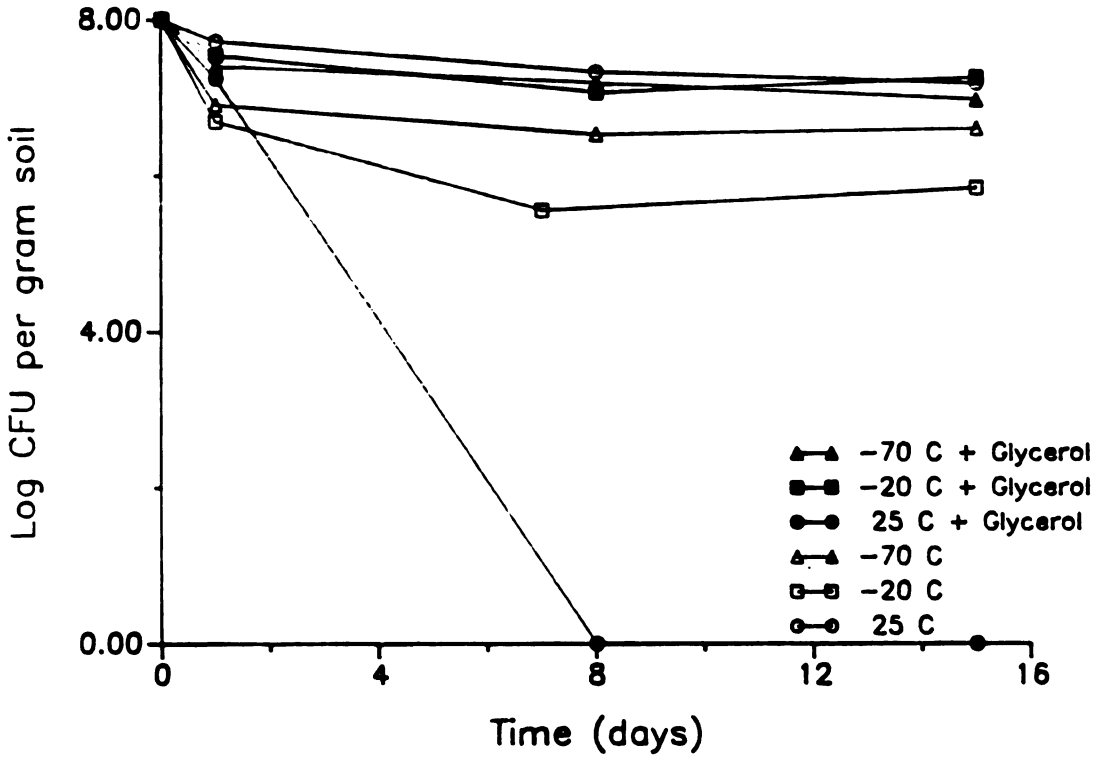

Fig. 2. Effect of different freezing treatments on culturability of PC-1 on selective medium. Data represent the average of samples from three replicate bags of soil for each treatment.

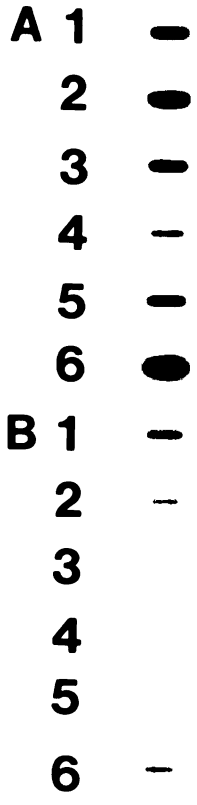

Fig. 3. Effect of different freezing treatments on detection of PC-1 by slot blot anaysis of DNA isolated from soil. Individual replicate bags of soil (70 g) inoculated at the same time with PC-1 were treated as follows: quick frozen and stored at -70 °C with or without glycerol, (1) and (2), respectively; stored at -20 °C with or without glycerol (3) and (4), respectively; or incubated at room temperature with or without glycerol (5) and (6) respectively. DNA was extracted from individual bags of soil after 1 day (A) or 12 days (B) storage. The same amount (10 ug) of DNA was added to each individual slot and the blot was probed with [^{32}P]-labeled *nptII*.

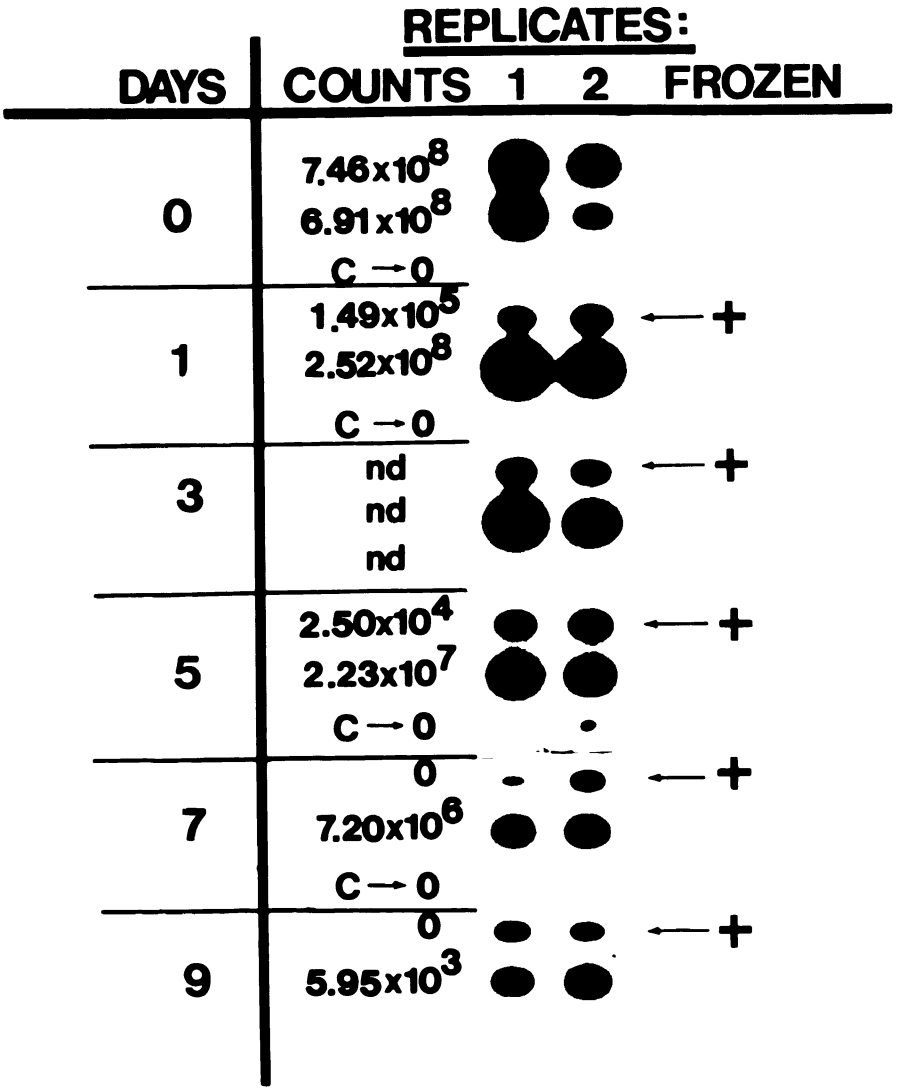

Fig. 4. **Autoradiogram from a slot blot with DNA isolated from soil inoculated with B8-1 and stored at -20 °C or incubated at room temperature, and the corresonding plate counts.** Soil inoculated with B8-1 was divided into three bags (500 g/bag). Two bags were stored at -20 °C and the third at room temperature and the soil in each bag was sampled every other day by DNA extraction and selective plating. The same amount (10 ug) of DNA was loaded onto individual slots and probed with [^{32}P]-labeled *nptII*. Simultaneously, soil samples were plated onto agar medium containing rifampicin and kanamycin.

differences in experimental conditions. For example, the same soil sample inoculated with B8-1 was repeatedly samples, which may have caused some partial thawing that could result in the decreased viability.

PC-1 and B8-1 were added to individual soil samples to directly compare their response to freezing treatments under the same experimental conditions. Samples were placed in plastic bags and frozen at -70° C or -20° C. Glycerol was not added because the previous experiment showed that it did not make a difference at -70° C, and we wanted to compare -70° C to the harsher -20° C freezing treatment. At 12 and 24 day intervals the total bacterial DNA was extracted and the pseudomonads were enumerated on selective media. The cell numbers determined by selective plating are given in Table 1 and the corresponding slot blot in Fig. 5. In this experiment, the plate count data was similar for both species, but the amount of DNA hybridization was less for B8-1. Both species showed a dramatic and continuous decline in numbers in the -20° C treatments. Freezing at -70° C resulted in an initial lesser decline that was follwed by a stabilization of the population.

The decrease in plate counts seen for B8-1 may correlate to cell death and lysis, since there is a corresponding decrease in DNA hybridization. Since the procedure for isolation of DNA relies on extraction of intact bacteria from soil, the lysed organisms would not be extracted, and the liberated DNA could bind to clay particles making it unextractable as well. Strain PC-1 however, did not show a parallel decrease in culturability and DNA hybridization. The plate

Table 1. Effect of different freezing treatments on culturability of PC-1 and B8-1 over time.

Day	Culture	Freezing Treatment °C	Log CFU/gdw soil
0	B8-1	-	8
	PC-1	-	8
12	B8-1	-20	4.9
	B8-1	-70	6.8
	PC-1	-20	5.1
	PC-1	-70	6.5
24	B8-1	-20	4.1
	B8-1	-70	6.4
	PC-1	-20	4.8
	PC-1	-70	7.0

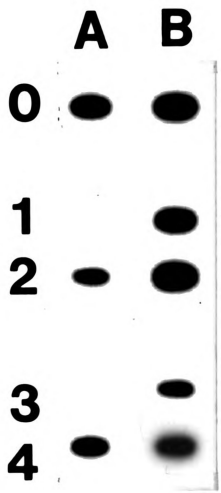

Fig. 5. **Autoradiogram from a slot blot with DNA isolated from soil inoculated with PC-1 or B8-1 and stored at -20 °C (1 and 3), or quick-frozen in liquid nitrogen and stored at -70 °C for 1 or 12 days** . Capac soil was inoculated with (A) B8-1, or (B) PC-1. Soil bacterial DNA was isolated at 0 days, (0); 1 day, (1 and 2); or 12 days, (3 and 4). DNA extracted after 1 or 12 days was stored frozen at -20 °C (1 and 3), or quick-frozen in liquid nitrogen and stored at -70 °C (2 and 4). The same amount (10 ug) of DNA was added to individual slots and the blot was probed with [^{32}P]-labeled *nptII*. Individual replicate bags of soil were sampled for each treatment and sampling date.

counts decreased much more rapidly. This result is an example of the use of gene probes to detect organisms that are non-culturable on selective media.

In conclusion, it appears that multiple soil samples can be stored by freezing at -20° C with the addition of glycerol, or at -70° C for later enumeration of bacteria by selective plating or DNA hybridization. However, a portion of the population is still lost and it is advisable to determine the extent of this loss. Freezing of pseudomonads at -20° C without glycerol was found to be too destructive to the soil population for use as a routine storage procedure.