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Characterization of Soil Microbial Communities Involved in Bioremediation by Molecular Biological Techniques

by

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Abstract

Two soil microbial communities was characterized by the reverse sample genome probe (RSGP) technique. A master filter containing 35 bacterial standards isolated from C5+ hydrocarbon contaminated soil was generated to study the effects of two hydrocarbons, degradable toluene and recalcitrant dicyclopentadiene (DCPD), on the C5+ degrading community. These standards were identified by comparing their partial 16S rRNA gene sequences with a database. Two toluene degraders were identified by RSGP. Their capacity of toluene degradation was 60% to 80% of that of the entire soil community. The potential for DCPD degradation of two standards, identified by RSGP, was determined by measuring mineralization and oxidation of DCPD.

An enriched soil community, able to completely dechlorinate tetrachloroethene (PCE) to ethene, was characterized by a similar approach. Bacterial standards with dechlorination activity were identified and their activity was confirmed. Nutrients required for dechlorinating activity by these bacteria were also defined.

Acknowledgements

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I am grateful to Les Stehmeier from Nova Research and Technology Corp. for providing me with his bacterial standards and for giving me a standard ethene gas for calibration, and to Dr. Francis, also from Nova, for giving me bacterial isolates for the master filter used to study the C5+ degrading community. The cultures from which PCE standards were isolated were graciously supplied by Dr. J. M. Odom at Du Pont.

Last and most of all, I would like to thank my family. Without the support and help of Fang Sun, my husband, I could not have smoothly completed this graduate program. The patience, encouraging, and understanding of Sarah (Yiyi) Sun, my daughter, and my mother during past three years are deeply appreciated.

To my family

..... and in memory of my dad

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Abbreviations and Symbols

A_{600}	absorbance at 600 nm wavelength
BTEX	benzene, toluene, ethylene, and xylenes
bp	base pairs
BSA	bovine serum albumin
BM	basal salts medium
BY	basal salts and yeast extract medium
ca.	approximately
C5+	low molecular weight aromatic hydrocarbons (≥ 5 C atoms)
Ci	Curie
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DCE	dichloroethene
DCPD	dicyclopentadiene
DNA	deoxyribonucleic acid
dNTPs	deoxynucleoside triphosphate
e^-	electron
e.g.	for example
EDTA	ethylenediaminetetraacetic acid
ETH	ethene
GC	gas chromatography
GC/MS	gas chromatography/mass spectrometry
g/L	grams per liter
h	hours
HDM	hydrocarbon degradation medium
HEPES	*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid
HGT	high gelling temperature
HP	Hewlett Packard
kb	kilobase pairs = 10^3 bp
λ	lambda
min	minute
MSM	mineral salts medium
MY	mineral salts and yeast extract medium

NMR	nuclear magnetic resonance
PCE	tetrachloroethene
PCR	polymerase chain reaction
RDP	ribosomal database project
PEG	polyethylene glycol
PSL	photostimulable luminescence
PVP	polyvinylpyrrolidone
PVPP	polyvinylpolypyrrolidone
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RSGP	reverse sample genome probing
SDS	sodium dodecyl sulfate
sp.	species (singular)
spp.	species (plural)
SSC	standard sodium citrate
TBE	Tris-boric acid-EDTA
TCA	tricarboxylic acid
TCE	trichloroethene
TE	Tris-EDTA
TOL	toluene degradation pathway
Tris	Tris(hydroxymethyl)methylamine
TY	tryptone-yeast extract
VC	vinylchloride
vs.	versus
v/v	volume per volume
w/v	weight per volume

Chapter 1. Introduction

Microorganisms rarely occur as pure cultures under natural conditions, but are normally found as part of a microbial community. When a bacterium is successfully colony purified in the laboratory, it exists in multiplied form as a group of similar individuals, called a population (Atlas & Bartha, 1993). A population is an assemblage of individual organisms with common characteristics. The microbial populations living together at a particular location interact with each other to form a microbial community (Atlas & Bartha, 1993). Major benefits can be gained by the coexistence of populations in nature. Populations in microbial communities interact with each other in both positive and negative ways. The balance of these interactions results in an optimal population density at maximal growth rate (Atlas & Bartha, 1993). However, interactions between diverse populations in a microbial community are too complex to be classified in detail. These interactions not only act to regulate the population densities or biomass of the individual component species of the community, but also act to prevent the establishment of invading species. Most microbial communities have many or an uncounted number of microbial populations.

The population levels of microorganisms in an ecosystem are determined by nutrients, physical and chemical parameters of the natural environment, such as pressure, temperature, pH, salt concentration, water availability, and redox potential. Soil is a favorable habitat for most types of microorganisms, including bacteria, fungi, algae, protozoa, and their associated viruses (Atlas & Bartha, 1993). The soil microorganisms are located in close association with soil particles. Their activities and interactions with other organisms largely depend on the soil microenvironment, the environment where the microorganism actually lives (Totsche, 1995). Many distinct microenvironments can be distributed throughout the soil particles, and these microenvironments can change rapidly

with time (Brock et al., 1994). For example, the inside of a soil particle can be completely anoxic while its outside layer can be oxic. These physicochemical conditions allow anaerobic, aerobic, and facultative microorganisms to coexist in such a soil particle (Brock et al., 1994). Therefore, microbial communities isolated from soil samples are usually characterized by a high species diversity. This diversity helps maintain community stability, allowing it to cope with environmental fluctuations within a broad tolerance range.

At present, it is not easy to characterize the species diversity of natural microbial communities, but it is becoming increasingly clear that these communities have their own characteristics and unique composition and that certain species dominate under given environmental conditions. Complete characterization of a microbial community includes defining its species composition, the metabolic activities of individual microorganisms, and the associations between different species.

Biodegradation in soil is a natural process that leads to decomposition of oil and other hydrocarbon pollutants. It relies on the combined metabolic activities of diverse microorganisms, especially bacteria, and their interactions with the natural environment (Bossert & Kosson, 1997). Aerobically, some oil hydrocarbons are readily attacked by a variety of microorganisms that utilize these hydrocarbons as both carbon and energy sources for growth. Such biodegradation is usually desirable, since it avoids the accumulation of toxic compounds in the environment. The microbial degradation of hydrocarbons is sometimes promoted by addition of inorganic nutrients (Brock et al., 1994). Procedures that aim to accelerate the natural biodegradation rate are referred to as bioremediation. This can involve, in addition to the supply of inorganic nutrients, the supply of oxygen, or cometabolic substrates (Morgan & Watkinson, 1989). Biodegradation activity in soil is difficult to measure directly, because of the complex soil environment. The most frequently used methods for studying the biodegradation activity in soil are to enumerate degrading microbes and to measure their metabolic

activities in the soil by analysis of hydrocarbon loss or appearance of products (e.g. CO_2; Bossert & Kosson, 1997).

The rate and extent of hydrocarbon removal from the environment by biodegradation depends on various factors, including the environmental conditions, the chemical nature of the hydrocarbon compounds, the composition of the resident microbial community and its adaptive response to the presence of hydrocarbons. The predominant environmental factors determining biodegradation rates in soil are temperature, oxygen and nutrient concentrations, moisture level and pH (Leahy & Colwell, 1990). With respect to the chemical nature of the target compounds, complete mineralization (conversion to CO_2) has been found for relatively low molecular weight saturated or aromatic hydrocarbons, such as *n*-alkanes, benzene, toluene, and naphthalene (Perry, 1984). However, high molecular weight aromatics and highly branched alkanes are much more recalcitrant. A given microorganism only utilizes a limited range of hydrocarbons, and biodegradation of hydrocarbon mixtures in the natural environment is, therefore, usually carried out by mixed microbial communities. The ability to degrade and/or utilize mixed hydrocarbons depends on the composition of microbial communities and their adaptation in response to hydrocarbon exposure (Leahy & Colwell, 1990).

Competition among microorganisms is intense, only the microorganisms able to endure the resident environmental stresses survive. Microorganisms have developed many means for survival. The adaptive response of a microbial community to hydrocarbon exposure is one of these. Adaptation includes (i) induction of specific enzymes, (ii) genetic changes which lead to altered enzymatic specificities or novel metabolic activities, and (iii) selective enrichment of a specific microorganisms able to transform the target compound (van der Meer et al., 1992). A characterization of microbial communities in the natural environment is, therefore, also important for understanding the nature and rate of adaptation processes.

The goal of this research is to characterize two soil microbial communities active in bioremediation by molecular biological methods. The effects of hydrocarbon addition on the microbial community structure, and the functions of the dominant populations in each community are also defined.

Chapter 2. Background and objectives of this research

The microbial communities studied in this research are soil microbial communities involved in (i) degrading low molecular weight aromatic hydrocarbons (C5+) and in (ii) reductively dechlorinating tetrachloroethene. To understand the possible fate of C5+ hydrocarbons and tetrachloroethene in the natural environment, previous research on this topic will be reviewed. The methods and techniques used for analysis of soil microbial communities will also be reviewed in order to define the experimental strategies for this research.

2.1 Biodegradation of C5+ hydrocarbons

The C5+ stream is formed as a byproduct in the pyrolysis of ethane to ethylene. C5+ is composed of a variety of petroleum chemicals, including BTEX (benzene, toluene, ethylbenzene, xylenes), styrene, cyclopentadiene, dicyclopentadiene as the major and many others as minor components (Szekeres et al., 1977). Pyrolysis plants contain soil and sludge with significant C5+ hydrocarbon contamination due to accidental releases in transport and handling. There is a strong interest in removal of C5+ hydrocarbons from these sites by bioremediation. Bioremediation field trials have indicated that the BTEX components are removed relatively rapidly following nutrient addition and auguring to provide air access, while dicyclopentadiene and higher molecular weight hydrocarbons are removed more slowly (Stehmeier, 1997). Research is, therefore, focused on DCPD removal because of its recalcitrant structure and pungent smell.

2.1.1 Biodegradation of BTEX

BTEX are low molecular weight, monoaromatic hydrocarbons that can be major environmental pollutants. BTEX are volatile. Upon spilling, they can evaporate into the atmosphere or remain in soils due to adsorption or physical entrapment. Removal of these

contaminants by biodegradation requires provision of adequate oxygen, which is utilized for ring activation and cleavage and serves as electron acceptor for the complete oxidation of aromatic hydrocarbons (Gibson & Subramanian, 1984). Aerobic biodegradation of BTEX is well understood. Of the group of BTEX compounds, the toluene metabolic pathway in bacteria has been studied most extensively and forms an excellent model system for further understanding of degradative pathways of other monoaromatic hydrocarbons (Marques & Ramos, 1993). The best understood aerobic toluene degradative pathway is the TOL plasmid (pWW0) pathway of *Pseudomonas putida* (Figure 2-1; Burlage et al., 1989). On the TOL plasmid pWW0, a set of catabolic genes encoding enzymes for toluene degradation are organized in two operons. One operon (*xyl*CMABN) encodes for the upper pathway enzymes, which oxidize the methyl side chain of the aromatic ring to carboxylic acid. The lower pathway or *meta* operon is composed of 13 genes which encode the enzymes for the degradation of benzoate to TCA cycle intermediates (acetaldehyde and pyruvate). Many *Pseudomonas* species are able to utilize a variety of aromatic hydrocarbons for growth, because they contain one or more degradation pathway operons on plasmids or their chromosomal DNA. The distribution of aromatic hydrocarbon degraders in the natural environment can be determined by using a TOL plasmid gene probe or more specific pathway gene probes that can be amplified by PCR.

Aromatic hydrocarbon degradation pathways encoded by plasmids and chromosomes show a certain degree of diversity. Nevertheless, genes encoding pathway enzymes with similar enzymatic functions share a high degree of similarity. Divergence of aromatic hydrocarbon degradative pathways while maintaining sequence similarity, may be caused by DNA rearrangements during the evolution of different pathways in the process of adaptation of microorganisms to particular hydrocarbon compounds (van der Meer et al., 1992). This feature of genomic similarity allows screening for BTEX degraders in environmental samples by use of function-specific gene probes.

In recent years, anaerobic biodegradation of BTEX has also been extensively studied,

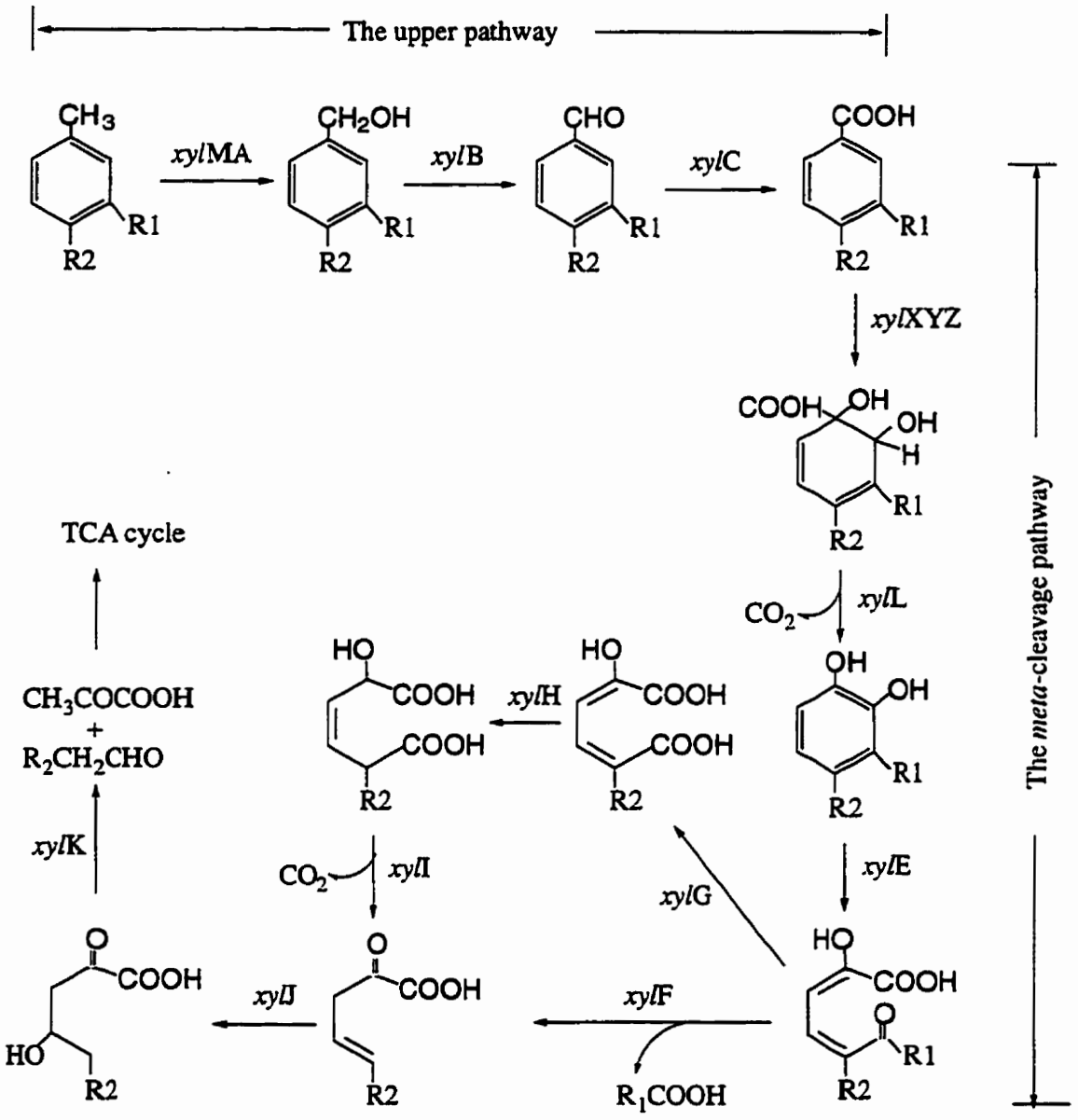


Figure 2-1. TOL plasmid pWW0 upper and *meta*-cleavage pathways for degradation of toluene and related hydrocarbons to TCA cycle intermediates. Toluene (R_1=R_2=H), *m*-xylene (R_1=CH_3, R_2=H), 3-ethyltoluene (R1=C2H5, R2=H), *p*-xylene (R_1=H, R_2=CH_3), and 1,3,4-triethylbenzene (R_1=R_2=CH_3) are all substrates of the pathway.

because oxygen availability is limited in many contaminated sites due to its low solubility in water and diffusion constraints in the soil and sediment matrix. Oxygen availability is often a rate-limiting factor for BTEX removal in aerobic treatments. Anaerobic degradation of BTEX has been observed under denitrifying (Evans et al., 1991), methanogenic (Grbic-Galic & Vogel, 1987), sulfate-reducing (Beller et al., 1992), and Fe(III)-reducing (Lovley & Lonergan, 1990) conditions, demonstrating that a wide range of electron acceptors (nitrate, carbon dioxide, sulfate, and ferric ion) can replace oxygen in anaerobic degradation of BTEX compounds. It should, therefore, be realized that both aerobes and anaerobes of soil microbial communities can play a role in the biodegradation of BTEX.

2.1.2 Biodegradation of dicyclopentadiene

Dicyclopentadiene (DCPD) is a dimer of cyclopentadiene (CPD), which dimerizes spontaneously at room temperature by Diels-Alder condensation to form primarily endo-dicyclopentadiene (Figure 2-2). DCPD is a liquid at room temperature with a persistent, highly camphor-like odor. DCPD is an alicyclic hydrocarbon, and these are usually more resistant to microbial attack than other groups of hydrocarbons (Trudgill, 1984). Among the alicyclic hydrocarbons, cycloalkanes are more difficult to degrade than their daughter hydrocarbons (cycloalkanols and cycloalkanones). Bacteria capable of utilizing simple alicyclic hydrocarbons like cyclohexanol and cyclohexanone as sole carbon and energy sources are well known (Trudgill, 1984). Anaerobic denitrifying bacteria which are able to completely degrade cyclohexanol and cyclohexanone have been also isolated (Dangel et al., 1988; Harder, 1997).

However, little information is available on microorganisms capable of utilizing simple cycloalkanes (e.g., cyclohexane) as sole carbon and energy source. The complete oxidation of [^{14}C]-cyclohexane by marine mud was demonstrated, but none of the bacterial strains isolated from this environment were able to utilize cyclohexane as sole carbon and energy source (Beam & Perry, 1974). Beam et al. suggested that cometabolism and

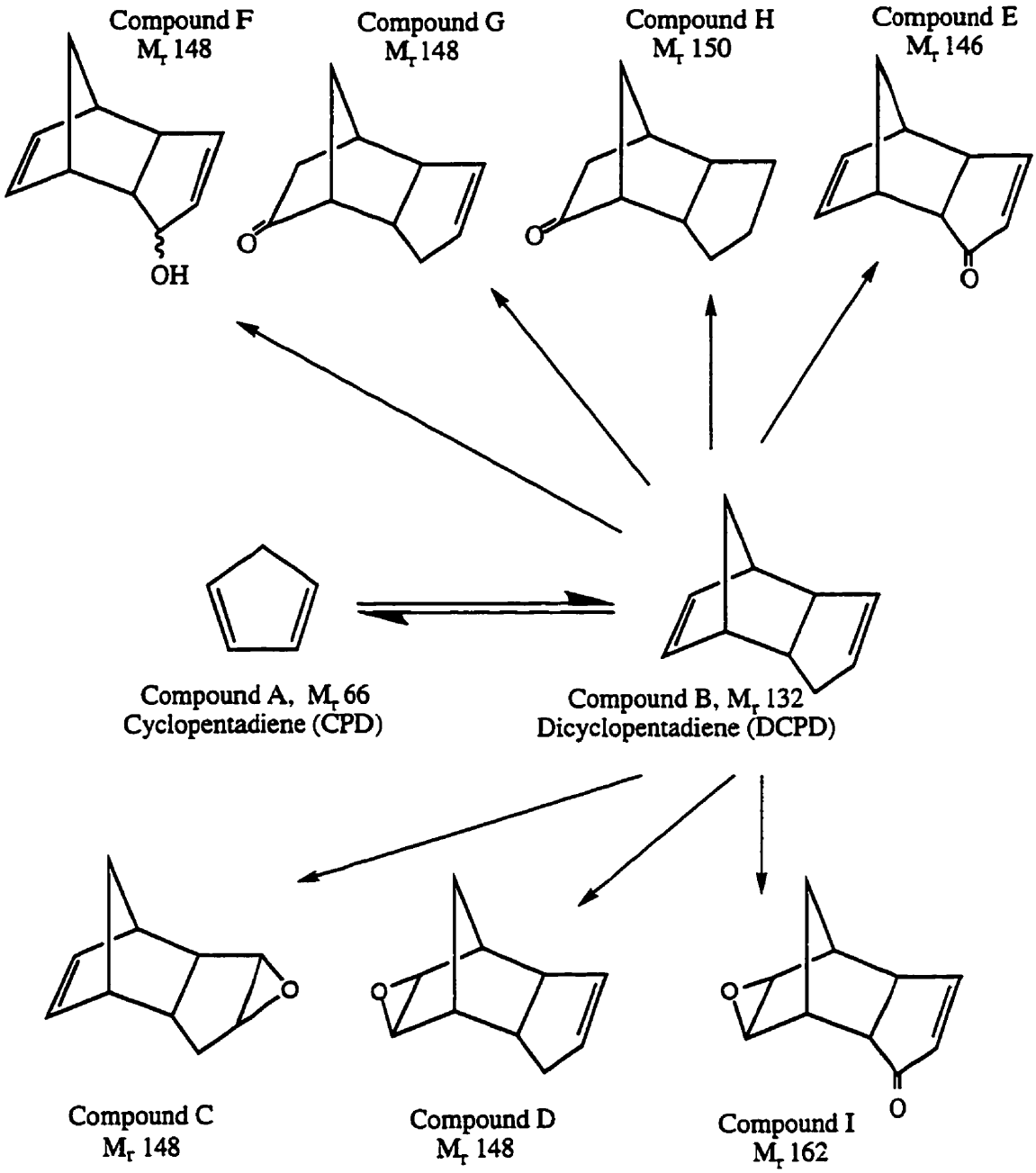


Figure 2-2. Possible initial steps for oxidation of dicyclopentadiene in the environment. Compounds C, and D are enzymatically oxygenated products (van Breemen et al., 1987), compounds G and H are oxygenated derivatives found in DCPD contaminated groundwater (van Breemen et al., 1987), compounds E and I are oxygenated derivatives found in DCPD contaminated surface water (van Breemen et al., 1993), and compound F is a biodegradation intermediate (Stehmeier et al., 1996).

commensalism might play an important role in the biodegradation of cyclohexane by the natural soil microbial community. Commensalism is a common positive interaction in microbial communities, in which the activity of one population primarily benefits a second population based on the combined physical and metabolic capacities that enhance the growth rate and reduce environmental stress (Atlas & Bartha, 1993). The relationships in a mixed culture of a *Nocardia* species and a *Pseudomonas* species capable of cyclohexane biodegradation was described by Slater (1978). The *Nocardia* species is able to metabolize cyclohexane, but requires biotin and factors produced by the *Pseudomonas* species. Although the *Pseudomonas* species is unable to utilize cyclohexane, it can grow on cyclohexanol that is a metabolic product provided by the *Nocardia* species.

So far no microorganisms have been reported that can use DCPD as sole carbon and energy source. The metabolism of DCPD or its possible transformation in the environment is, therefore, still largely unknown. Initial investigations were done by Spanggord et al (1979) on the environmental fate of DCPD in soil and natural water at the Rocky Mountain Arsenal in Colorado, which were contaminated by DCPD and other chemicals. They found that biotransformation of DCPD was very slow and estimated that conversion of 50% of DCPD concentration in the soil to carbon dioxide would require 4 to 7 years. This estimate was made from the rate of $^{14}CO_2$ production from [^{14}C]-labeled DCPD (Spanggord et al., 1979). A similar result was reported by Williams (1986), that is, a low percentage of $^{14}CO_2$ was produced from [^{14}C]-labeled DCPD in soil-containing media from the Rocky Mountain Arsenal after extended incubation (William, 1986). These results indicated that only a limited number of microorganisms present at this DCPD-contaminated site degrade DCPD to CO_2.

van Breemen et al (1987, 1993) studied the fate of DCPD at the Rocky Mountain Arsenal by analyzing oxygenated derivatives of DCPD extracted from ground and surface water with GC-MS and NMR methods. Derivatives with one, two, or three added oxygen atoms were extracted from ground water in the land adjacent to the Rocky Mountain Arsenal (van

Breemen et al., 1987). Two ketone derivatives were identified by comparison with synthetic standards (compounds G and H in Figure 2-2). These differed from two monoepoxide derivatives produced by incubation of DCPD with rabbit cytochromes P-450 (van Breemen et al., 1987; compounds C and D in Figure 2-2). Another two oxygenated derivatives of DCPD (compounds E and I in Figure 2-2) were identified by van Breemen et al. (1993) in DCPD-contaminated surface water. These authors indicated that bacterial metabolism might be responsible for producing these oxygenated derivatives in the ground water and photo-oxidation was considered a more likely mechanism in the surface water (van Breemen et al., 1987; 1993). DCPD is not considered to be a toxic or carcinogenic compound. The toxicities of oxygenated derivatives have not been investigated, and it is thus unknown whether the conversion of DCPD to these compounds is beneficial or hazardous.

Recent laboratory and field studies of DCPD biodegradation by Stehmeier et al. (1995) and Stehmeier (1997) indicated that DCPD may be biodegraded by a variety of microbial consortia instead of by single strains. The rate of DCPD mineralization was again found to be very slow. This rate could be enhanced by addition of inorganic nutrients (Stehmeier et al., 1995). Incubation of [^{14}C]-labeled DCPD with a variety of mixed cultures from soil and sludge samples produced up to 2.0% of $^{14}CO_2$ (after 25 days of incubation), and most of the remaining DCPD was converted to oxygenated derivatives (Stehmeier et al., 1996). A new oxygenated derivative of DCPD (Figure 2-2: compound F) was identified by comparison with a synthetic standard (Stehmeier et al., 1996). Further investigation is required to confirm these observations. Initial steps for DCPD oxidation pathway based on the data in the literature, are shown in Figure 2-2. Thus, previous studies of DCPD biodegradation have indicated that DCPD is a recalcitrant hydrocarbon, that is not readily utilized as sole carbon and energy source by single microorganisms. Commensalism between different microbial populations is required to completely degrade DCPD into CO_2.

Characterization of the microbial community involved in DCPD biodegradation in soil may help us to understand the process of microbial DCPD degradation in nature.

2.2 The fate of chlorinated hydrocarbons in the environment

The second organic compound targeted in this research is tetrachloroethene, also known as perchloroethene (PCE). PCE is a volatile chlorinated organic compound, and is widely used as a solvent and degreasing agent. Significant amounts of PCE have been released into the ground from industrial, commercial, and agricultural sources because of improper handling, storage, or disposal of this chemical. An extensive understanding of the fate of chlorinated ethenes in the environment is necessary to apply bioremediation for cleanup of contaminated sites.

The migration of chlorinated hydrocarbon in the environment is determined by the physical properties of these compounds (Table 2-1). Since chlorinated ethenes are denser than water (Table 2-1), they will migrate downward through aquifers and soil columns and accumulate at the groundwater table. This is why most chlorinated organic compound contamination is found in the groundwater. Chlorinated organic compounds may be either oxidized or reduced, depending on their structure, especially the number of chlorine substituents, and the local environmental conditions (e.g., the redox potential; Vogel et al., 1987). Increased chlorine substitution makes these compounds more susceptible to reduction, and less susceptible to oxidation (Vogel et al., 1987). For a given reductant, the rates of reduction of chlorinated compounds should correlate with their standard reduction potentials (Table 2-2), assuming no change in mechanism. More energy is released upon reduction of the more chlorinated compounds. Reduction generally leads to dechlorination.

Dechlorination, the removal of a chlorine substituent from a molecule by concurrent addition of electrons ($R\text{-}Cl + H^+ + 2e^- \rightarrow R\text{-}H + Cl^-$), requires an electron donor (reductant) and the absence of oxygen (Mohn & Tiedje, 1992). Organic compounds or hydrogen act as electron donors or reductants and become oxidized. The chlorinated compounds serve as

Table 2-1. Physical properties of chlorinated ethenes

Compounds	Mol wt	m.p[a].	b.p[b].	Density[c]	Solubility[d]
PCE	165.8	-19	121	1.6227	insoluble
TCE	131.4	-73	87	1.4642	slightly
1,1-DCE	96.9	-122	37	1.218	insoluble
cis-DCE	96.9	-80	60	1.2837	slightly
trans-DCE	96.9	-50	47	1.2565	slightly
VC (gas)	62.5	-153	-13	0.9106	slightly
ETH (gas)	28.0	-169	-103	0.00126	insoluble

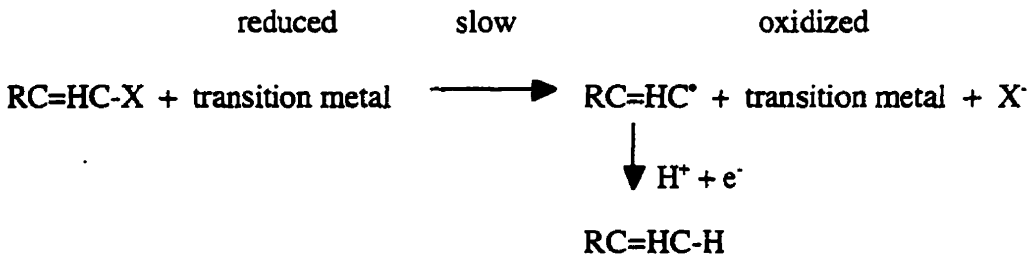
[a]Melting temperature (°C),[b]boiling temperature (°C), [c]density in g/cm^3, [d]solubility in water, (Weast, 1986).

Table 2-2. Standard reduction potentials ($E^{\circ'}$) of relevant acceptor and donor pairs

Electron acceptor (Oxidant)	Electron donor (Reductant)	E° (V)[a]
Vitamin B_{12} (oxidized)	Vitamin B_{12} (reduced)	-0.59 to -0.8
Acetate + CO_2 + $2H^+$	Pyruvate + H_2O	-0.70
Ferredoxin (oxidized)	Ferredoxin (reduced)	-0.43
$2H^+$	H_2	-0.42
$2H^+$ + CO_2	Formate	-0.42
Cytochrome P450 (Fe^{3+})	Cytochrome P450 (Fe^{2+})	-0.30
Acetaldehyde + $2H^+$	Ethanol	-0.20
Pyruvate + $2H^+$	Lactate	-0.19
Cytochrome c (Fe^{3+})	Cytochrome c (Fe^{2+})	+0.22
VC + H^+	ETH + Cl^-	+0.29
DCE + H^+	VC + Cl^-	+0.39
Nitrate + $2H^+$	Nitrite + H_2O	+0.42
Sulfate + $2H^+$	Sulfide + H_2O	+0.48
TCE + H^+ /	DCE + Cl^-	+0.49
PCE +H^+ /	TCE +Cl^-	+0.59
$2H^+$ + 1/2 O_2	H_2O	+0.82

[a] E° is the standard oxidation-reduction potential (pH7, 25°C) (Vogel et al., 1987 and Stryer, 1988). $E^{\circ'}$ refers to the partial reaction (Oxidant + e^- → Reductant).

electron acceptors or oxidants and are reduced in the process (Vogel et al., 1987). Highly chlorinated compounds have higher standard reduction potentials than their less chlorinated counterparts (Table 2-2 and Figure 2-3). Thus, more energy is released upon reduction of the more highly chlorinated compounds. Certain transition metals and transition metal complexes can also act as reductants. As a result, these metals and metal complexes are oxidized (Vogel et al., 1987), e.g.,



PCE, containing four chlorine substituents, is a stronger electron acceptor ($E^{o'}$ = +0.59 V) than nitrate ($E^{o'}$ = +0.42 V) and is thus likely to be reductively dechlorinated under most anoxic conditions. PCE is not biodegradable under aerobic conditions. Several biologically active electron donors, such as vitamin B_{12}, pyruvate, ferredoxin, hydrogen, formate, and ethanol have lower reduction potentials ($E^{o'} \leq 0$ V; Table 2-2) than do most of the chlorinated compounds ($E^{o'}$ = +0.2 to +0.59 V), and could, therefore, be involved in chlorine removal by reduction (Vogel et al., 1987). Many microorganisms also contain cytochrome P-450, which may be used to mediate reductive dechlorination. The presence of other electron acceptors (e.g., oxygen, sulfate, nitrate, and carbon dioxide) may compete with the chlorinated compounds for these electron donors. Differences in the concentrations of available electron donors and competing electron acceptors in PCE contaminated sites affect the rate of dechlorination and the species composition of PCE dechlorinating microbial communities.

2.2.1 Anaerobic biodegradation of tetrachloroethene

Anaerobic biodegradation of PCE has been demonstrated with pure cultures (Egli et al., 1987), mixed cultures (Bagley & Gossett, 1990), and soil columns (Vogel & McCarty, 1985). Yet little is known about the environmental conditions necessary to initiate and sustain dechlorination activity in a contaminated environment. Most of these studies described sequential reductive dechlorination of PCE under strictly anaerobic, particularly methanogenic conditions. The pathway for complete conversion of PCE to ethene through reductive dechlorination is shown in Figure 2-3. Ethene can be further reduced to ethane and finally mineralized to CO_2, where oxygen is available. The partial dechlorination of PCE to TCE as major intermediate and traces of DCE and VC was reported by Vogel McCarty (1985) in a continuous-flow, methanogenic column. Such incomplete dechlorination of PCE under anaerobic conditions has been frequently observed in laboratory scale systems (Vogel & McCarty, 1985; Freedman & Gossett, 1989; Bagley & Gossett, 1990), natural environments, and soil microcosms (Parsons et al., 1984; 1985). Trichloroethene (TCE), dichloroethene (DCE) isomers and vinylchloride (VC), are often accumulated as intermediates in the environment. These less chlorinated ethenes are also environmental pollutants. VC is worse than the parent compound because it is suspected to be a carcinogen (Infante & Tsongas, 1982). Therefore, it is important to achieve complete dechlorination of PCE to ethene in a bioremediation process.

Freedman and Gossett (1989) demonstrated that complete dechlorination of PCE to ethene is possible by a mixed culture under methanogenic conditions, although the rate of the final step (VC to ethene) is limited, causing significant amounts of VC to remain. Distefano et al. (1991) developed methanol-PCE enrichment cultures, which were able to convert PCE to ethene at high rates, having only small amounts of VC. Methanol served as the electron donor in these cultures. Complete dechlorination of PCE to ethene was also observed in anaerobic enrichment cultures with hydrogen as the electron donor (Distefano et al., 1992). A complete dechlorination of PCE to ethene and then to ethane was only observed in a

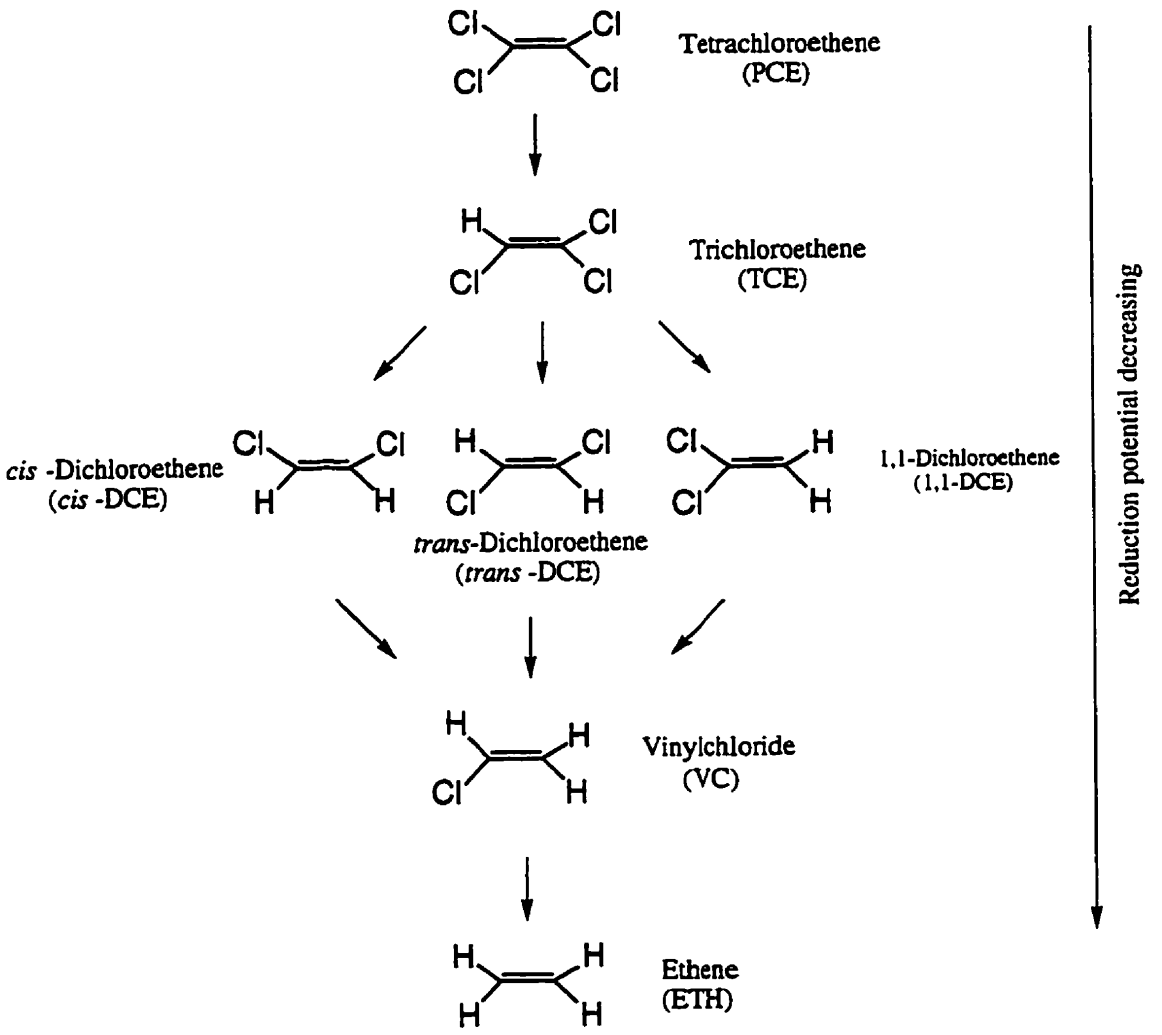


Figure 2-3. The pathway for complete reductive dechlorination of tetrachloroethene to ethene.

continuous-flow, fixed-bed column filled with Rhine river sediment and ground granular sludge (de Bruin et al., 1992). Lactate was used as an electron donor in this system with nearly no accumulation of partial dechlorinated ethenes. Hence, anaerobic mixed cultures have the potential to achieve complete dechlorination of PCE. It is unclear which electron donor can prevent the accumulation of less chlorinated intermediates. Since all of these studies were carried out in mixed cultures, it is also unknown which organisms are responsible for the dechlorination reactions.

Recently, a pure culture of an anaerobic bacterium, *Dechlorobacter restrictus*, coupling the reduction of PCE to growth has been isolated by Holliger et al. (1993; 1994). This organism reduces PCE to *cis*--dichloroethene (*cis*-DCE), with either hydrogen or formate as the electron donor. TCE can also be used as an electron acceptor for growth (Holliger et al., 1993). This organism requires some yeast extract for growth. Another strictly anaerobic PCE-dechlorinating organism, *Dehalospirillum multivorans* isolated by Scholz-Muramatsu et al. (1995), grew in defined medium with PCE and hydrogen as sole energy source and acetate as carbon source. In addition to hydrogen, this organism can utilize a variety of electron donors for dechlorination, such as pyruvate, lactate, ethanol, formate, and glycerol (Scholz-Muramatsu et al., 1995). A third anaerobe, which catalyzes dechlorination of PCE with a narrow electron donor range and was isolated by Wild et al. (1996), is closely related to *Dechlorobacter restrictus*. It uses hydrogen as the electron donor for reductive dechlorination of PCE or TCE to *cis*-DCE.

In addition to anaerobes, some facultative bacteria also catalyze reductive dechlorination of PCE. For example, Sharma and McCarty (1996) reported on a rapidly-growing facultatively aerobic bacterium (strain MS-1) that can transform near saturation levels of PCE via TCE to *cis*-DCE at high rates in a defined growth medium. Before this facultative bacterium was isolated, Kastner (1991) showed that PCE or TCE was reductively converted to *cis*-DCE within 4 days by an aerobic enrichment cultures, after transition from aerobic to anaerobic conditions. This dechlorination reaction was accompanied by a sulfide, and a

decrease in redox potential from 0 to -150 mV (Kastner, 1991). Since all pure aerobic and anaerobic strains isolated from this PCE dechlorinating mixed culture were unable to dechlorinate PCE, Kastner suggested that facultative bacteria may have been involved. His hypothesis was supported by Enzien at al. (1994) who found that PCE and TCE can be reductively dechlorinated in a sediment column with bulk aerobic conditions, where the oxygen concentration was maintained at no less than 1.6 mg/liter . These observations suggested that facultative bacteria may also be, at least partially, responsible for reductive dechlorination of PCE and TCE, though the role of facultative bacteria in dechlorination of PCE is not fully understood.

2.2.2 Biodegradation of intermediates of PCE dechlorination

The biotransformation potential for less chlorinated intermediates produced from incomplete reductive dechlorination of PCE is inversely proportional to its chlorine content with the exception of 1,1-DCE (Chang & Alvarez-Cohen, 1996). TCE can be degraded under both aerobic and anaerobic conditions. Microbial transformation of TCE by reductive dechlorination under anaerobic conditions usually leads to DCE and VC. TCE can also be degraded by a cooxidation reaction with toluene or other aromatic hydrocarbons under aerobic conditions. Several aerobic bacteria have been reported to be responsible for the aerobic conversion of TCE. For example, the methane monooxygenase of the methanotroph *Methylosinus trichosporium* OB3b (Tsien et al., 1989), the toluene-*ortho*-monooxygenase of *Pseudomonas cepacia* G4 (Shields et al., 1989), and the toluene dioxygenase of *Pseudomonas putida* F1 (Wackett & Gibson, 1988; Heald & Jenkins, 1994), are thought to be responsible for the co-oxidation reactions. The oxidation products of oxygenase mediated TCE degradation have toxic effects on cell growth, which might limit the bioremediation capacity of certain bacteria (Wackett & Householder, 1989).

Aerobic DCE and VC degradation have been observed with a wide range of microorganisms exhibiting monooxygenase activity. Therefore, aerobic, facultative, and anaerobic bacteria

may all contribute to the complete biodegradation of PCE in the natural environment, and different bacteria may participate in different steps of the overall dechlorination reaction. A proper spatially organized bacterial consortium, rather than a single species, would be important for complete reductive dechlorination and oxidation of chlorinated ethenes. In this work I will focus on the characterization of microbial communities involved in reductive dechlorination of PCE, in order to understand the mechanism of the complete reductive dechlorination of PCE in the natural environment. It is hoped that this information can eventually be used to improve clean up of chlorinated ethene contaminated sites.

2.3 Methods for studying soil microbial communities

Analysis of microbial community structure is still an outstanding problem in environmental microbiology. A variety of techniques is currently available for studying various aspects of microbial community structure. These can be divided into three general categories based on (i) laboratory cultivation or incubation, (ii) on direct microscopic observation, and (iii) on the direct extraction and analysis of nucleic or fatty acids (Ogram & Feng, 1997). Soil microbial communities probably are the most complex communities in natural ecosystems. There may be as many as 4,000 species per gram of soil (Torsvik et al., 1990). Based on the current estimation by Torsvik, et al., (1990), not all microorganism present in soil microbial communities are readily cultivated. It has been stated that less than 1% of soil microorganisms are readily cultivatable by current laboratory cultivation techniques. Most of the microorganisms in soil microbial communities may thus be hard to study in pure form, and many approaches focus on small, well-defined groups of microorganisms, such as those involved in the degradation of a particular hydrocarbon or those capable of utilizing a specific compound as electron donor or acceptor. Since most biodegradation studies are primarily concerned with bacteria, only the techniques used for characterizing bacterial communities will be considered.

2.3.1 Use of selective enrichment and isolation

The general strategy for laboratory cultivation methods includes the selective enrichment and isolation procedures to (i) boost the number of bacteria capable of utilizing the selected substrate in the initial sample, (ii) isolate bacteria able to grow on the selected substrate by further liquid enrichment, and (iii) isolate a pure culture with the capacity to use the selected substrate by plating techniques (Slater & Lovatt, 1984).

Most microbial communities can be dissected by this classic strategy using a variety of selected media. It should be realized that the bacteria isolated with such methods are usually those that grow fast under the selective conditions used. Slower growing bacteria which may be important in the natural environment will not be obtained because they will be overgrown by faster growing bacteria under these laboratory conditions. Optimal incubation times should be applied to overcome this disadvantage. Also many environmental bacteria, particularly *Pseudomonas* sp., may be very difficult to be separated by plating, because they secrete abundant amounts of extracellular polysaccharides when grown on synthetic media, causing different cells to stick together (Ogram & Feng, 1997). Serial dilution to extinction in liquid medium and then evenly spreading on solid medium may solve this problem. The purity of a culture of an isolated bacterium is usually confirmed by its consistent colony morphology, and its appearance under phase contrast microscopy. It should be noted that colony morphologies are highly dependent on the growth substrate (Ogram & Feng, 1997). Other methods, e.g., gene probe testing, can help to identify a bacterial strain with different colony morphologies on different growth medium plates.

Selective enrichment and pure culture isolation is the best method for studying the specific behavior of isolated bacterial species present in the microbial community. However, relatively few species present in an environmental sample can be isolated with this approach due to culturability problems.

2.3.2 Use of molecular biological techniques

To overcome the limitations and biases of laboratory cultivation methods, studies of microbial communities based on analysis of total nucleic acids such as DNA, RNA (Sayler & Layton, 1990a) and fatty acids (Vestal & White, 1989) extracted from environmental samples have been developed. DNA, RNA and fatty acids are indicator molecules which offer different information regarding the structures and activities of microbial communities. Analysis of these indicator molecules directly extracted from soil samples can reveal genotypic diversity and its change in natural microbial communities without culturing.

Information on the shifts in structure of soil microbial communities was obtained by studying the changes of fatty acid profiles (Frostegard et al., 1993). The limitation of this technique is that many factors involved in bacterial growth will affect the fatty acid patterns. This makes it difficult to correlate changes in fatty acid patterns with the dynamics of a microbial population (Ogram & Feng, 1997).

Gene probes are used to obtain microbial community information by their quantitative hybridization to nucleic acids directly extracted from environmental samples. They offer high sensitivity and specificity through rapid high-fidelity hydrogen bonding between target nucleic acid sequences and homologous complementary probe sequences. The gene probe can target either DNA or RNA, depending on what information is required. DNA-DNA hybridization is usually used to rapidly identify the presence and relative concentrations of genes encoding a particular function in the microbial community, while DNA-RNA hybridization is useful for indicating the relative activities of the target genes (Sayler & Layton, 1990a). Since DNA probes are made to detect specific species or strains, a variety of gene fragments can be designed as probes to directly identify and enumerate individual species or strains in environmental samples. For example, antibiotic resistance genes, toluene and naphthalene degradation genes, and genes encoding other specific functions are commonly used as probes to detect bacteria with these specific functions in soil samples (Sayler & Layton, 1990a).

The greatest advantage of using a gene probe technique to study soil microbial communities is that it can rapidly detect specific bacteria present in soil samples. The probe specificity is important in quantitative analysis of a microbial community. A low specificity probe may hybridize to nonspecific gene regions in the sample, resulting in an overestimation of the target species. A problem with application of gene probes targeting a "function" present in a soil sample is that genes encoding the functions are not completely conserved among different bacterial species. The conserved DNA sequences in a particular species or a gene family are often used to make gene probes. Since soil microbial communities are highly diverse, a lack of cloned genes representing specific functions of soil microorganism may prevent the detection and enumeration of interesting species. 16S rRNA genes, in which certain regions are highly conserved between genera and species, also allow the study of diverse populations of microorganisms in natural communities without cultivation (Ward et al., 1990). The rRNA gene probes can be made using universal primers to amplify these conserved regions of rRNA genes by PCR (Amann et al., 1992).

The application of rRNA gene probes in studying microbial communities may not allow identification of closely related bacterial species. As an example, *Bacillus globisporus* and *Bacillus psychrophilus* have identical 16S rRNA gene sequences, but their genomic DNA-DNA similarity is less than 50% (Fox et al., 1992). To analyze soil microbial communities more conveniently and more precisely, total genomic DNA has been used as a probe to identify specific bacteria in soil samples. Genomic gene probes can recognize all genomically similar species because they have a higher probability and better sensitivity than a single-copy gene (Sayler & Layton, 1990a). With a genomic DNA probe, a bacterium can be defined as the same genomic species when its DNA sequence similarity with the probe is 70% or greater (Schleifer & Stackebrandt, 1983).

The reverse sample genome probe (RSGP) method is a technique that uses genomic DNA hybridization to characterize environmental microbial communities (Voordouw et al., 1991; 1993). In this technique, the total genomic DNA is isolated from environmental samples,

combined with λ DNA, randomly labeled, and then hybridized with a master filter. The master filter contains known amounts (c_χ) of denatured bacterial genomic DNAs and a series of spots of known amounts (c_λ) of denatured λ DNA. The λ DNA is used as an internal standard in the RSGP method. Ideally, the bacteria have genomes that show little or no cross-hybridization. Species that have strongly cross-hybridizing genomes belong to the same standard. Different standards thus represent the collection of genomes with little or no cross-hybridization.

An advantage of RSGP is that it allows the simultaneous detection of a large number of standards by a single hybridization reaction. In Figure 2-4, an example is given in which an extracted DNA sample containing genomes 3, 5, 10, 14, 15, and 19 is mixed with λ DNA and randomly labeled. During the labeling reaction the amount of label allocated to each genome (including λ) is proportional to its weight fraction f_χ. Following hybridization of this mixed probe to the master filter, the hybridization intensity $I_\chi = k_\chi \times f_\chi \times c_\chi$, where k_χ is a hybridization constant. The hybridization of the internal standard will have an intensity $I_\lambda = k_\lambda \times f_\lambda \times c_\lambda$. From these equations the fraction of standard genome χ can be determined as:

$$f_\chi = (k_\lambda/k_\chi) \times (I_\chi/c_\chi) \times (I_\lambda/c_\lambda)^{-1} \times (f_\lambda) \qquad (1)$$

provided that (k_λ/k_χ) is determined for each standard as

$$(k_\lambda/k_\chi) = (f_\chi/f_\lambda) \times (I_\lambda/c_\lambda) \times (I_\chi/c_\chi)^{-1} \qquad (2)$$

Application of these equations allows quantitative analysis as shown in Figure 2-4. The number of isolated standards determines the scope of microbial diversity that can be studied (Figure 2-4) within the microbial community. Also, although the RSGP measurement itself

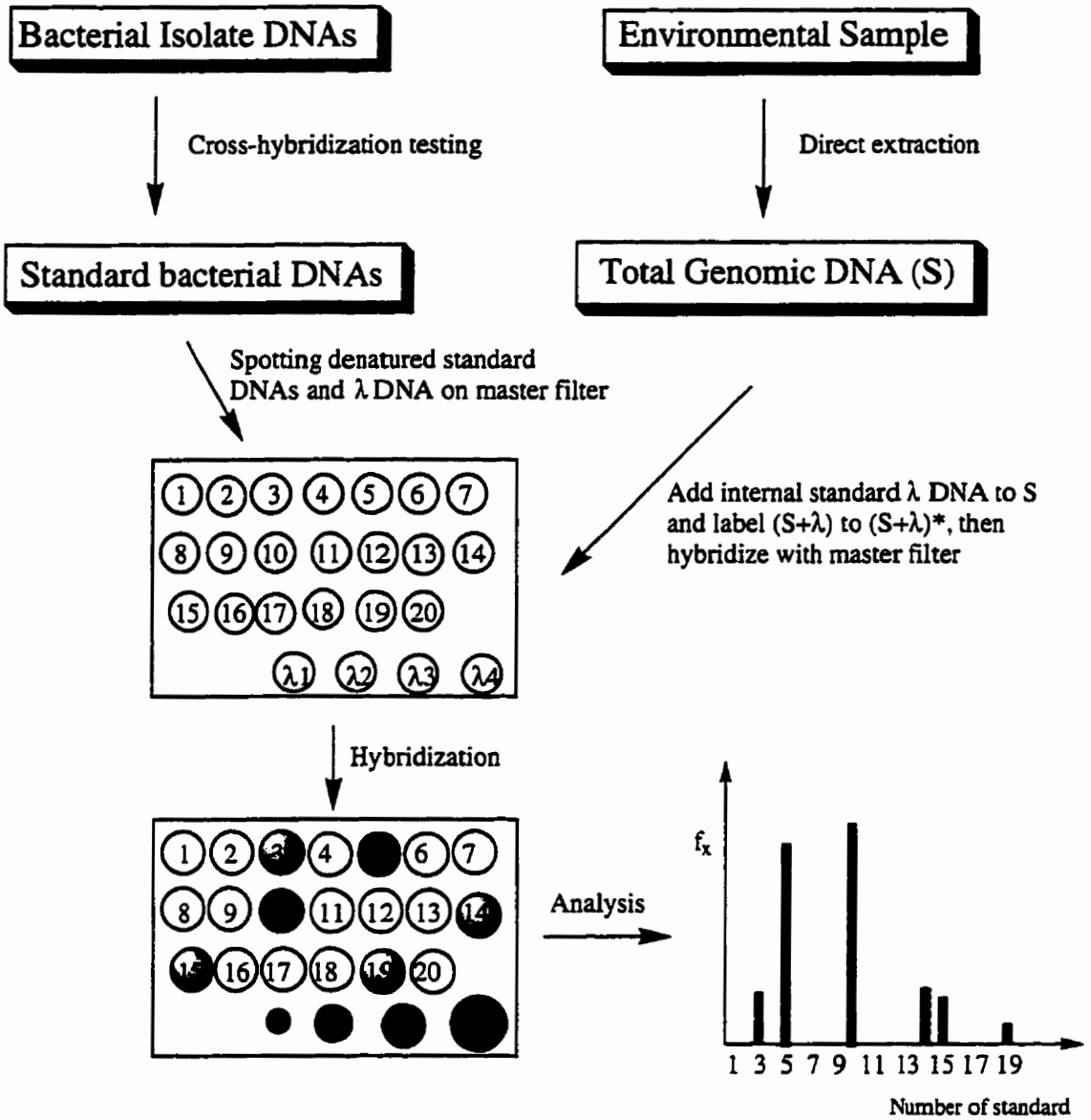


Figure 2-4. Principle of reverse sample genome probing (RSGP). The master filter contains denatured genomic DNAs from 20 bacterial standards and 4 spots of denatured λ DNA. The reverse sample genome probe (S+λ)* is hybridized with the master filter. Following analysis by equations (1) and (2), it appears that standard 3, 5, 10, 14, 15, and 19 are present in the environment sample at the fractions (f_x) shown.

does not involve cultivation, the standard bacteria are obtained as selective enrichment cultures of environmental samples and by colony purification. Therefore, the microbial community described by RSGP is only the cultivable group of bacteria present in the natural microbial world. An advantage is that it is possible to study further the specific functions and activities of these bacterial species in the community because they are already cultivated. The sensitivity and specificity of gene probe methods can be improved by increasing the yield and purity of total DNA or RNA extracted from soil samples. For example, the DNAs extracted from soil samples are always contaminated with humic substances, which will interfere with DNA detection and measurement. Humic substances can inhibit DNA hybridization (Steffan & Atlas, 1988) and enzymatic labeling reactions. To obtain DNA free from humic substances is therefore critical in the application of gene probe methods to soil community analysis.

There are other DNA-based methods used for studying microbial communities, such as comparison of the G+C contents of community DNA to analyze community structure shifts, and analysis of the total rRNA with oligonucleotide probes for characterization of the community structure (Ogram & Feng, 1997). Analysis of total rRNA, instead of rRNA genes (rDNA), extracted from soil samples has been applied to study the metabolic activity of bacteria in natural communities (Fleske et al., 1996). Each method has its limitations and biases, and it is important to select a suitable method for a particular application .

2.3.3 Identification of bacterial isolates

Various of methods are available for identification of environmental isolates. These can be based on determination of growth substrates, of metabolic functions, of cellular lipids, and of colony hybridization behaviors (Ogram & Feng, 1997). A precise method for identifying bacterial isolates from environmental samples is by sequencing their rRNA genes. Since rRNA molecules have both conserved and variable regions, determination of rRNA sequences can be used to determine the diversity of microbial communities, and to

identify bacterial isolates from the natural environment directly (Ward et al., 1990). The rRNA can be viewed as composed of structural domains within which sequence variation differs with respect to increasing phylogenetic distance. Particularly, 16S rRNA sequence analysis is valuable for characterization of microbial communities, because a large number of 16S rRNA gene sequences are available in a database for comparative purposes. Approximately 2,100 small subunit rRNA sequences for bacteria are available in the Ribosomal Database project (RDP; Maidak et al., 1994). The initial step in the characterization of an environmental isolate is extraction of its 16S rRNA or genomic DNA. The 16S rRNA has to be converted to cDNA and then cloned. Genomic DNA is used to amplify 16S rRNA genes by PCR, using primers targeting conserved regions of 16S rRNA genes. The amplified PCR product can then be cloned for nucleotide sequence determination, or can be directly sequenced. Sequencing of rRNA genes from some species may be difficult due to the presence of secondary structures which make the template DNA hard to denature under conditions of the sequencing reaction. Comparison of the nucleotide sequence obtained with an extensive database of 16S rRNA sequences, allows the species to be identified (Maidak et al., 1994).

This approach is limited by the 16S rRNA gene sequence data present in the databases. Because a majority of soil microorganisms have never been isolated, their 16S rRNA sequences are not available for comparison. Hence, many bacterial isolates can only be identified in terms of the highest similarity score with a sequence in the database. The identification will not be precise when the score is low.

2.4 Objectives of this research

The general objective of this research is to characterize two microbial communities involved in biodegradation of C5+ hydrocarbons and dechlorination of PCE in soil by molecular biological techniques. Specific objectives are to characterize the C5+ hydrocarbon degrading community and the community capable of complete reductive dechlorination of

PCE to ethene by RSGP, in order to identify bacteria involved in the degradation and dechlorination process. The degradation or dechlorination activity of these bacteria will then be compared with that of the entire soil consortium. These objectives will be reached by the strategies described below.

2.4.1 Identification of C5+ hydrocarbon degrading bacteria by RSGP

Microorganisms present in C5+ hydrocarbon contaminated soil will be isolated by enrichment and plating procedures. Genomic DNA will be isolated from all colony purified isolates. Cross-hybridization testing will define the set of standards, which will be used to make master filters. These master filters will be used to determine the effect of two C5+ hydrocarbons, toluene and DCPD, on the microbial community using the RSGP technique. The standards present on the master filter will be identified by partial sequencing and comparing their 16S rRNA gene sequences with those in the 16S rRNA database. Total genomic DNAs will be extracted from soil samples exposed to toluene or DCPD or from an untreated control. These DNAs will be labeled and hybridized with the master filters. The hybridization patterns will be analyzed and compared with the untreated control to define standards enhanced in the presence of DCPD or toluene.

2.4.2 Determination of C5+ hydrocarbon degradation activity

The C5+ hydrocarbon degradation activity potential of the enhanced standards will be determined in order to confirm whether they play a functional role in C5+ hydrocarbon degradation. Selected standards will be incubated with the degradable C5+ hydrocarbon toluene, or the recalcitrant C5+ hydrocarbon DCPD. Degradation activity will be measured as formation of oxidized derivatives or CO_2. The possibility that a mini-consortium of the enhanced standards is required for DCPD degradation will also be tested.

2.4.3 Identification of bacteria involved in reductive dechlorination of PCE

Bacterial isolates will be obtained by plating a PCE-contaminated soil sample and its enrichment cultures in the presence of PCE or DCE and incubating under aerobic and anaerobic conditions. Following cross-hybridization testing, master filters will be made by spotting denatured genomic DNAs of selected bacterial standards. Standards will be identified by sequencing their partial 16S rRNA genes and comparing their sequences with those in the 16S rRNA database.

The shifts in community structure will then be studied by quantitative RSGP. Total genomic DNAs will be isolated from a PCE contaminated soil sample incubated with or without PCE and DCE under different conditions. The dechlorination activity of the enhanced standards in the community will be studied further.

2.4.4 Determination of dechlorination activity

The dechlorination activity of standards indicated by quantitative RSGP analysis will be determined by incubating these standards individually in growth medium under anaerobic and aerobic conditions with either PCE or DCE. In the case of anaerobic PCE dechlorination, the disappearance of PCE and the appearance of products (TCE, DCE, VC, and ETH) will be monitored by gas chromatography.

The incubation conditions and nutrient requirements for complete dechlorination of PCE to ethene will be defined. The activities observed for individual standards will be compared with that of the entire soil consortium.

Chapter 3. Materials and Methods

3.1 Materials

3.1.1 Chemicals and reagents.

Chemicals. Dicyclopentadiene (95% pure) was obtained from Nova Research & Technology Corp. in Calgary and toluene (99.5% pure) was from BDH Chemical Co.. Tetrachloroethene (95% pure) and trichloroethene (99% pure) were purchased from Fisher Scientific Co., whereas *cis*--dichloroethene (97% pure) was obtained from Aldrich Chemical Co.. Vinyl chloride (99.0% pure; 200 mg/ml in methanol) and QTM volatile halocarbons mix (2,000 μg/ml of each component in methanol) were purchased from Supelco in 1 ml ampoules. Ethene calibration gas standard (1056 ppm ethene in nitrogen) was obtained as a gift from Nova Research & Technology Corp. in Calgary. Heptamethylnonane (98% pure) was from Aldrich Chemical Co. and polyvinylpolypyrrolidone (PVPP) was from Sigma Chemical Company. Most other chemicals were reagent grade from BDH, Fisher, or Sigma.

Biochemical reagents. All enzymes were purchased from Pharmacia, Boehringer Mannheim, Amersham, or Gibco BRL. Bacteriophage λ DNA (0.5 mg/ml) was obtained from Pharmacia and Hybond-N hybridization transfer membrane was purchased from Amersham. Molecular porous membrane tubing for electroelution of DNA from agarose gel was bought from Spectrum Medical Industries, Inc. Deoxyoligonucleotides, used as primers for PCR and sequencing reactions, and random hexanucleotides used in random extension labeling reactions were synthesized by the regional DNA Synthesis Laboratory of the University of Calgary. All other biochemical reagents were obtained from Sigma, Boehringer Mannheim, Difco Laboratories, Fisher, or BDH.

Radioisotopes [α^{35}S]dATP (10 mCi/ml; 400 Ci/mmol) was from Amersham and [α^{32}P]dCTP (10 mCi/ml; 3,000 Ci/mmol was from ICN. Both [^{14}C]-DCPD (151 μCi/ml) and [^{14}C]-toluene (10 μCi/ml) were obtained as universally labeled cyclopentadiene and toluene from Wizard Laboratories (Davis, California).

3.1.2 Environmental samples

C5+ hydrocarbon contaminated soil samples. Soil samples were collected from a soil pile constructed for a bioremediation project at an ethylene pyrolysis plant by personnel from Nova Research & Technology Corp. in Calgary. The designations NW, SW, NE and SE represent soil samples dug from different locations of the soil pile. The soil pile was constructed from soils with a significant C5+ hydrocarbon contamination, and used for field studies of C5+ hydrocarbon bioremediation. The soil was contaminated with ca. 70 μg of DCPD and 70 μg of BTEX per gram of soil at the start and ca. 30 μg of DCPD and 0 μg of BTEX at the end of the bioremediation project (Stehmeier, 1997).

PCE contaminated soil sample. This sample was obtained from the former Du Pont Plant landfill near Victoria, Texas, in which various solid and liquid wastes from industrial activities were dumped for more than 20 years. PCE waste and other chemicals placed in the landfill have migrated through the overlying layer into a water-bearing sand zone, resulting in significant PCE contamination in the groundwater and in the water of a canal adjacent to the landfill. The PCE contaminated soil sample was collected from this sand zone (Beeman et al., 1993).

3.1.3 Buffers and growth media

Buffers. TE buffer is 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0. TBE buffer for electroelution is 0.089 M Tris-borate, 0.089 M boric acid, and 0.02 M EDTA, pH 8.0. 1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.2.

Growth media. Tryptone yeast extract (TY, pH 7.4) medium, used as rich medium for soil sample enrichment contained 10 g of bactotryptone, 5 g of yeast extract and 5 g of NaCl per liter of H_2O. Liquid medium C and plating medium C and E for the growth of sulfate-reducers were made as described by Postgate (1984). The formulations of minimal salts medium (MSM, pH 7.0) and hydrocarbon degradation medium (HDM, pH 7.0) for studies of C5+ hydrocarbon degradation are listed in Table 3-1.

HDM medium was used for isolation of hydrocarbon degraders from soil samples by Dr. M. McD. Francis of NOVA Research & Technology Corp. in Calgary. PTYG medium, consisting of 1 g of tryptone, 2 g of yeast extract, 2 g of glucose, 0.6 g of $MgSO_4 \cdot 7H_2O$, and 0.07 g of $CaCl_2 \cdot 2H_2O$ per liter of H_2O, was used for the growth of bacterial isolates from soil samples by Dr. L. G. Stehmeier. Minimal glucose medium (MG, pH 7.0) for the growth of PCE dechlorinating bacterial species contained 50 ml of 10 x MG salts, 0.1% (w/v) of glucose, 0.5 mM $MgSO_4$, 0.05% (w/v) of thiamine, and 0.05 mM $CaCl_2$ per liter of medium. 10 x MG salts solution consists of 30 g KH_2PO_4, 60 g Na_2HPO_4, 5 g NaCl, and 10 g NH_4Cl per liter of solution.

Minimal salts and yeast extract medium (MY, pH 7.0), modified basal medium (BM, pH 7.0) and basal and yeast extract medium (BY, pH 7.0) used for studies of PCE reductive dechlorination were made according to the formulations listed in Table 3-2. The trace element solution for BM medium was composed of 8 ml HCl (12 M), 2,100 mg $FeSO_4 \cdot 7H_2O$, 30 mg H_3BO_3, 100 mg $MnCl_2 \cdot 4H_2O$, 190 mg $CoCl_2 \cdot 6H_2O$, 24 mg $NiCl_2 \cdot 6H_2O$, 2 mg $CuCl_2 \cdot 2H_2O$, 144 mg $ZnSO_4 \cdot 7H_2O$, and 36 mg $Na_2MoO_4 \cdot 2H_2O$ per liter of solution (Widdel & Bak, 1992).

Table 3-1. Media for growth of hydrocarbon degrading bacteria

Chemical	MSM (g/L)[a]	HDM (g/L)[a]
NH_4NO_3	-	2
$NaNO_3$	4.0	-
KH_2PO_4	1.5	1
K_2HPO_4	-	1
Na_2HPO_4	0.5	-
$FeSO_4 \cdot 7H_2O$	0.0011	0.001
$MgSO_4 \cdot 7H_2O$	0.2	0.3
$CaCl_2 \cdot 2H_2O$	0.01	0.001

[a] Grams of each chemical per liter solution.

Table 3-2. Media for growth of PCE dechlorinating bacteria

Chemical	MY (g/L)[a]	BM (g/L)[a]	BY (g/L)[a]
NH_4Cl	-	4.3	4.3
$(NH_4)_2SO_4$	-	0.5	0.5
KH_2PO_4	0.5	1.5	1.5
Na_2HPO_4	-	2.4	2.4
$MgCl_2 \cdot 6H_2O$	0.2	-	-
$MgSO_4 \cdot 7H_2O$	-	0.2	0.2
$CaCl_2 \cdot 2H_2O$	0.1	0.01	0.01
Yeast Extract	10	0.5	5
Trace element solution[b]	-	10 ml	-

[a] Grams of each chemical per liter solution.

[b] The trace element solution was autoclaved and then added directly.

3.2 Methods for studying soil microbial communities

The primary method for characterizing soil microbial communities used in this research was reverse sample genome probing (RSGP), which can be accomplished in five steps: (i) enrichment and isolation of bacterial strains, (ii) master filter preparation and identification of bacterial standards, (iii) DNA extraction from soil samples, (iv) labeling of extracted DNA and hybridization, and (v) quantitative analysis of hybridization data.

3.2.1 Enrichment and isolation of bacterial strains

In order to isolate bacterial strains from the soil samples, bacterial cells must be separated from soil particles. Separation was be done by gently suspending 1 g of soil sample into 5 to 10 ml of minimal salts medium. Following suspension, the supernatant containing bacterial cells was serially diluted with minimal salts medium. A 100 µl aliquot of each diluted sample was then spread on two of TY and Medium E agar plates, respectively. The spread plates were incubated at room temperature. Under aerobic conditions, incubation time was 1 or 2 days, but a longer time period was required for anaerobic incubation (about 1 or 2 weeks), because anaerobic bacteria usually grow slower than aerobic ones. Colonies with different morphology (e.g. shape, color, size, and the level of mucosity) were selected, and restreaked on the same medium and grown under the same conditions for further purification. This selection procedure was repeated until colonies with a single and consistent morphology were obtained. Purified colonies were then inoculated into 5 ml of growth medium. These cultures were used to generate 100 ml of liquid cultures of each colony purified bacterial isolate for genomic DNA isolation. The bacterial strains isolated from soil sample enrichments were stored in glycerol at -70°C.

Bacterial species present on the PCE dechlorinating master filter were isolated by similar procedures as described above. A PCE contaminated soil sample (1 g) was suspended in 5 ml of growth medium, and 100 µl of suspension was spread on TY, MG, and Medium E

solid medium plates, respectively. These plates were incubated under both aerobic and anaerobic conditions. Bacterial colonies with different morphologies were isolated from these plates. Colony purification and liquid culture were conducted as described above. Some bacterial species present on the PCE dechlorinating master filter were isolated by plating liquid enrichment cultures on MY agar plates, according to the enrichment procedure described in section 3.4.1.

3.2.2 Master filter preparation and identification of bacterial standards

Genomic DNA was isolated from 100 ml liquid cultures of colony purified bacterial isolates as described by Marmur (1961), modified by including digestion with DNase-free RNase and proteinase K (Voordouw et al., 1990). The DNA preparations were dissolved in TE at a concentration of 50 to 500 ng/µl, as determined by a fluorimetric method (Voordouw et al., 1993). A part of each DNA preparation was then diluted to 50 ng/µl with TE and 2 µl was spotted on a Hybond-N membrane for genomic cross-hybridization testing, using a dot blot procedure under high-stringency conditions (Voordouw et al., 1991). For probe preparation 100 ng of purified chromosomal DNA from a single bacterial species, 0.1 ng of λ DNA, 6 µl of primer extension mix containing random hexamers (Voordouw et al., 1992), 2 µl of Klenow polymerase (2U/µl), and 2 µl of [α^{32}P]dCTP were combined in a total volume of 30 µl labeling mixture. The primer extension mix was made by adding 44 µl of 0.9 M HEPES in 0.1 M $MgCl_2$ (pH 6.6), 25 µl of 1 M Tris-HCl (pH 7.4), 10 µl of 0.1 M dithiothreitol, 4 µl of 50 mM dGTP, 50 mM dATP and 50 mM dTTP, and 10 µl of random hexadeoxyoligonucleotides (10 µg/µl) in 100 µl. The hybridization conditions used are described in section 3.2.4. The hybridization patterns obtained were quantitatively analyzed to determined which species had greater than ca. 70% cross-hybridization. These

DNAs were considered to represent the same standard. If individual DNA preparations for a standard were in short supply they were pooled. Only bacterial DNAs with limited genomic cross-hybridization obtained from bacterial isolates were used as "standards" for making these master filters. The exact degree of cross-hybridization between the chromosomal DNA of these standards was determined later. Following the definition of standards, master filters were prepared by spotting 2 µl of each heat denatured standard DNA in a known pattern on Hybond-N membranes. The amounts of DNA (c_x) spotted for each standard are indicated in Table 4-1 and Table 5-1. The amounts of denatured bacteriophage λ DNA (10, 20, 40, 60, 80, 100, 200 and 400 ng) were also spotted in the bottom row of each filter (Figure 4-1). The filters were dried and baked for 10 min at 80 °C in a vacuum oven. DNAs were then further covalently linked to the membrane filters by irradiation with UV light (8,000 µW/cm^2, 312 nm) for 3 min (Voordouw et al., 1992).

Partial 16S rRNA gene sequences were used to determine the identities of all standards. A 1.4 kb product was obtained by amplifying the 16S rRNA genes with the polymerase chain reaction (PCR) using primers f8 (Hicks et al., 1992) and r1406 (Olsen et al., 1986), which are listed in Table 3-3. PCR amplification was performed with a Perkin Elmer GeneAmp 2400 PCR system by using 100 µl of PCR reaction mixture containing 100 ng of genomic DNA, 10 µl of 10 × Buffer (500 mM KCl, 15 mM $MgCl_2$, 100 mM Tris-HCl; pH 9.0), 3.0 µl of 50 mM $MgCl_2$, 4.0 µl of all four deoxynucleoside triphosphates (2.5 mM each), 2.0 µl of each of the primers f8 and r1406 (10 pmol/µl), and 0.5 µl of *Taq* polymerase (5 U/ µl). Amplification was performed with two consecutive sets of 15 cycles. The first set of 15 cycles was template DNA denaturation at 94°C for 30 sec and primer annealing at 60°C for 30 sec (with the temperature decreasing by 0.5°C per cycle), and primer extension

at 72°C for 60 sec. This was immediately followed by 15 cycles of 94°C for 30 sec, 54°C for 30 sec and 72°C for 60 sec. After amplification, 60 µl of 20% (w/v) polyethylene glycol (PEG 8000) and 2.5 M NaCl was added to the PCR reaction mixture, followed by incubation at 37°C for 10 min. The PCR product was collected by centrifugation, washed with 70% ethanol, and then dried in air. The amplified DNAs were dissolved in 10 µl of TE and visualized on a 1% (w/v) HGT agarose gel with ethidium bromide (0.1 µg/ml).

The amplified 16S rRNA genes were sequenced without cloning using [α^{35}S]dATP with the Promega fmol cycle sequencing system with primer EUB338 (Amann et al., 1992) or primer p76 targeting the conserved regions of 16S rRNA genes. The sequences of both primers are listed in Table 3-3. Approximately 200 nucleotides of most 16S rRNA genes were obtained by this method. Identification of microorganisms from these sequences was done by comparison of the sequences obtained with those available in the RDP with the program SIMILARITY_RANK (Maidak et al., 1994) or in the GenBank database with the program BLAST (Altschul et al., 1990).

3.2.3 DNA extraction from soil samples

DNA was isolated from soil samples by a modification of the technique described by Tsai & Olson (1991), which includes three cycles of freezing and thawing. Soil samples (5 g) were combined with 1 g of acid-washed polyvinylpolypyrrolidone (PVPP) and suspended in 20 ml of 0.1% (w/v) sodium pyrophosphate, and homogenized by stirring for 20 min at 4°C. The acid-washed PVPP was prepared as described by Holben et al. (1988). Soil particles and acid-washed PVPP were removed by centrifugation at 1,000 × g for 10 min at 4°C. The soil particles were washed twice with 0.1% (w/v) sodium pyrophosphate and the combined supernatants were centrifuged at 15, 000 × g for 20 min at 4°C to collect bacterial cells.

Table 3-3. Sequences and target sites of 16 rRNA gene primers

Primer	Sequence (5' → 3')	Target position[a]
f8	TGAGCCAGGATCAAACTCT	8-26
r1406	ACGGGCGGTGTGT(A/G)	1406-1392
EUB338	GCTGCCTCCCGTAGGAGT	338-355
P76	GCCAGC(A/C)GCCGCGGT	517-531

[a]Target positions correspond to nucleotide positions of the *Escherichia coli* 16S rRNA gene.

Table 3-4. Incubation of a PCE contaminated soil sample with PCE and DCE

Inoculum	Medium	Substrates[a]	Incubation conditons[b]
soil (1 g)	MY (10 ml)	PCE	mixed gas (5% H_2, 10% CO_2 and 85% N_2)
soil (1 g)	MY (10 ml)	none	mixed gas (5% H_2, 10% CO_2 and 85% N_2)
soil (1 g)	MY (10 ml)	PCE	nitrogen (100% N_2)
soil (1 g)	MY (10 ml)	none	nitrogen (100% N_2)
soil (1 g)	MY (10 ml)	DCE	air
soil (1 g)	MY (10 ml)	none	air

[a]Addition of 100 µl of 2 M PCE or DCE in heptamethylnonane into sealed 125 ml serum bottles.
[b]Gas atmosphere in the headspace of each inoculated serum bottle.

The collected bacterial cell pellet was resuspended in 5 ml of lysis buffer (0.15 M NaCl, 0.1 M EDTA, pH 8.0) containing lysozyme (15 mg/ml), and incubated in a 37°C water bath for 1 hour with agitation at 15 min intervals. Following incubation, 2.5 ml of 25% (w/v) sodium dodecyl sulfate (SDS) was added. Three cycles of freezing in a -70°C dry ice-ethanol bath for 30 min and thawing in a 65°C water bath for 30 min were conducted in order to release DNA from microbial cells by thermal shocks. After the freezing and thawing procedure, 0.6 ml of 5 M sodium perchlorate and 3 ml of a chloroform-isoamylalcohol mixture (24:1) were added, DNA was extracted with gentle end-over-end inversions on a wheel for 60 min. The mixture was then centrifuged at 6,000 × g for 10 min at 4°C, the top aqueous phase was collected, and precipitated with 2 volumes of cold ethanol (95%) at -70°C for 1 hour or at -20°C overnight. Finally, the crude DNA pellet was obtained by centrifugation at 12, 000 x g for 15 min at 4°C. This was brownish as a result of the coextraction of some of the humic acids in the soil sample that were not removed by the PVPP.

The crude DNA pellet was redissolved in 500 µl TE buffer, and purified by electrophoresis and electroelution from an agarose gel. The crude DNA was loaded on a 1% (w/v) high-gelling-temperature (HGT) agarose gel containing 0.1 µg of ethidium bromide per ml. The humic acids migrated faster than the DNA by electrophoresis in the agarose gel. The separated DNA band was excised from the agarose gel and placed in dialysis tubing (molecular porous membrane tubing, MWCO: 6-8,000). The tubing was filled with 0.5 × TBE buffer and placed in an electrophoresis tank perpendicular to the current. The electroeluted DNA was extracted twice with phenol to remove agarose and ethidium bromide and precipitated with ethanol. The purified DNA was redissolved in 50 µl TE and its concentration was determined prior to the labeling reaction.

The DNA extraction method for PCE contaminated soil was slightly modified because this soil sample had approximately 90% sand content. 10 g of soil was used for DNA extraction and was suspended in 20 ml of 0.1% (w/v) sodium pyrophosphate solution with stirring. After homogenization, the soil suspension was allowed to settle on ice for 1 min and the supernatant was collected. The soil sediment was then extracted twice and the supernatants were combined (total volume about 55 ml). PVPP (0.1 g) was suspended into the supernatant and mixed by stirring for 10 min to complex and remove humic acids. Later, PVPP was removed by centrifugation at 1,000 × g for 10 min at 4°C and the supernatant was collected. Incubation with SDS (2.5% SDS per ml) and three cycles of freezing-thawing were conducted with the collected supernatant for cell lysis as described above. The DNA extraction and purification were then carried out by following the same procedures.

3.2.4 DNA labeling and filter hybridization

Genomic DNA probes were prepared by combining 100 ng of purified genomic DNA isolated from soil samples, and 0.1 ng λ DNA and labeling with [α^{32}P]dCTP by the random hexamer extension method (Voordouw et al., 1992). The λ DNA served as the internal standard in the hybridization reaction (Voordouw et al., 1993). In some cases, larger amounts of λ DNA (up to 2.5 ng) were added in the labeling reaction. After primer extension for 3 to 5 hours at 22°C, the resulting genomic DNA probe was boiled for 2 min and hybridized with a prehybridized master filter for 16 hours with the high-stringency procedure (Voordouw et al., 1990; 1991). The master filter was prehybridized in hybridization solution for at least 2 hours at 68°C before the probe was added The hybridization solution was made by combining 30 ml of 6 × SSC, 5 ml of 10% (w/v) SDS, 10 ml of 50 x Denhardt solution (5 g Ficoll 400, 5 g PVP, and 5 g BSA in 100 ml water) ,

and 0.5 ml of 10% (w/v) of denatured salmon sperm DNA in a total volume of 100 ml. After hybridization, the master filters were washed in 1 × SSC at 22°C for 15 min, and then washed in 1 × SSC with 0.2% (w/v) SDS at 68°C for 60 min. After being washed, the master filters were air dried on 3MM paper and wrapped in Saran Wrap for quantitative analysis.

3.2.5 Quantitative analysis of hybridization data

The hybridized dot blots on the master filter were exposed to BAS-III Imaging plates which were scanned with a Fuji BAS1000 Bio-Imaging Analyzer. The hybridization intensity of each dot was determined by MacBas 2.2 software in units of photostimulable luminescence (PSL). An adjacent area was measured for all hybridized spots as the local background level (PSL-B). Net hybridization intensity (ΔPSL), obtained by subtraction of this local background (PSL-B) for all dots, was referred to as I_χ and I_λ for further calculations. The weight percentage f_χ of each standard DNA in the community DNA was calculated with equation 1 (Voordouw et al., 1993), given in section 2.3.2

The hybridization intensity observed for λ DNA (I_λ) increased linearly with the amount of denatured λ DNA spotted on the master filter (c_λ) in the range 10 to 60 ng. (I_λ / c_λ) values obtained for this range were averaged for all calculations. Under given hybridization conditions, the hybridization constant k_χ for each chromosomal DNA is dependent on its genome complexity. Because the genome complexity is unknown, relative hybridization constants (k_λ/k_χ) were determined for each individual standard in duplicate by labeling its chromosomal DNA together with λ DNA, and hybridizing the resulting mixed probe with a master filter. Relative hybridization constants (k_λ/k_χ) were then determined with equation

2, given in section 2.3.2. If 100 pg of internal standard λ DNA was used to spike 100 ng of chromosomal DNA for standard χ in the labeling reaction, then f_χ =0.999 and f_λ = 0.001 were used in the calculation. In cases, where 200 pg or more λ DNA was used in the labeling reaction, f_χ and f_λ values were adjusted accordingly. Duplicate measurements of (k_λ/k_χ) were done for all standards present on the master filter for studying the C5+ degrading microbial community. The average (k_λ/k_χ) values derived for each bacterial standard genome were used for the calculation of f_χ values for all standards in experiments where extracted community DNAs were labeled and hybridized with a master filter. The hybridization results for individual standard genomic DNAs were also shown in bar-diagrams by plotting percentage hybridization (with the standard used as the probe taken as 100%) versus standard number. These diagrams were reproducible in duplicate incubations and allowed the degree of cross-hybridization of the labeled DNA with the other chromosomal DNAs on the filter to be derived.

3.3 Methods for monitoring biodegradation of C5+ hydrocarbons

3.3.1 Soil incubations

Soil samples obtained from either the NW or NE end of the soil pile constructed for a C5+ bioremediation project were used. 10 g of each of these were placed in sterile 100 ml glass beakers covered with aluminum foil. After addition of 10 ml of MSM, the beakers were placed in glass dessicators containing a saturated atmosphere of either DCPD and H_2O, toluene and H_2O, or H_2O only. The dessicators were incubated at room temperature in the dark for 4 to 8 weeks. A 200 µl sample of the top layer of the incubated soil-medium mixture was transferred to 10 ml fresh MSM for further enrichment under the same

conditions. The soil-medium mixture was then centrifuged at 10,000 × g for 10 min. The soil-cell pellet was used for DNA isolation by the method described in section 3.2.3 and the supernatant from the DCPD incubated soil-medium mixture was used for extraction and measurement of oxygenated DCPD derivatives.

The DNAs isolated from soil-cell pellets were loaded on 0.7% (w/v) HGT agarose gels, electrophoresed and stained with ethidium bromide (0.1 μg/ml) and visualized under UV light. The amount of extracted DNA also served as an indicator of bacterial growth in the incubated soil. This gave some indication on the occurrence of biodegradation activity. The amounts of DNA isolated were quantitatively analyzed by comparing the DNA band intensities with those of λ DNA/*Hind*III molecular markers loaded on the same agarose gel.

For studying DCPD degradation by specific strains, steam-sterilized soil was used. This was prepared by autoclaving at 120°C for 30 min, leaving at room temperature overnight, and repeating this procedure five times in order to kill spore-forming bacteria. Specific bacterial strains were grown to saturation in TY medium before 10 to 20 μl amounts were inoculated into 5 to 10 g of sterilized soil.

3.3.2 Analysis of oxygenated DCPD derivatives

The supernatants collected from DCPD incubated soil-medium mixtures were saturated with sodium chloride, and extracted three times with a total volume of 45 ml ethyl acetate. Extracts were combined and concentrated using a rotary evaporator at 35°C. For quantitative analysis of oxygenated derivatives of DCPD, the concentrated ethyl acetate extracts (about 0.5 ml) were nearly dried with a stream of nitrogen and redissolved in 0.2 ml dichloromethane containing 20 μg of *p*-dichlorobenzene as the internal standard. Quantitative analysis of oxygenated DCPD derivatives was performed by gas chromatography (GC), using a Hewlett Packard 5890 GC equipped with a flame ionization

detector (FID) and an OV-1, fused silica capillary column (15 m × 0.32 μm). Helium was used as the carrier gas, and data were integrated with a HP 3392A integrator. The oven temperature was programmed for 2 min at 60°C, and then increasing temperature at 10°C/min to 250°C. 1 μl of each sample dissolved in dichloromethane with 10% (w/v) *p*-dichlorobenzene was injected directly into the gas chromatography.

Oxidized derivatives of DCPD were identified by comparing their mass spectra with those published before (van Breemen, et al., 1987; 1993; Stehmeier et al., 1996). GC/MS spectra were obtained on a Hewlett Packard 5890 gas chromatography equipped with a 5971 mass selective detector in electron impact mode. The GC column used in GC/MS experiments was a liquid phase, fused silica capillary column (DB-1701, 30 m × 0.25 μm). The temperature program was 100°C for 2 min followed by a 10°C/min ramp rate to a final temperature of 250°C. 5 μl of each sample dissolved in dichloromethane was used for GC/MS analysis.

3.3.3 Mineralization of [^{14}C] DCPD and [^{14}C] toluene

Mineralization experiments were carried out essentially as described by Bazylinski et al. (1989). The bacterial strains used in mineralization studies were selected based on RSGP analysis and grown to saturation in TY medium. 20 μl of each saturated culture was inoculated into 5 ml of minimal salts medium with 1 g of the sterilized soil and 10 μl of [^{14}C]-DCPD (2 μl of 0.15 μCi/ μl uniformly labeled DCPD diluted with 8 μl of unlabeled DCPD) or [^{14}C]-toluene (2 μl of 0.06 μCi/ μl uniformly labeled toluene diluted with 8 μl of unlabeled toluene) in a 20 ml ampoule which was then flame sealed. Duplicate ampoules for each strain were incubated with gentle shaking at 22°C in the dark. After incubation for four weeks, the sealed ampoules were cooled in an ice bath for at least 1 hour. They were

then connected to a CO_2 collection apparatus which consisted of two serially connected test tubes containing 10 ml of 0.6 M KOH each. The top of the ampoule was then broken and 1 ml of 1 M HCl was added. The produced $^{14}CO_2$ was collected in the KOH tubes by bubbling with a gentle stream of nitrogen for 30 min. The contents of the KOH tubes were transferred to two scintillation vials, mixed with 5 ml EcoLite™ scintillation fluid (ICN) and counted with an LKB 1215 RACKBETA liquid scintillation counter for 2 or 10 min. The count values of all samples were corrected with a blank value obtained by incubating an ampoule with sterilized soil, MSM, and the [^{14}C] labeled compound, but without bacterial inoculum. This background value was subtracted from the count values. Non-sterilized soil samples without added inoculum were also used in some experiments.

3.4 Methods for measuring dechlorination activity

Since chlorinated ethenes are highly volatile, all experiments were conducted in 125 ml serum bottles which were sealed by crimping aluminum rings with PTFE-faced/Tan silicone septa (Kimble Glass Inc.). These bottles contained 50 ml of sterilized medium. Incubation was performed at 22°C and inoculum was added with a disposable 1 ml tuberculin syringe (Becton Dickinson & Company). All substrates were introduced into the sealed bottles using a gas tight 10 µl microliter syringe (Hamilton Company).

3.4.1 Enrichment procedures

A PCE contaminated soil sample (1 g) was added to 125 ml serum bottles with 50 ml MY medium. Three sets of duplicate sample bottles were equilibrated in a different gas atmosphere before substrates were introduced (Table 3-4). One set of bottles, sealed under anaerobic conditions (5% H_2, 10% CO_2 and 85% N_2), received 100 µl 2 M PCE in heptamethylnonane. The headspace of the second set of sealed bottles was changed to 100% N_2, after which 100 µl 2 M PCE in heptamethylnonane was added. A third set of

bottles was sealed in air, after which 100 µl 2 M DCE in heptamethylnonane was added. Sample bottles without substrate addition with the same gas atmosphere were also incubated in these experiments. After incubation for 2 weeks, 100 µl was withdrawn from each of the bottles and serially diluted with MY medium. The serial dilutions were spread on MY medium plates and incubated in the same gas atmosphere for isolation of bacteria with possible PCE dechlorinating activity. Following incubation, the isolation and purification of bacterial stains from these MY plates were conducted similarly as described in section 3.2.1. Total genomic DNAs were extracted from all sample bottles for RSGP analyses after 4 weeks of incubation.

3.4.2 Assay of dechlorination activity

Bacterial strains identified by RSGP analyses were tested for their ability to dechlorinate reductively PCE in MY medium, BM medium and BY medium containing 100 to 200 ppm of PCE. A culture enriched in the presence of PCE was also used as an inoculum for testing of dechlorination activity. This culture was obtained by inoculating 1 g of a PCE contaminated soil sample in 500 ml MY medium with 50 ppm of PCE per ml and incubating at 22°C for three months. A 1 to 2% (v/v) inoculum of this culture was used in these experiments and in subsequent culture transfers. The dechlorinating activity was quantified by monitoring the time-dependent depletion of the substrate (PCE, TCE, or DCE) and production of end products (ETH) by gas chromatography. Medium (50 ml) was added to each of the 125 ml serum bottles, which were sealed in an anaerobic atmosphere for PCE and TCE as substrates and sealed in air for DCE as substrate. The sealed bottles were autoclaved at 120°C for 30 min. Chlorinated substrates (PCE, TCE or *cis*-DCE) were introduced into the sealed serum bottles with a Hamilton syringe. The bottles were gently shaken in inverted position (to prevent loss of chlorinated ethenes through the septum) at 22°C for 24 hours to reach equilibrium of chlorinated ethenes in the

medium. Before inoculation, the initial substrate concentration of each bottle was measured by analyzing 100 µl of headspace from each bottle. Saturated bacterial cultures in MY medium (200 to 500 µl) were then inoculated into the serum bottles. The dechlorination activity was monitored by measuring substrate and end product concentrations in the headspace of the incubation bottles, using gas chromatography.

3.4.3 Analytical methods

Concentrations of volatile chlorinated ethenes (PCE, TCE, DCE, and VC) and the end product ethene were determined by headspace analyses. 100 µl of headspace gas was withdrawn from the incubation bottles using a gas-tight syringe (Hamilton Co.) and analyzed with a Hewlett Packard 5890 gas chromatography equipped with a flame ionization detector and a fused silica capillary column (30 m × 0.32 µm). The temperature program was as follows: 40°C for 1 min after injection, then run from 40°C to 150°C at 10°C/min. The retention times and peak areas were determined by a HP 3392A Integrator supplied with the GC. Identification and confirmation of chlorinated ethenes and ethene was made by injecting a standard of gaseous pure chemicals into the same GC system under the same operating conditions, and matching the retention times of standard chemicals with the peaks from headspace samples of the bacterial cultures. Identification was also confirmed by injection of the headspace of QTM volatile halocarbons mix (2,000 µg/ml of each component in methanol) containing all chlorinated ethenes. This allows the evaluation of possible shifts of the retention time for each chlorinated ethene in the mixed state.

The concentration of chlorinated ethenes and ethene were obtained by comparing the peak areas from headspace samples of the incubations with a standard curve. Standards of PCE, TCE and DCE were preparation by adding a series of different volumes of chlorinated

ethenes (2,000 μg/ml in methanol) into a 125 ml aluminum crimp-sealed serum bottles containing 50 ml of sterilized MY medium (Table 3-5). The bottles were gently shaken in inverted position at 22°C overnight, 100 μl headspace was withdrawn from each of these bottles, and measured by GC under the same operation conditions as that for bacterial cultures. Different volumes of the ethene gas calibration standard (1,056 ppm ethene in nitrogen) were directly injected in the GC as described above. Standard curves were generated by plotting peak areas versus the concentration of chlorinated ethenes and ethene.

Table 3-5. Volumes of standard chlorinated ethenes used for standard curve measurement

Bottle	MY (ml)	PCE (µl)[a]	TCE (µl)[a]	*cis*-DCE (µl)[a]	*trans*-DCE (µl)[a]	VC (µl)[a]
1	50	5	5	5	5	5
2	50	15	15	15	15	15
3	50	20	20	20	20	20
4	50	30	30	30	30	30
5	50	40	40	40	40	40
6	50	50	50	50	50	50
7	50	60	60	60	60	60
8	50	70	70	70	70	70

[a] 2,000 µg/ml in methanol of each chlorinated ethene was added into 125 ml sealed serum bottle containing 50 ml of MY medium.

Chapter 4. Results on a C5+ degrading microbial community

A soil microbial community involved in C5+ hydrocarbon degradation was analyzed using the RSGP method and the degradation activity of either the community or some individual species have been studied.

4.1 Isolation of bacterial species from C5+ contaminated soil

A total of 12 colony purified bacterial isolates was obtained by spreading samples derived from C5+ contaminated soil on TY and Medium E plates. Genomic DNAs were extracted and purified from the liquid cultures of these bacterial isolates. After cross-hybridization testing 7 of these 12 isolates, Q7 to Q13 (Table 4-1: standards 27 to 33) were chosen as bacterial standards. Standards 27, 28, and 29 were isolated on TY medium plates under aerobic conditions, while standards 30 to 33 were grown in TY medium under anaerobic conditions (gas atmosphere of 5% H_2:10% CO_2:85% N_2).

Standards 1 to 20 and 22 to 24 were isolated from C5+ contaminated soil under aerobic conditions by Dr. L. G. Stehmeier. These were selected from a set of 44 colony purified isolates obtained by streaking on PTYG medium plates and incubating at room temperature. The genomic DNAs of these standards used for master filter preparation were purified from cultures in 300 ml PTYG medium. Standards 21, 25, and 26 were isolated on aerobic HDM plates grown in the presence of benzene, naphthalene or styrene vapor, respectively, by Dr. M. McD. Francis. Genomic DNA was isolated for each of these standards from 1 liter liquid cultures in HDM plus these hydrocarbons. Standards 34 and 35 were isolated on Medium E plates and their DNAs were purified from cultures in liquid Medium C. These standards had been obtained in earlier work on soil samples from a petroleum hydrocarbon contaminated site.

Table 4-1. Bacterial standards present on the master filter of the C5+ degrading community

Pos[a]	Name[b]	Medium[c]	C[d]	S[e]	Closest RDP homolog[f]
1	LQ1	PTYG	164	0.802	*Bordetella bronchiseptica*
2	LQ5	PTYG	156	0.375	*Pseudomonas syringae*
3	LQ6	PTYG	200	0.396	*Azospirillum* sp.
4	LQ10	PTYG	110	0.598	*Sphingomonas parapaucimobilis*
5	LQ11	PTYG	148	0.769	*Bacillus macroides*
6	LQ14	PTYG	132	0.615	*Xanthomonas campestris*
7	LQ15	PTYG	116	0.580	*Bacillus pseudomegaterium*
8	LQ16	PTYG	176	0.861	*Pseudomonas syringae*
9	LQ17	PTYG	152	0.931	*Agrobacterium rubi*
10	LQ19	PTYG	152	0.349	*"Flavobacterium" lutescens*
11	LQ20	PTYG	156	0.816	*Pseudomonas syringae*
12	LQ21	PTYG	148	0.791	*Bordetella parapertussis*
13	LQ26	PTYG	130	0.865	*Bordetella parapertussis*
14	LQ27	PTYG	130	—	(*Bordetella* sp.)[g]
15	LQ29	PTYG	152	0.843	*Bordetella parapertussis*
16	LQ30	PTYG	140	0.935	*Sphingomonas yanoikuyae*
17	LQ33	PTYG	180	—	nd[h]
18	LQ34	PTYG	138	0.969	*Pseudomonas flavescens*
19	LQ35	PTYG	148	0.972	*Pseudomonas flavescens*
20	LQ36	PTYG	136	0.965	*Pseudomonas flavescens*
21	Q1	HDM, Benzene	172	0.851	*Rhodococcus* sp.
22	Q2	PTYG	148	0.879	*Bacillus cereus/thuringiensis*
23	Q3	PTYG	184	0.763	*Nocardioides luteus*
24	Q4	PTYG	160	0.683	*Flavobacterium ferrugineum*
25	Q5	HDM, naphthalene	160	0.779	*Pseudomonas syringae*
26	Q6	HDM, styrene	116	0.836	*Rhodococcus globerulus*
27	Q7	TY	172	0.741	*Pseudomonas syringae*
28	Q8	TY	148	0.879	*Bacillus benzeovorans*
29	Q9	TY	146	0.809	*Bacillus polymyxa*
30	Q10	TY, anoxic	146	0.504	*Bacteroides distasonis*
31	Q11	TY, anoxic	240	0.498	*Bacteroides heparinolyticus*
32	Q12	TY, anoxic	152	0.846	*Clostridium xylanolyticum*
33	Q13	TY, anoxic	128	0.337	*Clostridium* sp.
34	Q14	Medium C, anoxic	172	0.582	*Desulfovibrio longus*
35	Q15	Medium C, anoxic	146	0.571	*Desulfovibrio desulfuricans*

Footnotes to Table 4-1:

[a]Position of denatured genomic DNA on the master filter.

[b]Name assigned at time of isolation.

[c]Medium used for isolation, standards 1 to 30 were isolated under aerobic conditions.

[d]Amount of denatured genomic DNA (ng) spotted on the filter.

[e]Similarity coefficient for query and matching sequences (Maidak et al., 1994).

[f]Closest homolog in the RDP database as determined by using the program SIMILARITY_RANK (Maidak et al., 1994).

[g]Based on its cross-hybridization with other *Bordetella* spp. genomes on the master filter.

[h]Not determined.

4.2 Characterization of the C5+ degrading microbial community

4.2.1 Development of a master filter

The master filter used for studying the C5+ degrading microbial community contained the 35 bacterial standards listed in Table 4-1. These were selected from 61 bacterial isolates obtained from the C5+ hydrocarbon contaminated soil samples (see section 4.1) by eliminating species with strong genomic cross-hybridization in dot blot hybridization tests. More than two hundred master filters were made by spotting 2 μl of denatured chromosomal DNAs for all 35 standard bacteria and 2 μl of denatured λ DNA in a series of increasing concentrations as shown in Figure 4-1. The amount of each standard DNA spotted on the filter is listed in Table 4-1. These values were calculated based on the average concentration of each DNA preparation, estimated for three different dilutions by a fluorimetric method (Voordouw et al., 1993).

4.2.2 Identification of bacterial standards

Most (33 out of 35) standards present on the master filter were identified by sequencing of PCR-amplified rRNA genes and comparison of the sequences obtained with those available in the RDP database. The closest RDP homolog and the similarity coefficient (S) for the similarity between standard and homolog determined by the program SIMILARITY_RANK are listed in Table 4-1. Not enough sequence data were obtained for standard 14 to give a definitive assignment. However, possible placement in the genus *Bordetella* sp. was suggested by cross-hybridization of its genome probe with other *Bordetella* species genomes on the filter. Standard 17 was not identified due to failure of its 16S rRNA gene to be amplified by PCR. All of the 16S rRNA gene sequences obtained were aligned by using the PILEUP program of the GCG (Genetic Computer Group) sequence analysis software package, and the pairwise alignments of these sequences were plotted in a tree shown in Figure 4-2. This tree shows the similarity

between all the sequences as calculated by PILEUP and represented in the clustering order.

4.2.3 Cross-hybridization of selected bacterial standards

The relative genome complexity values (k_λ/k_χ) for all 35 standard bacteria were determined by hybridizing a labeled standard genomic and λ DNA mixture with the master filter. All of the k_λ/k_χ values were measured in duplicate. The averaged k_λ/k_χ values and deviation of the average values are listed in Table 4-2. These values reflect the difference in genome complexities among the standard bacteria. The higher the k_λ/k_χ value of a bacterium is, the larger genome size it should have. This resulted in the observation of less hybridization intensity. For example, standard 21 (Q1) has the highest and standard 5 (LQ11) has the lowest k_λ/k_χ value of 35 bacterial standards. When the same amount of chromosomal DNA (100 ng) and λ DNA (150 pg) were labeled and hybridized with the master filters, the hybridization patterns shown in Figure 4-3 were obtained. The differences in k_λ/k_χ make that the hybridization intensities of a hybridization pattern cannot be directly converted to the fractions (f_χ) of standards in the community, if total genomic DNA extracted from an environmental sample is used as a probe. The average k_λ/k_χ value for each individual standard must therefore be used in equation 1 for calculating the fractions (f_χ) of standard genomes in the extracted environmental DNAs. The derived bar diagrams (f_χ versus standard number) were used for analyses of microbial communities in this research.

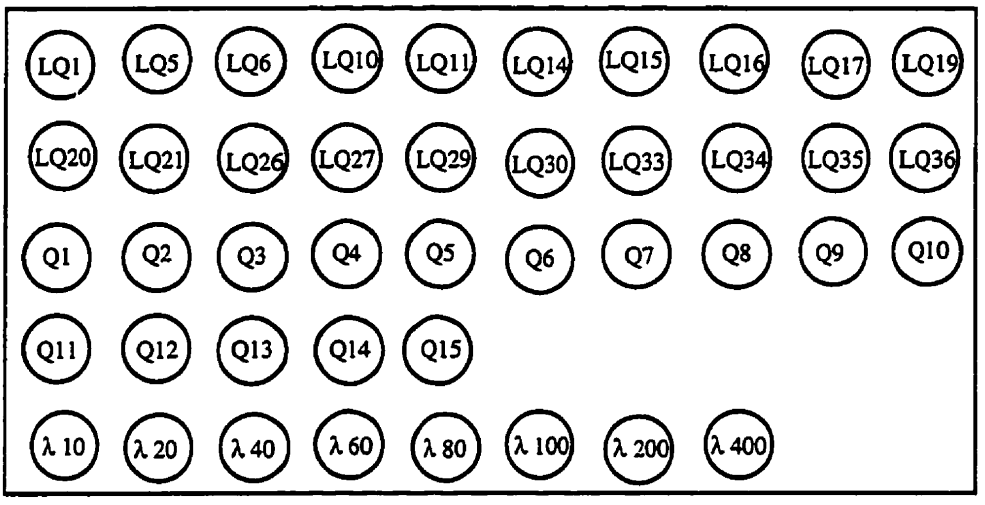


Figure 4-1. Layout of master filter used for studying the C5+ hydrocarbon degrading microbial community. The denatured genomic DNAs of 35 standard bacteria were spotted on the filter in the order shown. See also Table 4-1. The bottom row contained denatured λ DNA in the amounts indicated (ng).

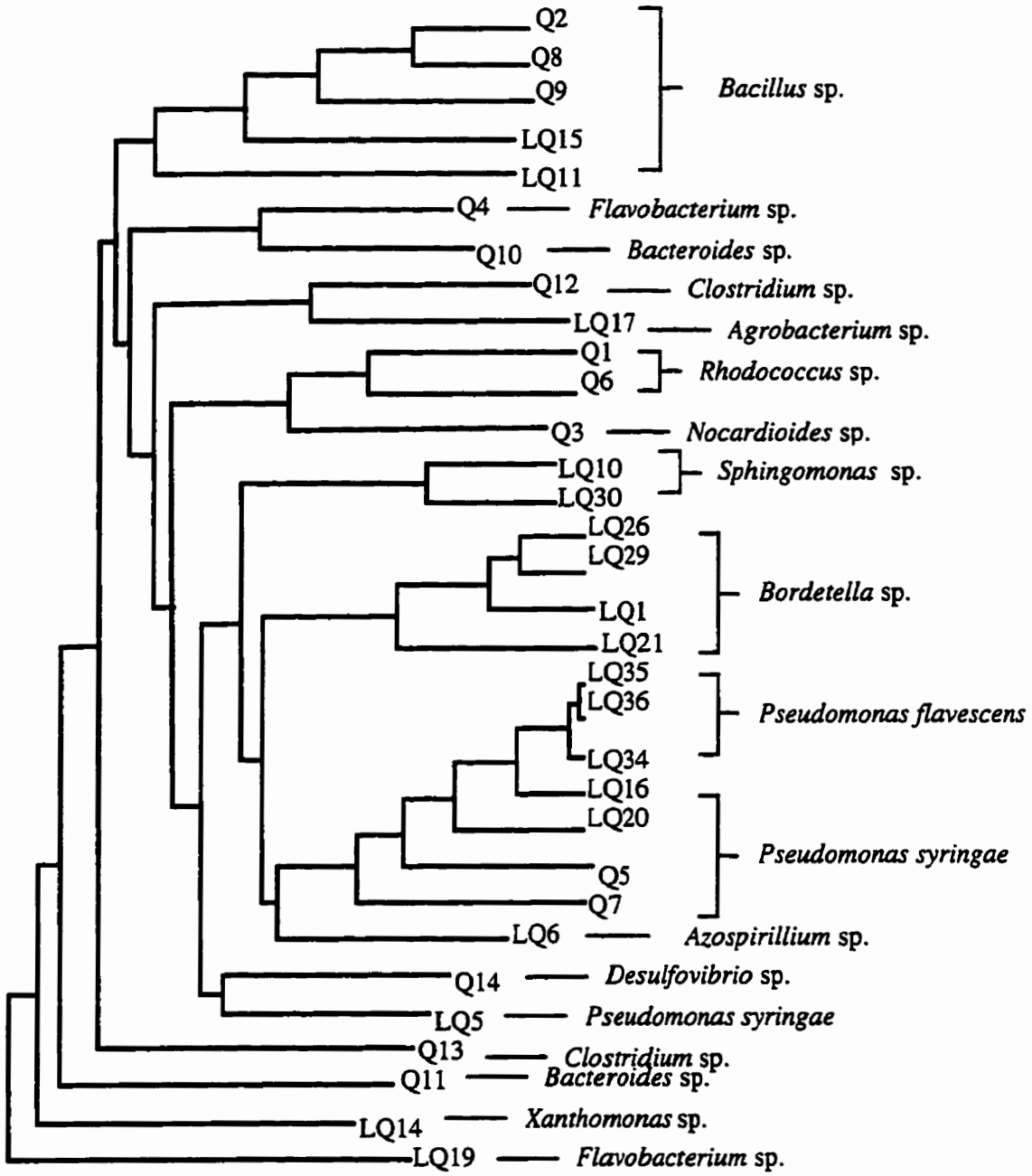


Figure 4-2. The similarity relationships between 16S rRNA gene sequences of the standards present on the master filter for studying C5+ community. This tree was generated by PILEUP program of GCG, based on pairwise alignments. The identifications were obtained by comparison of these sequences with those in RDP database.

Table 4-2. The k_λ/k_χ values for the bacterial standards

Standard[a]	Name[b]	k_λ/k_χ (1)[c]	k_λ/k_χ (2)[c]	k_λ/k_χ (avg.)[d]	σ[e]
1	LQ1	257	218	238	20
2	LQ5	190	170	180	10
3	LQ6	279	286	283	4
4	LQ10	122	101	112	11
5	LQ11	44	47	46	2
6	LQ14	72	49	61	12
7	LQ15	91	44	68	24
8	LQ16	96	94	95	1
9	LQ17	107	116	112	5
10	LQ19	170	143	157	14
11	LQ20	180	240	210	30
12	LQ21	294	294	294	0
13	LQ26	190	141	166	25
14	LQ27	185	113	149	36
15	LQ29	240	176	208	32
16	LQ30	204	195	200	5
17	LQ33	262	235	249	14
18	LQ34	157	183	170	13
19	LQ35	165	154	160	6
20	LQ36	103	115	109	6
21	Q1	672	476	574	98
22	Q2	54	66	60	6
23	Q3	298	362	330	32
24	Q4	83	103	93	10
25	Q5	145	138	142	4
26	Q6	197	206	202	5
27	Q7	138	163	151	12
28	Q8	57	44	51	7
29	Q9	65	80	73	7
30	Q10	61	82	72	10
31	Q11	88	72	80	8
32	Q12	59	71	65	6
33	Q13	78	44	61	17
34	Q14	121	133	127	6
35	Q15	85	102	94	8

Footnotes to Table 4-2:

[a]Position of denatured genomic DNA of standards on master filter.

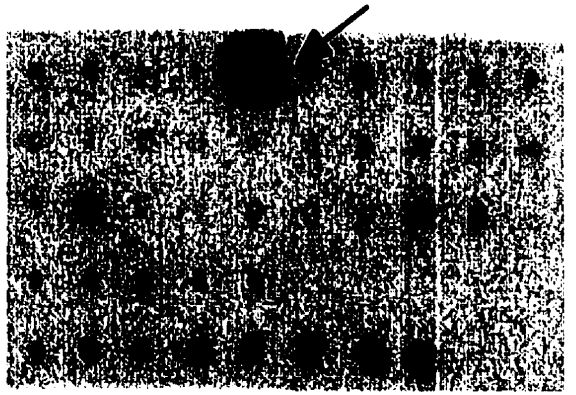
[b]Name assigned at time of isolation.

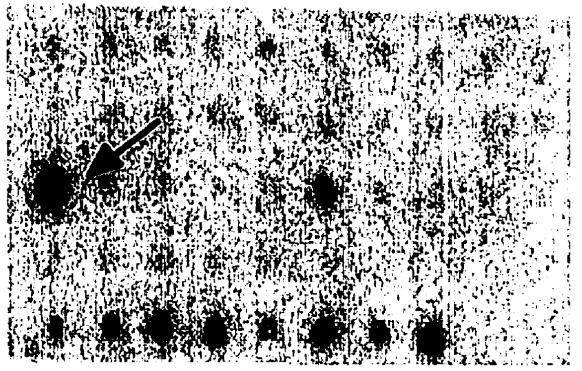
[c]Ratio of hybridization constants for bacteriophage λ and genomic DNA χ from equation 2, given in section 2.3.2.

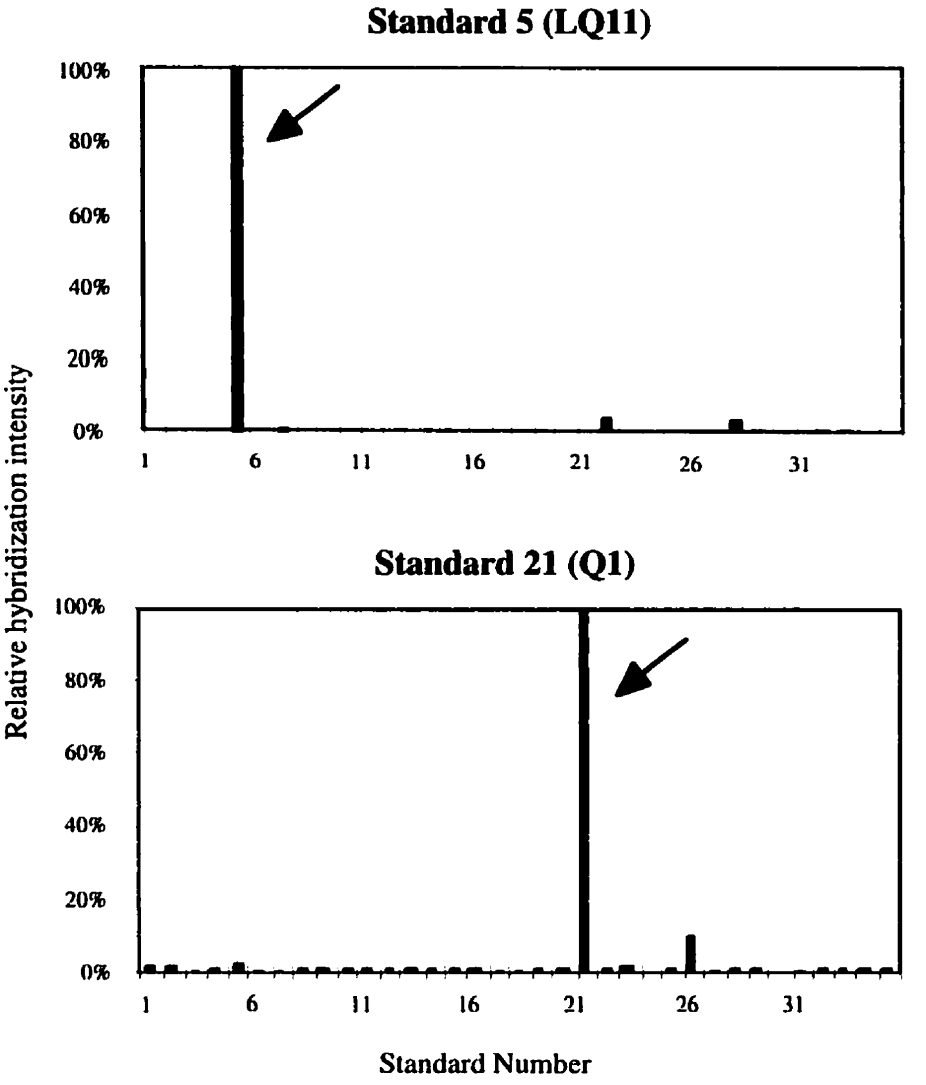
[d]Average k_λ/k_χ value obtained from duplicate experiments (1) and (2).

[e]Deviation from average k_λ/k_χ value.

Figure 4-3. Hybridization patterns and derived bar diagrams for standard 5 and 21. Relative hybridization intensity I_χ is on y axis, and the standard number is marked on the x axis. The arrow indicates self-hybridization, which is taken as 100% in the bar diagrams. Note that relative to λ DNA the standard 5 genome hybridizes much stronger than the standard 21 genome, indicating a lower (k_λ/k_χ) values.






Standard 5 (LQ11)
Standard 21 (Q1)
Relative hybridization intensity
100%
80%
60%
40%
20%
0%
1
6
11
16
21
26
31
Standard Number

The levels of cross-hybridization between a standard genome and the other 34 bacterial standard genomes present on the master filter were determined by quantitatively analyzing the hybridization patterns as in Figure 4-3. All cross-hybridization data for the 35 standard genomes are displayed as a matrix in Figure 4-4, where the diagonal represents self-hybridization and each off-diagonal square is shaded in proportion to the percentage of cross-hybridization. The degree of cross-hybridization varied among different standards of the same genus. Less than 5% cross-hybridization was displayed by the five *Bacillus* standards (Figure 4-5; LQ11, LQ15, Q2, Q8, and Q9), while up to 30% cross-hybridization was observed in five *Pseudomonas* standards (Figure 4-5; LQ5, LQ16, LQ20, Q5, and Q7). These data can be used to correct for cross-hybridization in quantitative analysis of hybridization patterns when all component genomes in a sample are known. Unfortunately, this is not usually the case in the analysis of environmental samples.

4.3 Analysis of the C5+ degrading microbial community

Soil samples obtained from the NW and NE end of a C5+ contaminated soil pile, were used for studying the C5+ degrading microbial community. The composition shifts of the microbial community were analyzed by quantitative RSGP, after these samples were incubated with two C5+ components: degradable toluene and recalcitrant dicyclopentadiene. An agarose gel of DNAs extracted from these incubated soil samples is shown in Figure 4-6. The amounts of DNAs extracted from the incubated soil samples could be estimated by comparison of the DNA band intensities of the extracted DNAs with the λ DNA/*Hind*III size markers, which were loaded on the same gel (total 1,000 ng λ DNA digested). The amounts of DNA extracted from soil samples incubated with a DCPD (lane 3, about 1.65 μg/g soil) and a toluene atmosphere (lane 4, about 2.1 μg/g soil) are 2.5 to 3.5 times higher than those extracted from the soil incubated without

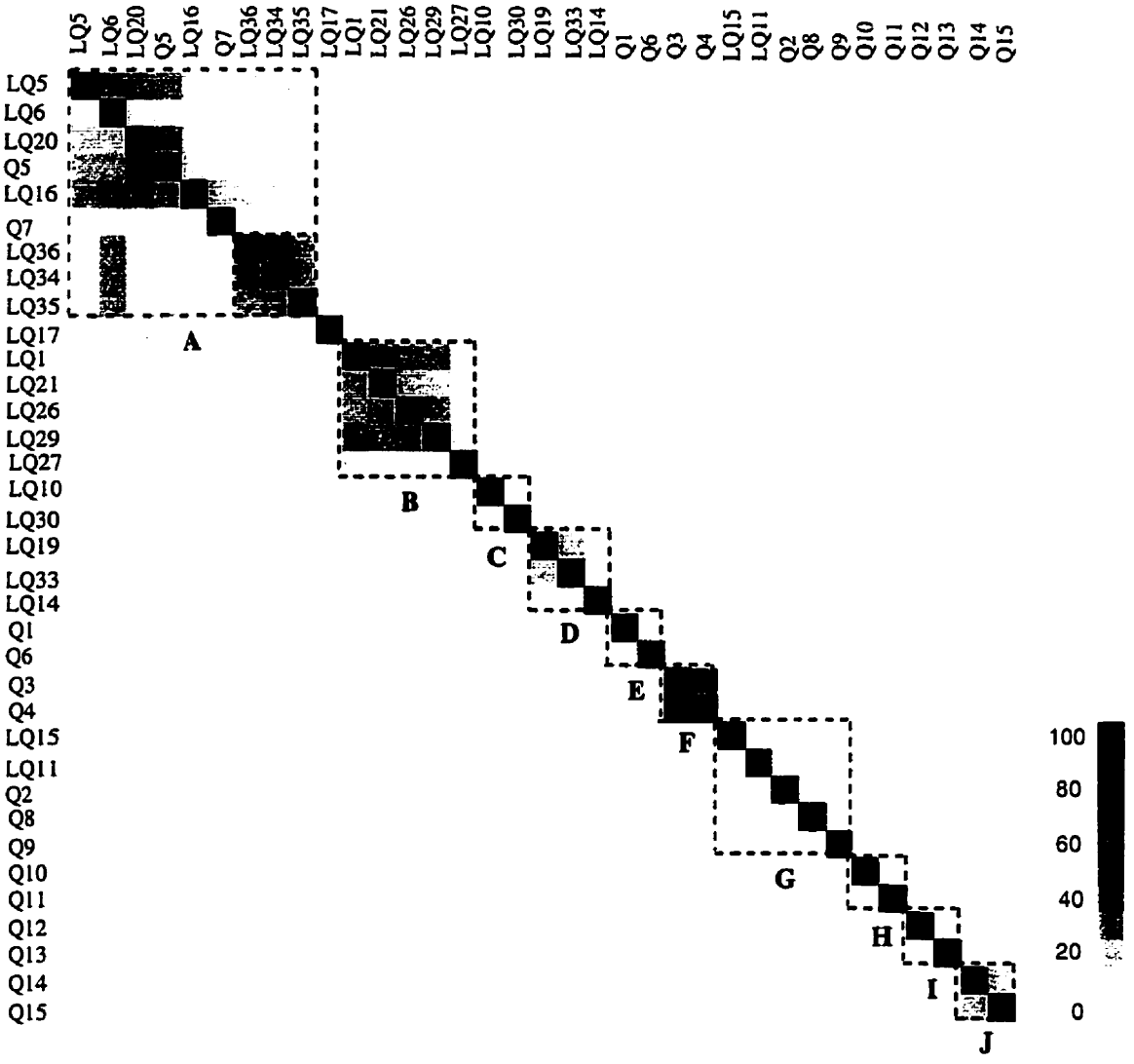
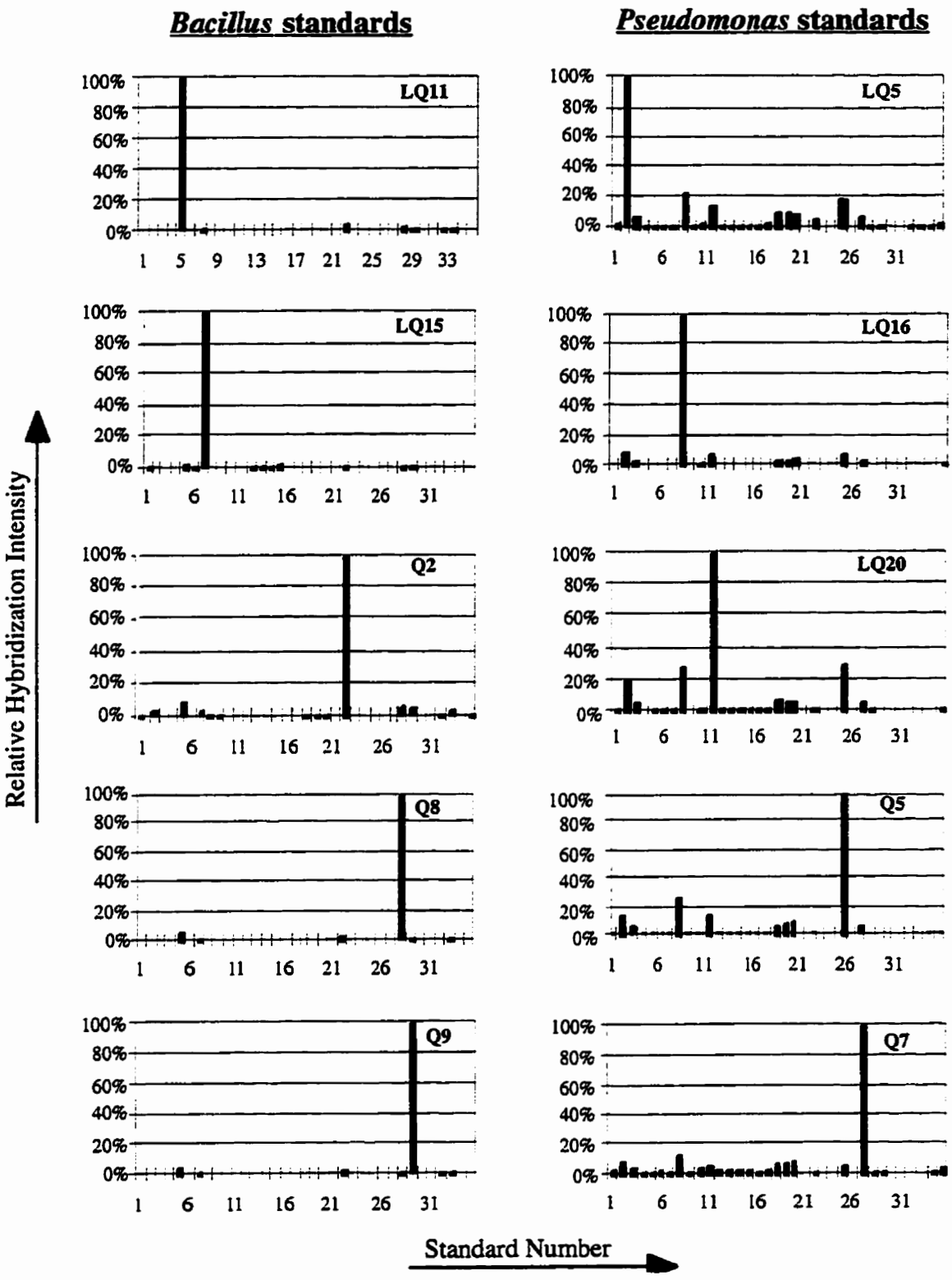


Figure 4-4. Cross-hybridization matrix for the genomes of all 35 bacterial standards. Each square in the matrix represent its percentage of cross-hybridization with the DNA on the diagonal aquare in the column, according to gray scale provided. The order of columns and corresponding rows was changed to bring genomes with similar 16S rRNA phylogeny in close proximity. The squares dash line enclosed represent: (A) *Pseudomonas* spp. (*P. flavescens* spp inside and *P. syringae* spp. outside the smaller square), (B) *Bordetella* spp., (C) *Sphingomonas* spp., (D) various, (E) *Rhodococcus* spp., (F) various (species Q3/Q4 is anomalously high), (G) *Bacillus* spp., (H) *Bacteroides* spp., (I) *Clostridium* spp., and (J) *Desulfovibrio* spp.

Figure 4-5. Hybridization of individual genomic DNAs with the master filter representing the C5+ microbial community. The relative hybridization intensity I_x is plotted vs. standard number (the same order as in Table 4-1). Self-hybridization of each genomic probe was taken as 100%.


Bacillus standards
Pseudomonas standards
LQ11
LQ5
LQ15
LQ16
Q2
LQ20
Q8
Q5
Q9
Q7
100%
80%
60%
40%
20%
0%
1 5 9 13 17 21 25 29 33
1 6 11 16 21 26 31
Relative Hybridization Intensity
Standard Number

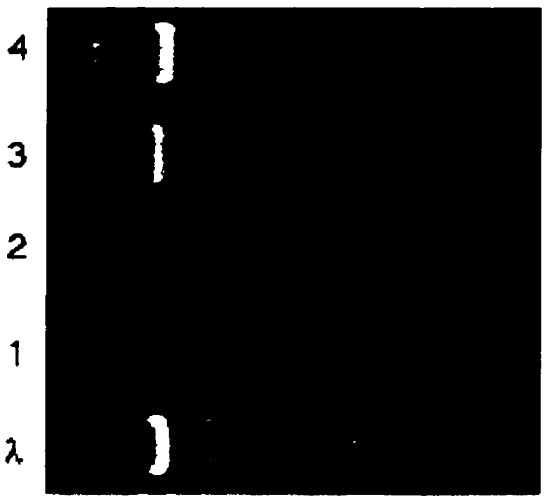


Figure 4-6. Agarose gel electrophoresis of the genomic DNA extracted from soil. Lane 1 is the DNA extracted from soil before incubation. Lanes 2, 3, and 4 are DNAs extracted from 10 g of soil after four weeks of incubation at room temperature with 10 ml MSM in an atmosphere saturated with water (Lane 2), water plus DCPD (Lane 3) and water plus toluene (Lane 4). Lane λ is DNA molecular size markers (bacteriophage λ DNA restricted with *Hind*III, bands from left to right represent fragments of 23.1, 9.4, 6.6, 4.4, 2.3, and 2.0 kb, respectively.

hydrocarbon (lane 2, about 0.6 μg/g soil). Without any incubation, this soil sample gave about 0.05 μg/g soil (lane 1). The DNA extraction efficiency was measured by extracting DNAs from sterilized soil to which a known volume of bacterial culture had been added. The experimental data for duplicate measurements are listed in Table 4-3. On average the DNA extraction efficiency from soil was about 34% for three different bacterial species present in the C5+ degrading community. Identical RSGP patterns were obtained when DNA fractions obtained by subsequent extraction of the soil pellet were used as probes (data not shown). Although the microbial community profile would be affected by differences in DNA extraction efficiency from different standards, it is thought that this error should be the same from experiment to experiment.

4.3.1 Effect of toluene on community composition

Toluene is a readily degradable hydrocarbon compound. The effect of toluene on the community composition was determined by analysis of NE and NW soil samples. The RSGP patterns are shown in Figure 4-7. The community profiles of NE and NW soil samples (A and B in Figure 4-7) were very similar before exposure to toluene. Bacterial standards were broadly distributed with calculated f_χ values of 0.1 to 2.2%. Similar RSGP patterns were obtained for NE and NW soil samples incubated with MSM without a toluene atmosphere (not shown). Significant shifts in both the NE and NW community profiles were observed upon incubation with a saturated toluene atmosphere. Standards identified as homologs of *Pseudomonas* species were enriched to different levels as shown in Figure 4-7 C and D. Standard 27 (Q7) in the NE soil community (Figure 4-7C) and Standard 11 (LQ20) in the NW soil community (Figure 4-7D) were dominant with calculated f_χ values of 9.5 and 39%, respectively. Both of these are homologs to *Pseudomonas syringae* species (Table 4-1). Other *Pseudomonas* species, standards 2, 8,

and 25 (LQ5, LQ16, and Q5) in both soil communities and standard 18, 19, and 20 in the NE community, have been increased in their calculated f_χ values to a lesser degree. These increases are caused in part by cross-hybridization. This can be seen by comparison with the patterns in Figure 4-5.

4.3.2 Effect of dicyclopentadiene on community composition

The effect of DCPD on the community composition was determined by the same procedure for both NE and NW soil samples. Upon incubation in a DCPD atmosphere, significant shifts of the RSGP patterns were observed in both soil communities. These were distinctly different from those observed for toluene incubation. Standard 16 (LQ30, with highest RDP homology to *Sphingomonas yanoikuyae*) was enriched in both soil communities after incubation for 4 weeks as shown in Figure 4-8 C and D. The calculated f_χ values for standard 16 were around 6 to 8%. Further liquid enrichment of the supernatant obtained from the NW soil-MSM culture without soil led to a significantly different RSGP pattern, shown in Fig 4-9 B, in which standard 25 (Q5) was dominant. Separation of bacteria from this enrichment culture by serial dilution and plating gave a colony purified strain, which was very similar to standard 25 shown in Figure 4-9 C. Standard 25 (Q5) has the highest homology to *Pseudomonas syringae* in the RDP database. Thus, two bacterial standards, LQ30 and Q5, could be enriched by exposure to a DCPD atmosphere. Their DCPD degrading potential should be further tested.

4.4 Toluene degradation by *Pseudomonas* species

Standard 11 and four other standards with *P. syringae* homologs were abundant in soil incubated with a toluene atmosphere (Figure 4-7C). In order to confirm whether these

species are toluene degraders, their degradation activity has been investigated by measuring the production of [^{14}C]-CO_2 from uniformly labeled [^{14}C]-toluene.

4.4.1 Mineralization of [^{14}C]-toluene

The experiments for mineralization of [^{14}C]-toluene by the five *P. syringae* homologs were conducted in duplicate. A saturated bacterial culture in TY medium for each strain was inoculated into MSM containing sterilized soil and [^{14}C]-toluene. A NW soil sample was similarly incubated with [^{14}C]-toluene in MSM. The experimental data for mineralization of [^{14}C]-toluene after 4 weeks of incubation are listed in Table 4-4. Percentages of mineralization M (%) were calculated according to

$$M = \frac{\text{Counts}_{\text{recovered } [^{14}\text{C}]\text{-}CO_2} - \text{Counts}_{\text{no inoculum control}}}{\text{Counts}_{[^{14}\text{C}]\text{-toluene added to each sample}}} \times 100\% \qquad (3)$$

Average mineralization (M_r) relative to the NW soil community are also listed in Table 4.4 and plotted for the five *P. syringae* homologs in Figure 4-7 E. For individual standards, the highest M_r values of [^{14}C]-toluene were achieved by strains LQ20 (standard 11) and Q7 (standard 27), which were approximately 80% and 62% of the entire NW soil community. The lowest average M_r values were observed in strains LQ5 (standard 2) and LQ16 (standard 8), which were only 6% and 7% relative to the NW soil community.

4.5 DCPD degradation activity of species Q5 and LQ30

Although species Q5 and LQ30 were enriched by incubation in a DCPD atmosphere, their ability to oxidize or mineralize DCPD remains to be proven. The degradation activity of both strains individually and in combination have been investigated by measuring the end product (CO_2) and oxidized derivatives of DCPD both with and without soil.

Table 4-3. The efficiency of DNA extraction from cultures and soil mixtures

	Q5[a]	LQ15[a]	LQ30[a]	Average[b]
1ml culture (1)	5.6 μg	2.9 μg	2.8 μg	3.8 μg
1ml culture (2)	4.2 μg	4.4 μg	3.2 μg	3.9 μg
1ml culture + 1 g soil (1)	2.4 μg	1.0 μg	0.6 μg	1.3 μg
1ml culture + 1 g soil (2)	2.0 μg	1.2 μg	0.8 μg	1.3 μg
Extraction efficiency (av.)[c]	45.1%	30.2%	22.6%	**34.5%** [d]

[a]Culture of bacterial strain used for DNA extraction.

[b]Averaged value of DNAs extracted from the cultures of three different bacterial strains.

[c]Averaged extraction efficiency of DNA from individual bacterial culture and soil mixture.

[d]Averaged value of DNA extraction efficiency from three different bacterial culutres.

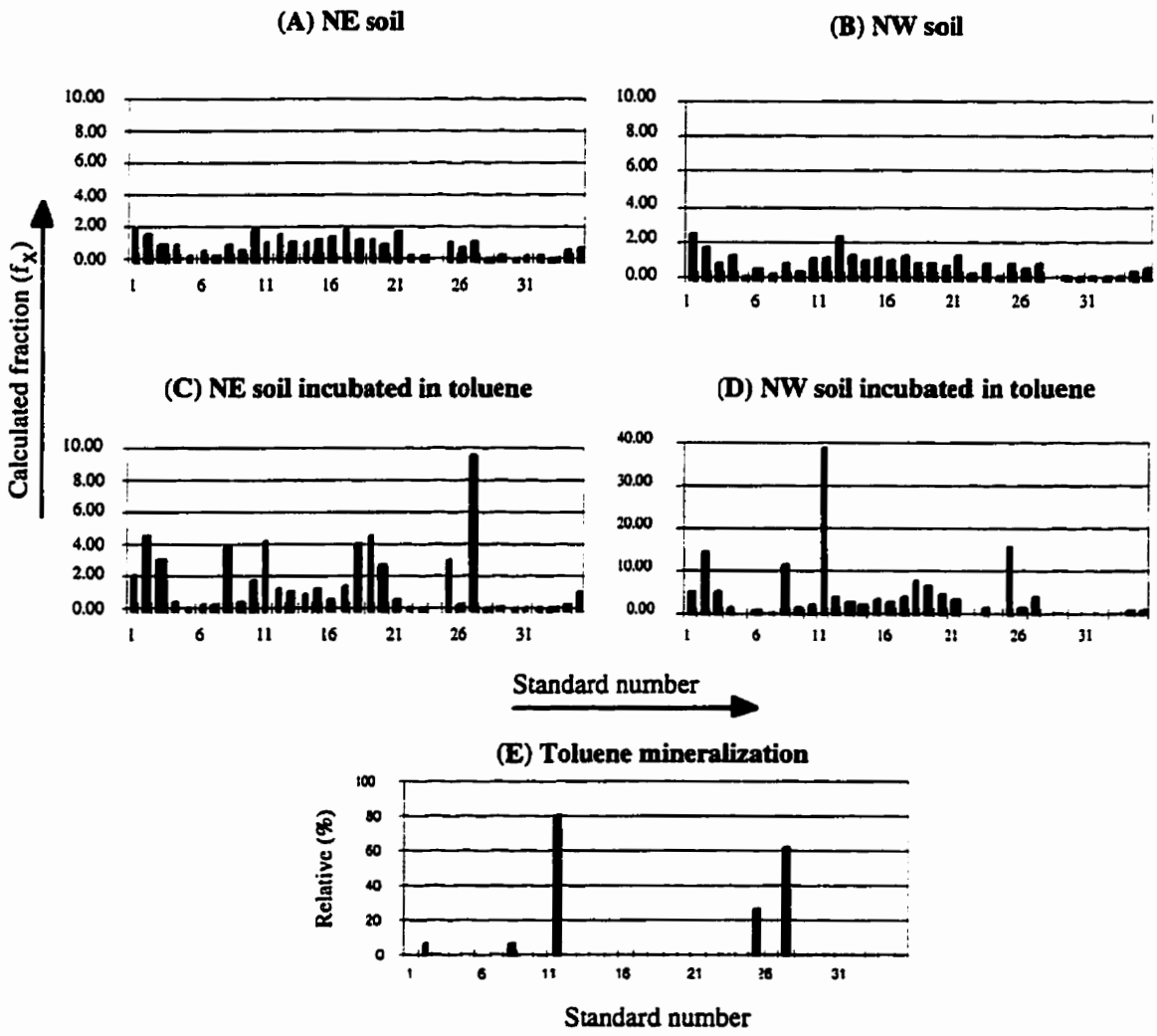


Figure 4-7. RSGP patterns of the C5+ community DNAs from NE and NW soil samples. (A) and (B) are before incubation. (C) and (D) are NE and NW soil samples incubated with MSM in a saturated toluene vapor. The calculated fraction of each species (f_x in %) is plotted vs. standard number. (E) Percentage of toluene mineralization by individual species relative to mineralization by whole NW soil community. Data are plotted for five *Pseudomonas syringae* species (standards 2, 8, 11, 25, and 27).

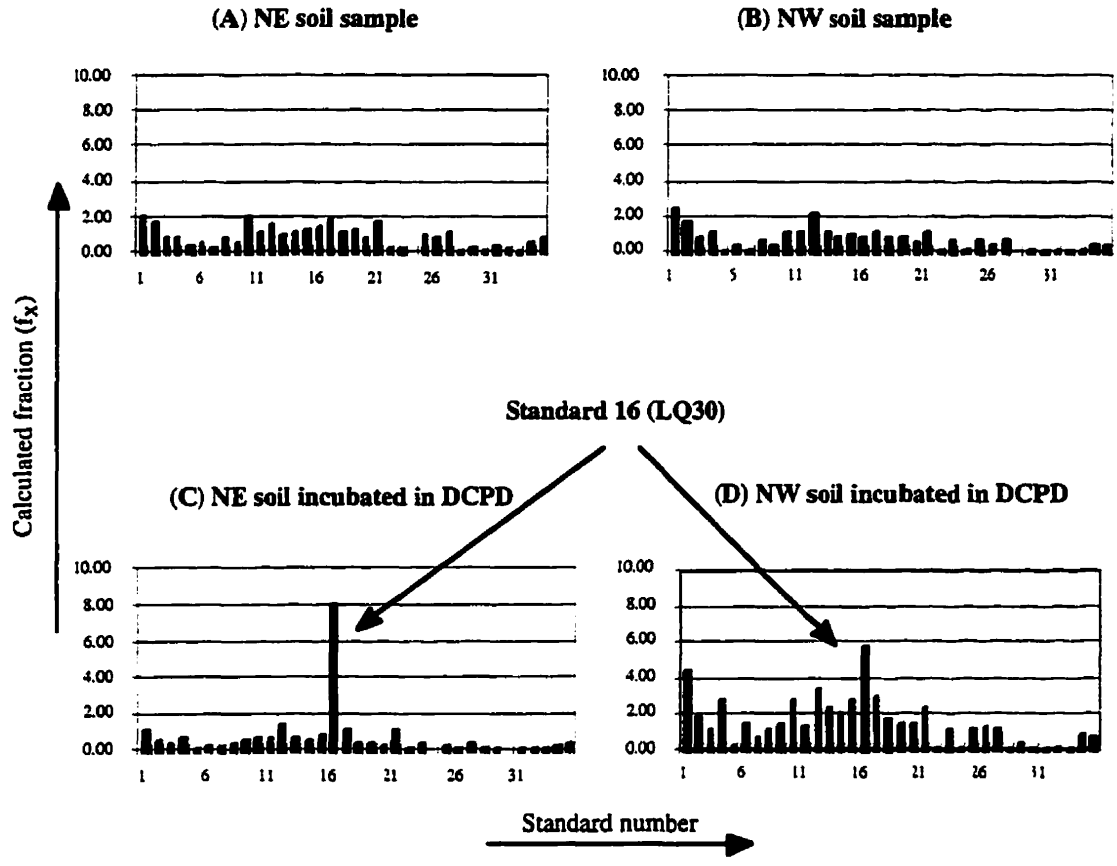


Figure 4-8. RSGP patterns of the C5+ degrading community DNAs from NE and NW soil samples. (A) and (B) are soil samples prior to incubation. (C) and (D) are NE and NW soil samples incubated with MSM in a saturated DCPD atmosphere. The calculated fraction of each species (f_x in %) in the community is plotted vs. standard number. Standard 16 (LQ30) was enhanced in both NE and NW soil samples incubated with a DCPD atmosphere.

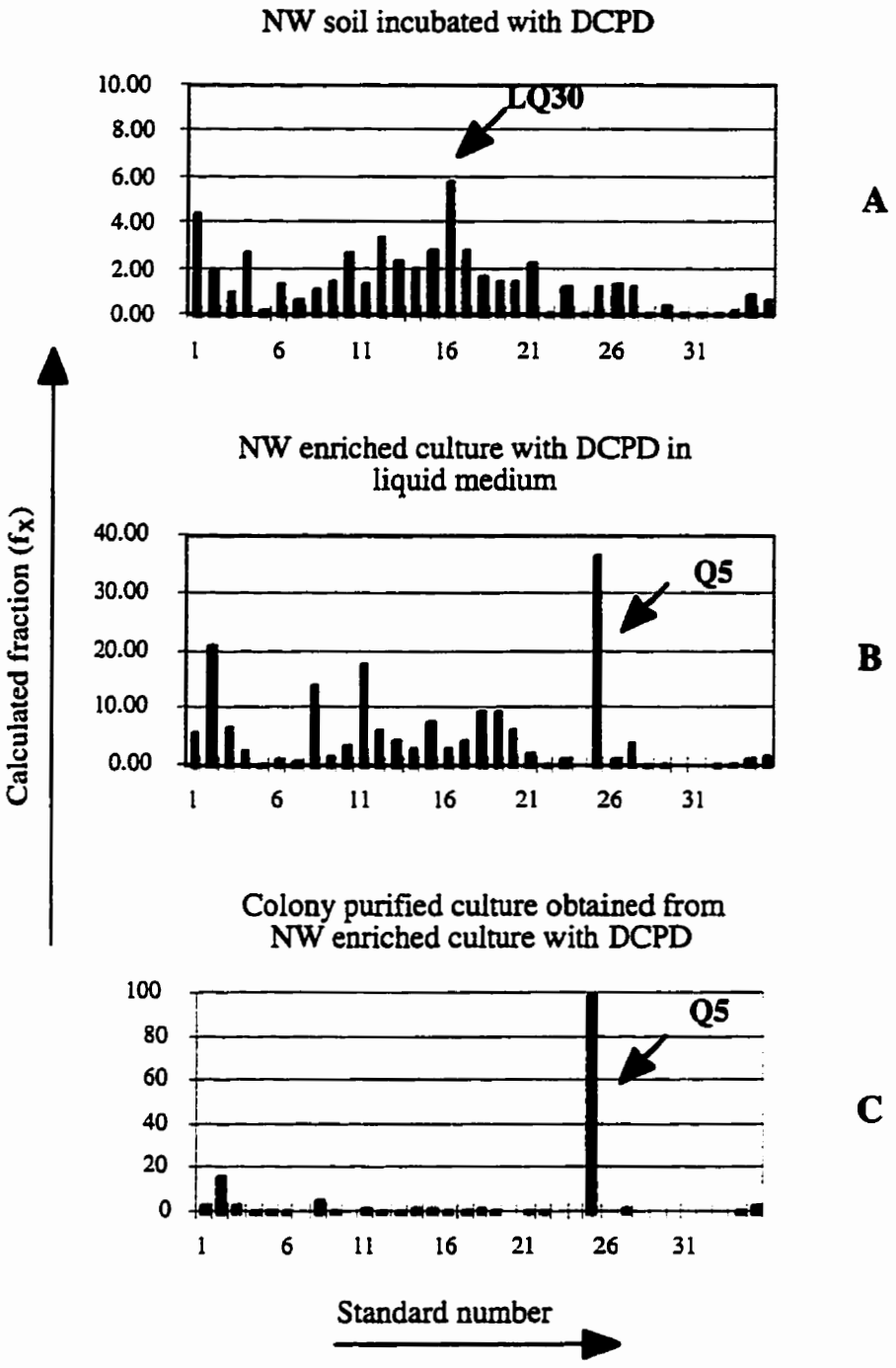


Figure 4-9. RSGP patterns of (A) NW soil and MSM incubated with DCPD and (B) NW enrichment culture incubated with DCPD in MSM. (C) A colony obtained by plating the enrichment culture, serially diluted until a single colony morphology was observed. The calculated fraction for each species (f_x in %) in the community is plotted vs. standard number in (A) and (B). (C) is relative to standard 25 (100%).

Table 4-4. Mineralization of [^{14}C]-toluene by *P. syringae* strains

Inoculum	M_1 (%)[a]	M_2 (%)[a]	M (%)[b]	M_r (%)[c]
LQ5	0.33	0.37	0.35	6.5
LQ16	0.30	0.46	0.38	7.1
LQ20	4.2	4.44	4.31	80.4
Q5	1.6	1.32	1.46	27.2
Q7	3.2	3.45	3.32	61.8
NW soil	5.7	5.07	5.36	100

[a]Percentage rate of [^{14}C]-CO_2 recovery from duplicate measurements.

[b]Average mineralization.

[c]Mineralization relative to that by the NW soil community.

Table 4-5. [^{14}C]-DCPD mineralization by strains Q5 and LQ30

Inoculum	M_1 (%)[a]	M_2 (%)[b]
Q5	0.21	0.15
LQ30	0.09	0.11
Q5 + LQ30	0.19	0.14
NE soil	-	0.12

[a]Percentage of [^{14}C]-CO_2 recovery from samples incubated with sterilized soil.

[c]Percentage of [^{14}C]-CO_2 recovery from samples without soil. Average of duplicate measurements, except for NE soil.

4.5.1 Mineralization of [^{14}C]-DCPD

Mineralization of [^{14}C]-DCPD by strains Q5, LQ30 and a Q5 plus LQ30 combination is summarized in Table 4-5. After 4 weeks of incubation with uniformly labeled [^{14}C]-DCPD in MSM, both with and without sterilized soil, the net amounts of [^{14}C]-CO_2 collected in the alkali traps were 0.1 to 0.2% of added [^{14}C]-DCPD for all inocula. NE soil sample itself gave a similarly low result, with 0.12% recovered as [^{14}C]-CO_2.

4.5.2 Growth in DCPD vapor

The growth of strains Q5 and LQ30 and their combination in MSM in the presence of a saturated DCPD atmosphere were determined by measuring the optical density of each culture at 600 nm. As shown in Figure 4-10, all three inocula grew during the first 14 days, but the cell density gradually decreased after that, and decreased to near the initial level over 56 days of incubation. This result suggested that strains Q5 and LQ30 were able to grow in the presence of a DCPD atmosphere for a limited time.

The growth of bacterial strains Q5 and LQ30 incubated with sterilized soil was not determined in this study. However, to confirm that no extra bacteria were introduced in the incubations with sterilized soil, total DNAs were extracted from these incubated soil-MSM mixtures after 7 weeks of incubation. The DNAs extracted were labeled and probed against the master filter of the C5+ degrading community. The RSGP patterns (Figure 4-11) of these extracted DNAs indicated that only the introduced bacteria were present in these incubations.

4.5.3 Accumulation of oxidized DCPD derivatives

Oxidized derivatives of DCPD were extracted with ethyl acetate from the supernatants of incubated bacterial cultures after centrifugation to remove bacterial cells. The concentrated extracts were analyzed by GC and GC/MS. The peaks on gas chromatograms were analyzed by comparing their mass spectra with those of oxidized

DCPD derivatives published before. Accumulation of oxidized DCPD derivatives was observed in the incubations of strain Q5, LQ30 and Q5 plus LQ30 in 20 ml MSM with 10 g sterilized soil in a DCPD atmosphere for 7 weeks. GC patterns for all of the extracts derived from these incubations were very similar. The mass spectra of peaks observed in GC/MS analysis of the ethyl acetate extracts were not completely identical to those published for oxidized DCPD derivatives, although all contained a fragment of m/z 66. This fragment corresponds to the monomer cyclopentadiene, and is present in all of the published mass spectra of oxidized DCPD compounds. GC peaks with m/z 66 in their mass spectra are therefore likely oxidized DCPD derivatives. The amounts of oxidized DCPD derivatives extracted from the soil-MSM incubations are listed in Table 4-6. These were estimated by comparing the major peaks (with m/z 66 in their mass spectra) with the known amount of the internal standard *p*-dichlorobenzene. More oxidized DCPD derivatives were obtained from the incubation of strain Q5 than from the incubation of strain LQ30 and from the combination of both strains. Oxidized DCPD derivatives were also extracted from bacterial cultures incubated in MSM for only two weeks (Table 4-7). The GC patterns for incubation without inoculum and for the three incubations with inocula were very similar. Figure 4-12a is the GC pattern obtained for the incubation with strain Q5 in the presence of a saturated DCPD atmosphere for two weeks. The mass spectra of the peaks indicated in Figure 4-12a are shown in Figure 4-12b. These are nearly identical with published MS spectra of DCPD and oxidized DCPD derivatives. Compounds C, D, and E are also shown in Figure 2-2. The quantitative amount of each oxidized derivative were estimated by comparison with the internal standard and are listed in Table 4-7.

The amounts of oxidized derivatives obtained from the incubations with inocula were higher than the amount obtained from incubation without inoculum, except for derivatives III (compound E in Figure 2-2) in the incubation with strain LQ30. Double the amount of oxidized derivatives (137 μg) was extracted from incubation with strain

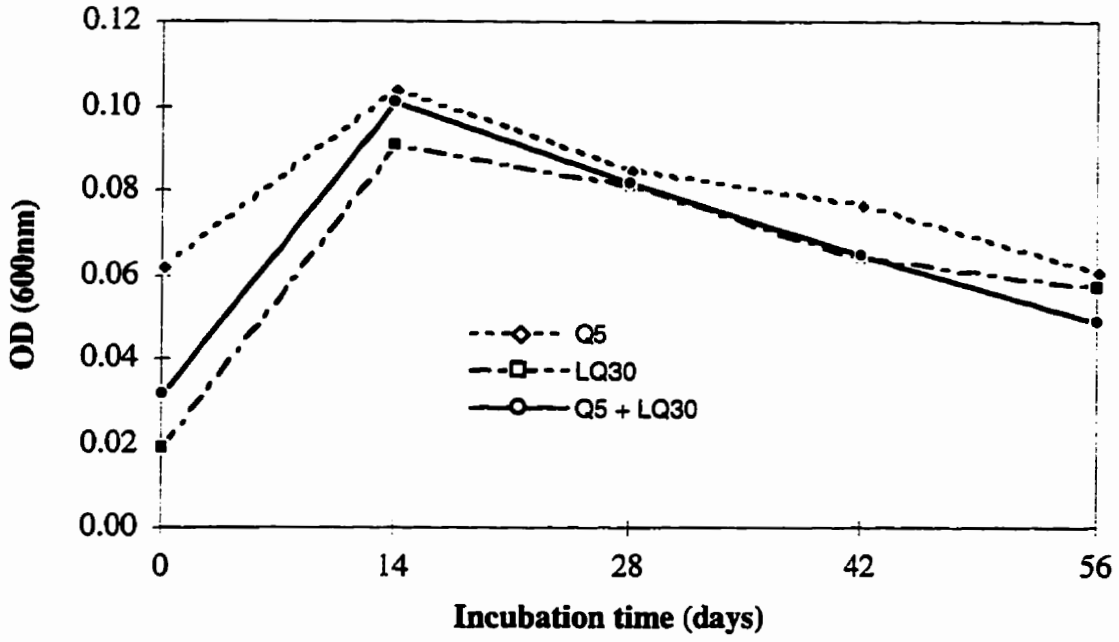
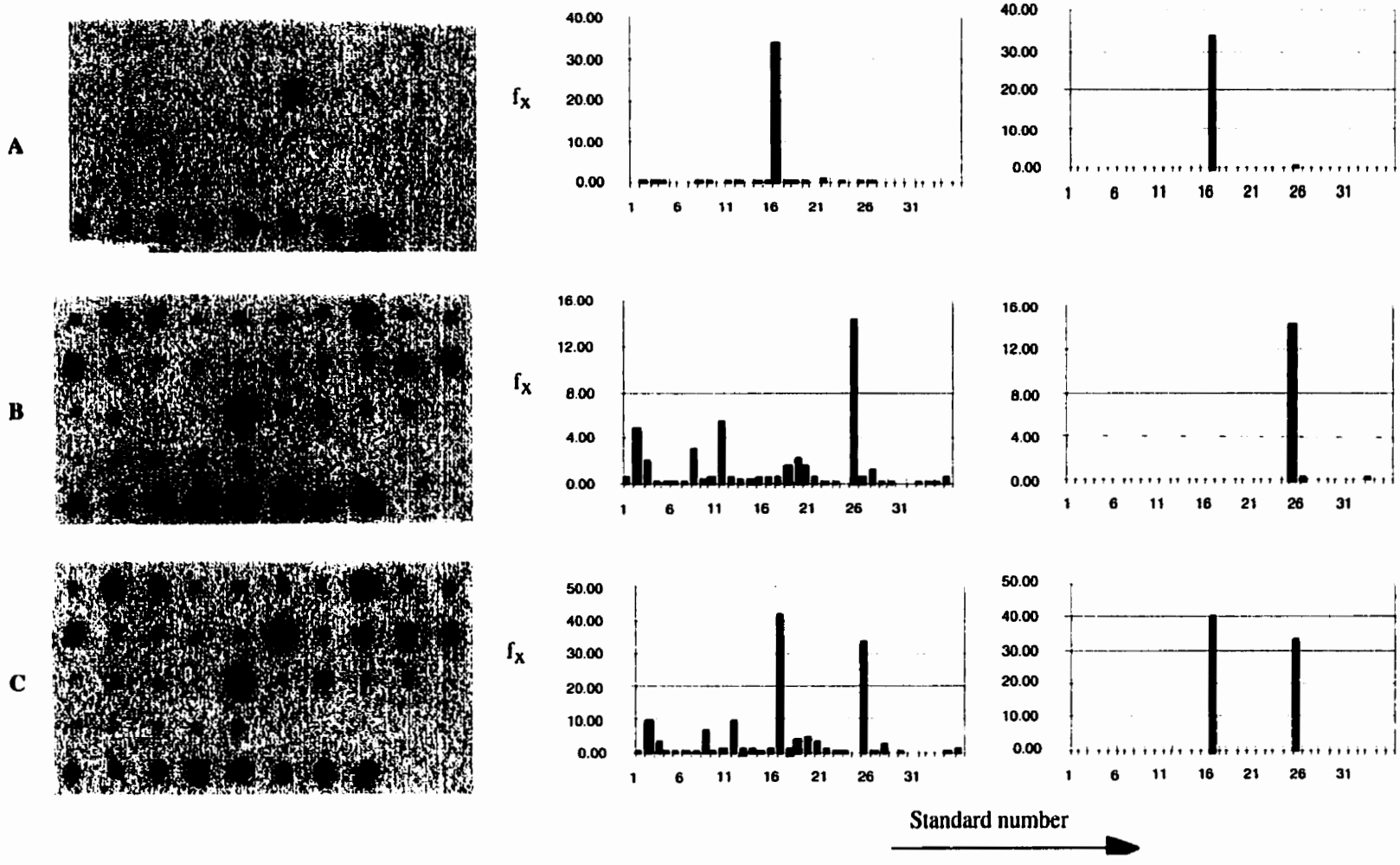


Figure 4-10. Growth of strains Q5, LQ30, and Q5 plus LQ30 in MSM incubated in a DCPD atmosphere in a single dessicator. The cell densities were determined by measuring absorbance at 600 nm, and are plotted vs. incubation time in days.

Figure 4-11. RSGP patterns of DNAs extracted from sterilized soil incubated with (A) LQ30, (B) Q5, and (C) LQ30 and Q5. The hybridization patterns are shown on the left, the derived community profiles (calculated f_χ vs. standard number) in the middle, and the community profiles corrected for cross-hybridization on the right.


A
B
C
f_x
40.00
30.00
20.00
10.00
0.00
16.00
12.00
8.00
4.00
50.00
1 6 11 16 21 26 31
Standard number

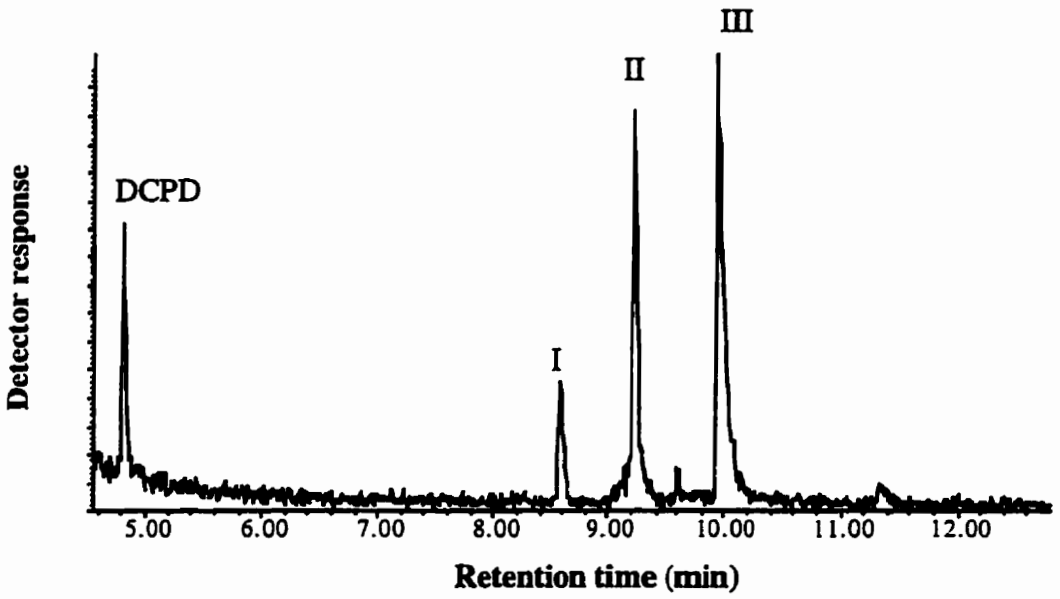


Figure 4-12a. Gas chromatogram of the ethyl acetate extracts obtained from incubation of Q5 in MSM with a saturated DCPD atmosphere for 2 weeks in the absence of soil. The mass spectra of peaks I, II, and III are shown in Figure 4-12b.

Figure 4-12b. Mass spectra of dicyclopentadiene (DCPD) and its oxidized derivatives. Oxidized DCPD derivatives I, II, and III were observed in GC/MS analysis of the ethyl acetate extracts obtained from the incubations of strain Q5, LQ30, and Q5 plus LQ30 in MSM with a saturated DCPD atmosphere.

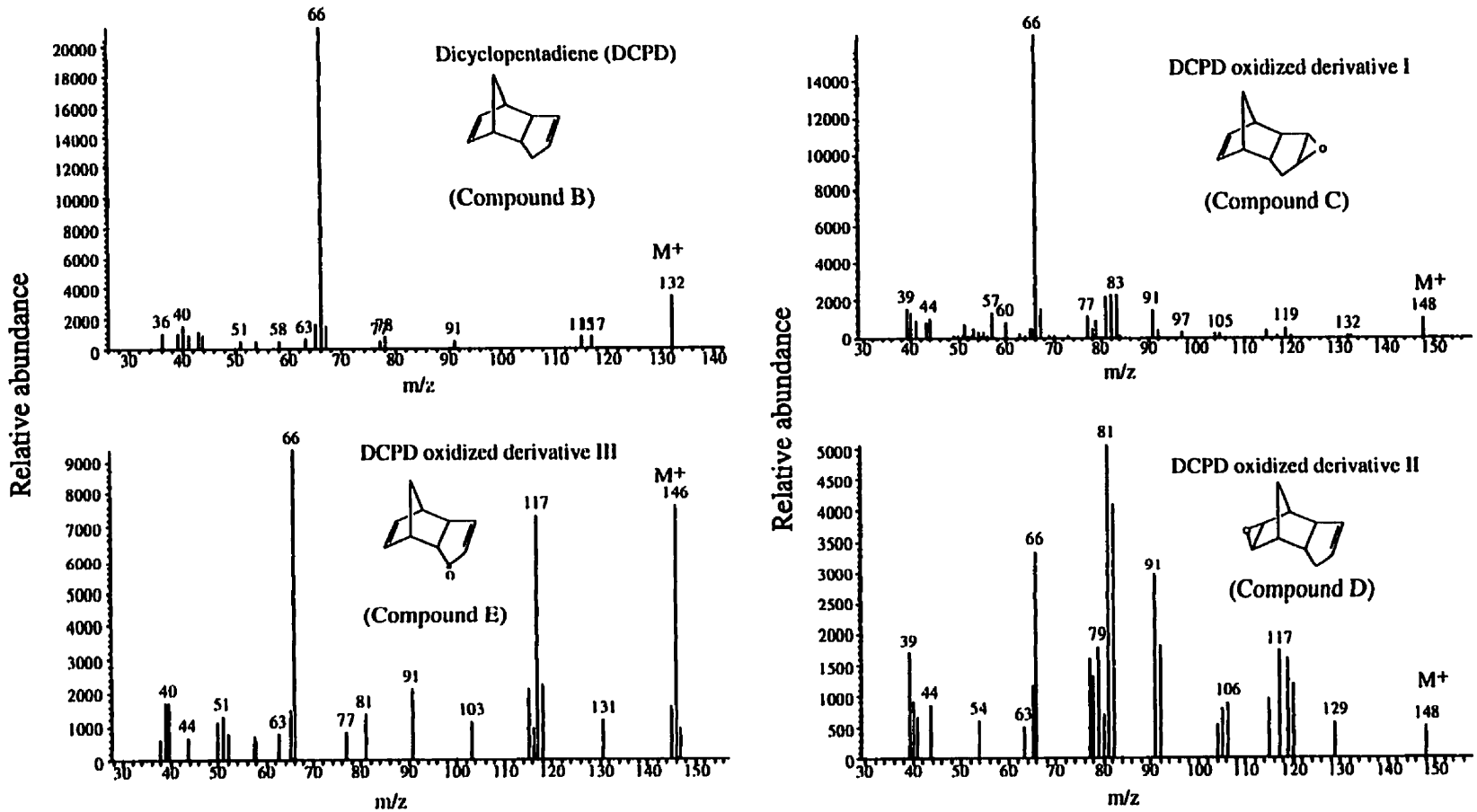

Relative abundance
Dicyclopentadiene (DCPD)
(Compound B)
M+
132
66
m/z
DCPD oxidized derivative I
(Compound C)
M+
148
66
m/z
DCPD oxidized derivative III
(Compound E)
M+
146
117
66
m/z
Relative abundance
DCPD oxidized derivative II
(Compound D)
M+
148
81
66
91
117
m/z

Table 4-6. Oxidized DCPD derivatives extracted from soil-MSM incubations

Soil	Inocula[a]	Oxidized derivatives (μg)[b]
sterilized soil	none	37
sterilized soil	Q5	103
sterilized soil	LQ30	58
sterilized soil	Q5 + LQ30	88
NW soil	none	162

[a]Saturated bacterial cultures of strain Q5 (10 μl), LQ30 (20 μl), and Q5 plus LQ30 (10 and 20 μl) were introduced in the incubations.

[b]major peak areas of suspected oxidized DCPD derivatives were calculated and converted into μg through comparison with the internal standard.

Table 4-7. Oxidized derivatives extracted after incubation with DCPD for 2 weeks

Inoculum[a]	Derivative I (μg)[b]	Derivative II (μg)[b]	Derivative III (μg)[b]	Total amount (μg)[c]
None	8.7	12.1	47.4	68.3
Q5	24.1	33.3	79.8	137.3
LQ30	13.0	18.5	33.5	65.0
Q5 + LQ30	15.1	23.3	59.8	98.2

[a]Saturated bacterial cultures of strain Q5 (100 μl), LQ30 (100 μl) and Q5 plus LQ30 (50 μl each) were inoculated into 10 ml MSM and incubated in a dessicator with a saturated DCPD atmosphere.

[b]Amounts of oxidized DCPD derivatives I, II, and III extracted from the incubations, as estimated by comparison with the internal standard.

[c]Total amounts of oxidized DCPD derivatives extracted from in the incubations with each inoculum.

Q5 as compared with incubation without inoculum (68 μg). The total amounts of oxidized derivatives extracted from the incubation with strain LQ30 (65 μg) were nearly the same as those extracted from the non-inoculated control (68 μg).

4.6 Discussion

Bioremediation of hydrocarbons in soil leads to the partial or complete destruction of these compounds, and is catalyzed by diverse microorganisms present in the contaminated site. The attacked hydrocarbons are used as a source of carbon, and/or energy for the growth of the microorganisms. Isolating and characterizing bacterial isolates obtained from soil samples is a preliminary step in the study of microbial communities involved in the bioremediation of hydrocarbons. In this study, a soil microbial community involved in C5+ hydrocarbon degradation has been characterized using the RSGP technique. A general strategy of this study was (i) isolation of an extensive set of bacteria on rich medium plates, (ii) development of a master filter with genomes having limited cross-hybridization, (iii) identification of possible hydrocarbon degraders based on their responses to the introduced hydrocarbon compounds, and (iv) characterization of degradation activity of the possible hydrocarbon degraders by mineralization studies and GC-MS analysis of oxidized derivatives. Although no definite DCPD degrader was identified, two toluene degraders (LQ20, and Q7 in Figure 4-7 C and D) have been identified and confirmed following this approach.

A master filter generated for studying the C5+ degrading microbial community contains a total of 35 bacterial isolates; these were derived by elimination of isolates with similar genomes (high level of cross-hybridization). Most of the resulting standards present on the master filter had a low level (< 20%) of cross-hybridization with other standards (Figure 4-4), but some standards, representing species of the same genus, had relatively high cross-hybridization (up to 30%), e.g., *Pseudomonas* sp. and *Bordetella* sp. shown in

the squares (A) and (B) enclosed by the dashed lines in Figure 4-4. The data shown in Figure 4-4 can be used for correction of cross-hybridization in analysis of RSGP patterns hybridized by mixed genomic DNA probes, if the components of genome mixture are known. Figure 4-11 shows an example of this correction by subtraction of the percentage of cross-hybridization from I_χ values before calculation of f_χ values according to equation 2. It is clear that the correction is useful to more precisely calculate f_χ values, by comparing the derived bar diagrams without correction (in the middle) and with correction (on the right) shown in Figure 4-11. But it has not been applied to analyze RSGP patterns obtained from environmental samples, because not all genomes of the environmental sample are represented on the filter.

Identification of standards was completed by comparing partial sequences of their 16S rRNA genes with those in the RDP database (Table 4-1). Since the number of sequences in the RDP database is limited, bacterial standards could only be identified based on the highest similarity coefficient between query and matching sequences, which varied from 0.337 to 0.972 (Table 4-1). The identification of standards with lower similarity coefficients (0.3 to 0.5) may be incorrect because a properly matching sequence is absent from the database.

The sequences obtained for 33 standards can be further analyzed by comparison of their similarities through the pairwise alignment to produce a tree of clusters, which indicate which sequences are most related. Figure 4-2 shows the final alignment. Although five *Bacillus* sp. present on the master filter had very low similarities in their genomic DNAs (Figure 4-5), their 16S rRNA genes are very conserved as shown by the closely clustered 16S rRNA gene sequences in Figure 4-2. Seven of the eight *Pseudomonas* sp. homologs with similarity coefficients 0.74 to 0.97 clustered together in Figure 4-2. Strain LQ5, which was identified as a homolog of *Pseudomonas syringae* with a very low similarity coefficient (0.375), did not group in this cluster, but its genomic DNA shares 20%

identity (Figure 4-5) compared with that of LQ20 (standard 11), a *Pseudomonas syringae* homolog. A lower similarity coefficient obtained for strain LQ5 might be the result from poor sequencing. The two *Rhodococcus* sp., the two *Sphingomonas* sp. and the four *Bordetella* sp. also clustered together in Figure 4-2. Strain LQ10 (standard 4) was identified as a *Sphingomonas* sp. RDP homolog with a low similarity coefficient (0.598). However, its 16S rRNA gene was related to that of another *Sphingomonas* sp. homolog, strain LQ30, with a similarity coefficient of 0.935. Therefore, whether the initial identifications are correct can be confirmed by alignment of all 16S rRNA sequences. For example, the 16S rRNA genes of strains Q12 (standard 32) and LQ17 (standard 9) were clustered together in Figure 4-2, and their identifications were *Clostridium* sp. for Q12 and *Agrobacterium* sp. for LQ17. It seems impossible that the 16S rRNA gene of a Gram-positive bacterium (*Clostridium* sp.) is closely related to that of a Gram-negative bacterium (*Agrobacterium* sp.). Since the similarity coefficients for both identifications were quite high (0.846 and 0.9310), the problem is not due to errors in sequencing. Possible the database sequences are in error. It may be necessary to submit these sequence data to another rRNA gene database for identification.

DNAs extracted from soil samples were labeled and hybridized with the master filter. The average DNA extraction efficiency found in this study was 34.5%. Thus more than half of the chromosomal DNA of bacteria present in the soil samples was not extracted. DNA extraction efficiency is highly dependent on cell lysis efficiency of indigenous bacteria present in soil samples, and the cell lysis efficiency is mainly determined by soil characteristics and bacterial community composition (Zhou et al., 1996). For example, a low DNA extraction efficiency is usually observed for the Gram-positive bacteria in the soil community due to a low cell lysis efficiency. Although the RSGP pattern may not be affected significantly by overall variation in DNA extraction, the f_x values will be affected by different cell lysis efficiencies. Complete disruption of cellular structure and release of all DNA is the goal of DNA extraction from soil samples. Therefore,

incubation with lysozyme and SDS plus three freeze and thaw cycles were applied in the DNA extraction procedure to enhance the efficiency of cell lysis. The amounts of DNA extracted from soil samples to which bacteria were added were used as an indicator of extraction efficiency (Table 4-3). The accuracy of this indicator is still doubtful because many factors will affect DNA recovery from incubated soil samples. Hydrocarbon vapor (e.g., DCPD) may affect the cell wall structures of bacteria incubated, resulting in easier DNA release from cells than from the bacteria incubated without hydrocarbons.

Most of the standards present on the C5+ degrading soil community master filter were obtained by plating and culturing on rich medium. Only three were isolated in mineral salts media with a hydrocarbon (benzene, naphthalene, or styrene) as sole carbon and energy source (Table 4-1). Toluene was not used as a carbon and energy source in these isolations, but species LQ20 and Q7 (Figure 4-7 D) were enriched to 40% and 10% of total community DNAs extracted from incubated soil samples, which were exposed to toluene in the presence of MSM for 4 weeks. Both species LQ20 and Q7 were identified as homologs of *Pseudomonas syringae* The degradation of toluene by *Pseudomonas* spp. is well known. *Pseudomonas* species often carry one or more large catabolic plasmids (Sayler et al., 1990b). The toluene degradation activity of species LQ20 and Q7 was confirmed by their capacity to mineralize [^{14}C]-toluene to [^{14}C]-CO_2. As shown in Figure 4-7 E, species LQ20 and Q7 could mineralize [^{14}C]-toluene up to 80% and 60% of the levels achieved by the entire NW soil community. Other species (LQ5, LQ16, and Q5) identified as homologs of *Pseudomonas syringae* were also enriched to certain degrees after exposure to toluene. Species Q5 had 27% of relative mineralization and species LQ5 and LQ16 had 6 to 7% of the relative mineralization (Table 4-4). The five *Pseudomonas syringae* standards had higher levels of cross-hybridization (Figure 4-5). The f_χ values shown in the RSGP patterns for LQ5 and LQ16 (Figure 4-7 C and D) may have resulted in part from cross-hybridization, for which no correction was made.

Soil microbial communities probably are the most complicated natural communities, and there may be as many as 4,000 species per gram of soil as estimated by Torsvik et al. (1990). The members of the community used in this study are limited to 35 bacterial standards obtained by selective enrichment. They may only cover a small fraction of the natural community and may not be representative. However, with this simplified representation we were still able to record community shifts in response to hydrocarbon compound exposure. Yet it is likely that some bacteria present in the incubated soil samples may not have been included in the C5+ degrading master filter. From the mineralization of [^{14}C]-toluene (Table 4-4), we can see that more [^{14}C]-CO_2 was collected from the NW soil sample than from any of the best standards LQ20 or Q7. This suggests that the biodegradation of toluene by the natural microbial community is more efficient than that of single species, although the difference is not huge.

The amount of DNA extracted from a soil sample incubated with a DCPD atmosphere is about 2.5 times higher than that extracted from soil without DCPD incubation (Figure 4-6). This suggested growth of the bacteria present in the soil sample during incubation with a saturated DCPD atmosphere. The same approach used for identification of toluene degraders was applied to characterize degraders of the extremely recalcitrant hydrocarbon DCPD. The interpretation of the results obtained is more complicated. Although species LQ30 was enriched for in both the NE and NW soil sample after incubation with a DCPD atmosphere for 4 weeks (Figure 4-8 C and D), it only represented 6 to 8% of the total community DNA extracted. During liquid culture enrichment of the NW soil-MSM culture, species LQ30 was not enriched for. It was doubtful, therefore, if species LQ30 was able to utilize DCPD for growth. There are growth factors in the soil samples, such as organic carbon, nitrogen and phosphorus. The ratio of added organic C:N:P in the DCPD contaminated site was 13:11:1 (Stehmeier, 1997). Species LQ30 may use carbon sources present in the soil sample, that become available when sterilized soil is added. Species Q5 did not become enriched in the incubation of soil samples with a DCPD

atmosphere, but it was greatly enriched in the subsequent liquid enrichment of a NW soil culture in MSM with a DCPD atmosphere, representing a calculated 38% of the extracted total community DNA (Figure 4-9 B). However, the mineralization of [^{14}C]-DCPD by both species LQ30 and Q5 was disappointing, only 0.1 to 0.2% of [^{14}C]-CO_2 was recovered from [^{14}C]-DCPD (Table 4-5). A similar result was obtained for the incubation of a NE soil sample (Table 4-5). These experimental data indicated that species LQ30, Q5, and the entire NE soil community are unable to utilize DCPD efficiently as sole carbon and energy source for their growth under the incubation conditions.

Some of the *Pseudomonas* species are able to metabolize multiple aromatic hydrocarbons for growth, because these strains contain a nonspecific toluene dioxygenase or monooxygenase with a broad substrate range for initial ring activation (Haigler et al., 1992). *Sphingomonas* species are known as an unique class of aromatic hydrocarbon degraders that are capable of growth on both monocyclic and polycyclic aromatic hydrocarbons, e.g., *Sphingomonas yanoikuyae* B1 and *Sphingomonas paucimobilis* (Kim et al., 1996; Fredrickson et al., 1995). Partial degradation of recalcitrant hydrocarbon compounds frequently occurs in natural microbial communities. It may be possible that partial degradation of DCPD is carried out by aromatic hydrocarbon degraders with a broad substrate range. Since Q5 and LQ30 were capable of some growth in the first 20 days of incubation (Figure 4-10), they may be able to metabolize DCPD to a certain degree, despite the failure to demonstrate mineralization. Evidence for conversion of DCPD to oxidized derivatives by a variety of microbial consortia has been obtained by Stehmeier et al. (1996), although van Breemen et al (1993) suggested that oxidized derivatives are formed by non-microbial, especially photochemical mechanisms. The oxidized DCPD derivatives extracted from incubated cultures were analyzed by capillary GC and GC/MS. It was thought at first that soil particles may be required by the bacteria as a matrix for growth and DCPD oxidation. Saturated cultures of bacterial strains in TY medium (10 to 20 ml) were, therefore, inoculated into a mixture of 10 ml MSM and 5 g

sterilized soil in a DCPD atmosphere, because growth of both Q5 and LQ30 was very slow in MSM alone. Oxidized DCPD derivatives were extracted from these incubated mixtures and analyzed by GC/MS. The problem in assigning the peaks of possible oxidized DCPD derivatives shown in GC chromatograms by their mass spectra is that closely matched reference mass spectra could not always be found. Also the introduction of sterilized soil may have given rise to organic materials not derived from DCPD. The growth curves shown in Figure 4-10 were obtained in incubations using larger inocula without soil, and should not have this problem. Three main peaks of oxidized DCPD derivatives shown in the GC chromatograms (Figure 4-12a) were obtained in the ethyl acetate extracts from 2 week incubations. The mass spectra of these peaks (I, II, and II) shown in Figure 4-12b are identical to those identified by van Breemen (1987, 1993). Because the incubation was conducted in the dark, it is unclear why oxidized DCPD derivatives are also present in uninoculated control (Table 4-6 and Table 4-7). These cannot been formed by a photochemical mechanism as suggested by van Breemen (1993). Both uninoculated and inoculated samples were incubated in a big dessicator with a saturated DCPD atmosphere. The liquid phase and gas phase should have a saturated concentration of DCPD and oxidized DCPD derivatives because they are volatile compounds. The concentration of DCPD and oxidized DCPD derivatives would eventually be equilibrated between the two phases if no further changes occurred. The presence of more or less of these oxidized derivatives in inoculated compared to uninoculated samples may be due to utilization or production of these compounds, dissolved in the liquid phases, by bacteria. Double the amount of oxidized DCPD derivatives was extracted from the incubation of Q5 in MSM and sterilized soil for 7 weeks (Table 4-6) and the incubation of Q5 in MSM for 2 weeks (Table 4-7) than extracted from other incubations. These results imply that the oxidized DCPD derivatives are produced by species Q5. The data in Figure 4-10 indicate that this organism is capable of limited growth with DCPD as sole carbon and energy source,

despite failure to demonstrate mineralization. Perhaps the different way of delivery of [^{14}C]-DCPD in the mineralization experiments (in hexamethylnonane rather than as the vapor) caused the failure to mineralize.

Species Q5 was originally isolated from HDM medium using naphthalene as sole carbon and energy source. This species may have a fairly broad substrate range and may be able to metabolize DCPD or impurities present in DCPD to maintain its growth. Longer incubation times (up to 12 weeks) have been conducted for both Q5 and LQ30, and under these conditions cell densities decreased almost to original levels. Therefore, both Q5 and LQ30 were unable to continually utilize DCPD as sole carbon and energy source for growth.

Chapter 5. Results on a dechlorinating microbial community

5.1 Enrichment and isolation of bacterial species

Bacteria were isolated from a soil sample by plating on rich medium (TY), minimal glucose, or Medium E plates, and incubation under aerobic or anaerobic conditions. A total of 22 bacterial isolates was obtained, based on different colony morphologies. After genomic cross-hybridization testing, 15 isolates were selected as bacterial standards. These are listed in Table 5-1 (number 1 to 15). Bacteria were also isolated from cultures of the soil sample in MY medium in the presence of PCE and DCE. The enrichment conditions are described in Table 3-4. After one week of incubation, 100 µl of DCE enrichment culture was withdrawn with a syringe from the sealed serum bottles, and diluted serially with MY medium. 100 µl of each dilution (10^4, 10^5 and 10^6) was spread on MY agar plates and incubated in a closed container under the same conditions. Cultures grown anaerobically in the presence of PCE were diluted and spread in the same way after two weeks of incubation. Another 16 bacteria with different colony morphology were isolated from these MY plates. Of these isolates 8 with little genomic cross-hybridization are listed as standards 16 to 23 in Table 5-1.

5.2 Characterization of the dechlorinating microbial community

The structure of the dechlorinating microbial community enriched in the presence of PCE and DCE was analyzed by RSGP. Some of the standards were identified by comparing their partial 16S rRNA gene sequences or by determining genomic DNA similarities. The growth of bacterial standards, which were enriched in the presence of PCE as shown by RSGP, was also determined.

Table 5-1. Bacterial standards present on the master filter for the PCE dechlorinating community

No[a]	Name[b]	Medium[c]	C[d]	Colony morphology[e]
1	A1	TY, aerobic	120	large, brown/yellow, wet
2	A2	TY, aerobic	180	orange, wet
3	A3	TY, aerobic	200	round, cream
4	A4	TY, aerobic	100	round, bright yellow
5	A5	Medium E, anaerobic	100	mucoid, cream
6	A6	Medium E, anaerobic	100	round, bright orange
7	A7	MG, aerobic	200	yellow, wet
8	A8	MG, facultative	100	small, round, cream
9	A9	MG, aerobic	320	round, cream
10	A10	MG, aerobic	100	round, cream, wet
11	A11	MG, aerobic	120	small, round, cream
12	A13	MG, facultative	100	round, wet
13	A14	TY, facultative	100	small, round, cream
14	A15	TY, faculative	50	small, cream
15	A17	Medium C, facultative	40	small, yellow-tint
16	Y1	MY, anaerobic	120	large, round, cream
17	Y2	MY, anaerobic	100	large, round, wet
18	Y3	MY, anaerobic	100	small, round, cream
19	Y4	MY, anaerobic	120	small, round, wet
20	Y5	MY, aerobic	40	large, round, yellow
21	Y6	MY, aerobic	110	large, round, cream
22	Y7	MY, aerobic	100	small, round, wet
23	Y8	MY, facultative	100	small, round, cream

[a]Position of the standards on the PCE dechlorinating master filter.

[b]Name assigned after cross-hybridization testing.

[c]Medium used for isolation, facultative bacteria can grow on the medium under both aerobic and anaerobic conditions.

[d]Amount of denatured chromosomal DNA (ng) spotted on the filter.

[e]Colony morphologies of standards on solid medium plates.

5.2.1 Master filter preparation

The denatured chromosomal DNAs of bacterial standards were spotted on a filter in the order listed in Table 5-1. Denatured bacteriophage λ DNA was also spotted on the bottom row of each filter in amounts of 10, 20, 30, 40, 50 and 60 ng, respectively. The concentrations of the chromosomal DNAs used for master filter preparations were determined before denaturation and are listed in Table 5-1. A total of 41 master filters was made, and 23 of these were used to measure (k_λ/k_χ) values for all standards. The (k_λ/k_χ) values listed in Table 5-2 were obtained by labeling a mixture of chromosomal DNA of each standard with 200 ng λ DNA and hybridizing the resulting probe with the master filter. The (k_λ/k_χ) value of 26401 for standard 1 (A1) is anomalously large. Excluding this value, the highest (k_λ/k_χ) value (380) was found for standard 5 (A5), and the lowest value (27) was for standard 22 (Y7). These data represent the genome complexities of standards present on the master filter. The chromosomal DNA of standard 15 (A17) did not hybridize with the master filter. The hybridization reaction may have been inhibited by impurities present in the chromosomal DNA preparation. The (k_λ/k_χ) value for standard 15 is therefore not included in Table 5-2.

The degree of cross-hybridization of each standard DNA with all others was determined by quantitative analysis of the hybridization patterns obtained in these genomic DNA hybridization experiments. The cross-hybridization data for 21 standards (excluding standard 1 and 15) were arranged in a matrix and are shown in Figure 5-1, in which each square is shaded in proportion to its percentage of cross-hybridization. The percentages of cross-hybridization for most standards were low (< 5%) relative to self-hybridization (100%). One anomalously strong cross-hybridization (about 200%) was observed between A10 and A7 (Figure 5-1). The cross-hybridization data in this matrix can be used for the interpretation of calculated f_χ values in the analysis of PCE dechlorinating communities.

Table 5-2. Identification of bacterial standards present on the master filter

No[a]	Name[b]	k_λ/k_χ [c]	Similarity[d]	Closest homolog[e]
1	A1	26401		*Pseudomonas* sp[f].
2	A2	38		n.d[g].
3	A3	49		n.d[g].
4	A4	86		n.d[g].
5	A5	380		n.d[g].
6	A6	50		n.d[g].
7	A7	116		n.d[g].
8	A8	119	0.716	*Xenorhabdus bovienii*
9	A9	146		*Pseudomonas* sp[f].
10	A10	192		n.d[g].
11	A11	66		n.d[g].
12	A13	76		*Pseudomonas* sp[f].
13	A14	122		n.d[g].
14	A15	40		n.d[g].
15	A17	n.d[g].		n.d[g].
16	Y1	69		*Aeromonas* sp[f].
17	Y2	55		*Clostridium* sp[f].
18	Y3	35	0.739	*Enterococcus saccharolyticus*
19	Y4	67		n.d[g].
20	Y5	58		n.d[g].
21	Y6	93		n.d[g].
22	Y7	27		n.d[g].
23	Y8	127	0.887	*Bacillus thuringiensis*

[a]Position of the bacterial standard on the master filter.

[b]Name assigned for bacterial isolates after cross-hybridization testing.

[c]Ratio of hybridization constants for bacteriophage λ and standard genomic DNA χ from equation 2; the value for A1 is likely to be anomalous.

[d]Similarity coefficient for query and matching sequences.

[e]Closest homolog in the RDP database as determined by SIMILARITY_RANK (Maidak et al., 1994).

[f]Identification was done based on its cross-hybridization with identified bacterial isolates on another master filter. [g]Not determined due to failure of labeled DNA to hybridize.

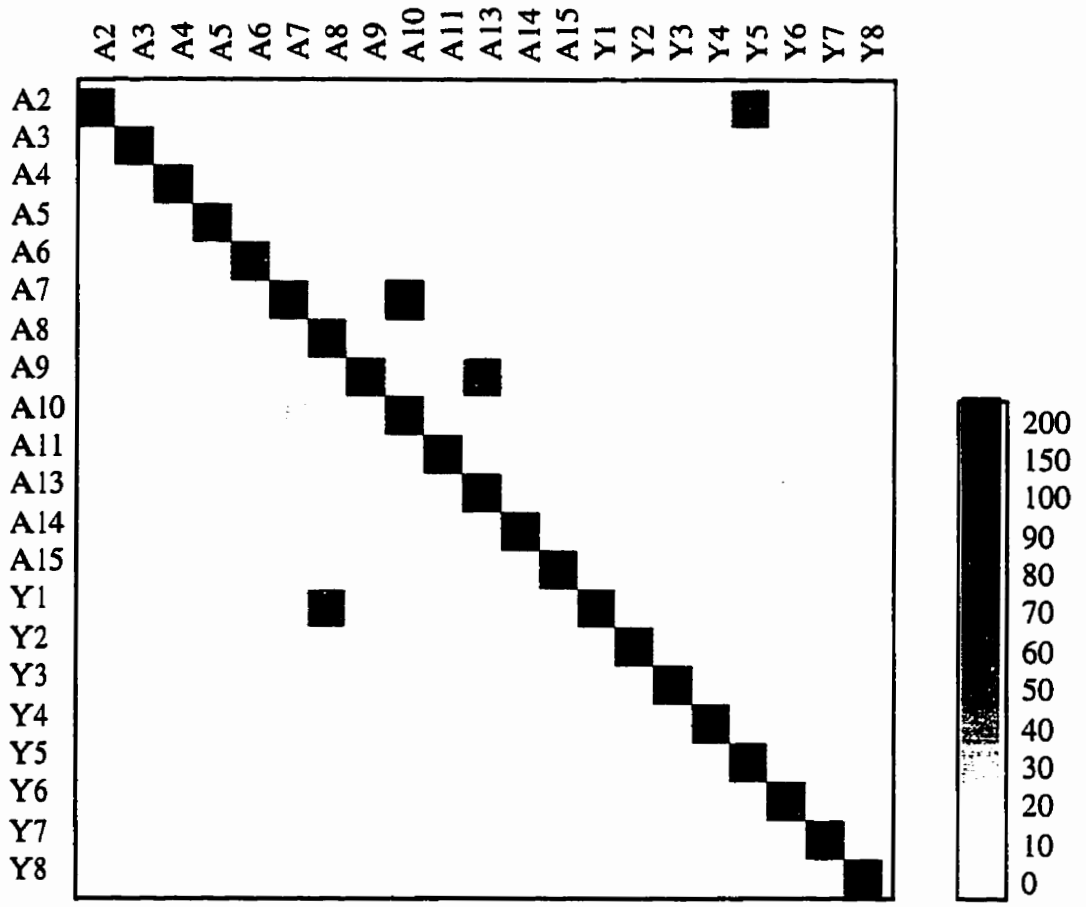


Figure 5-1. Cross-hybridization matrix for bacterial standards present on the master filter of the PCE dechlorinating community. Hybridization data obtained by labeling chromosomal DNA of each standard and hybridizing with a master filter were arranged as columns in the matrix. Each square in the matrix is shaded in proportion to its percentage of cross-hybridization, according to the gray scale provided. 100% was taken for each self-hybridization.

5.2.2 Identification and characterization of selected standard bacteria

Only 8 of the standards present on the master filter have been identified. Standards 8, 17, and 23 (A8, Y3, and Y8) were identified by comparing their partial 16S rRNA gene sequences with those available in the RDP database, using the program SIMILARITY_RANK. The closest RDP homologs and the similarity coefficients obtained are listed in Table 5-2. Standards 1, 9, 12, 16, and 17 (A1, A9, A13, Y1, and Y2) were identified based on high cross-hybridization (> 80%) of their genomic DNAs with those of bacterial isolates obtained and identified before.

5.2.3 Effects of PCE and DCE on the soil microbial community

The effects of PCE or DCE on the soil microbial community were analyzed by RSGP. Chromosomal DNAs were directly extracted from cultures in MY medium incubated for 4 weeks in three different gas atmospheres in the presence or absence of PCE or DCE, as described in Table 3-4. Hybridization of the labeled community DNAs extracted from these incubated samples with the master filter and quantitative analysis of the hybridization patterns gave the bar diagrams shown in Figure 5-2. The fraction contributed by standard 1 (A1) in all RSGP patterns shown in Figure 5-2, was set to zero in view of its anomalously high k_λ/k_χ value (26,401). Standards 8 (A8) and 16 (Y1) were greatly enriched after incubation with PCE and without PCE in mixed gas (Figure 5-2 A and B). The calculated fractions of A8 and Y1 in the extracted community DNAs were 1.5 to 2 times higher in the presence of PCE than in the absence of PCE. Incubation in 100% nitrogen as the gas atmosphere, gave a significant increase of standard 23 (Y8) in the presence of PCE compared to the absence of PCE (Figure 5-2 C and D). Standard 21 (Y6) was greatly enhanced by incubation in the presence of DCE in air compared to the absence of DCE (Figure 5-2, E and D). None of these standards (indicated as arrows in Figure 5-2) have significant cross-hybridization with other standards present on the

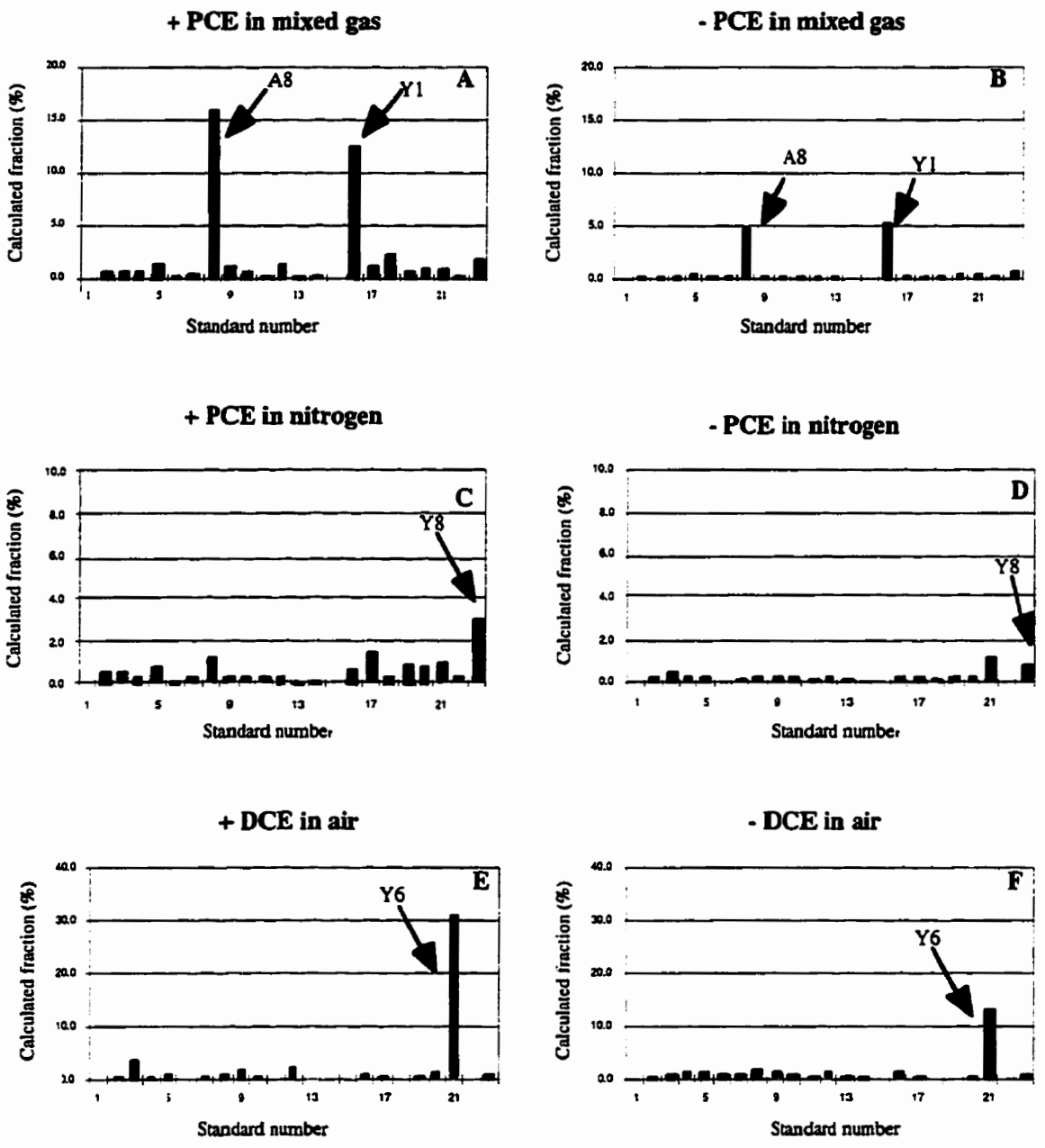


Figure 5-2. RSGP patterns of the PCE dechlorinating community after incubation with PCE and DCE under anaerobic condition in mixed gas, under anaerobic conditions in nitrogen, and under aerobic conditions. The enriched species are indicated by arrows in the bar diagrams, which are obtained by plotting calculated fractions (f_χ) for each standard vs. standard number.

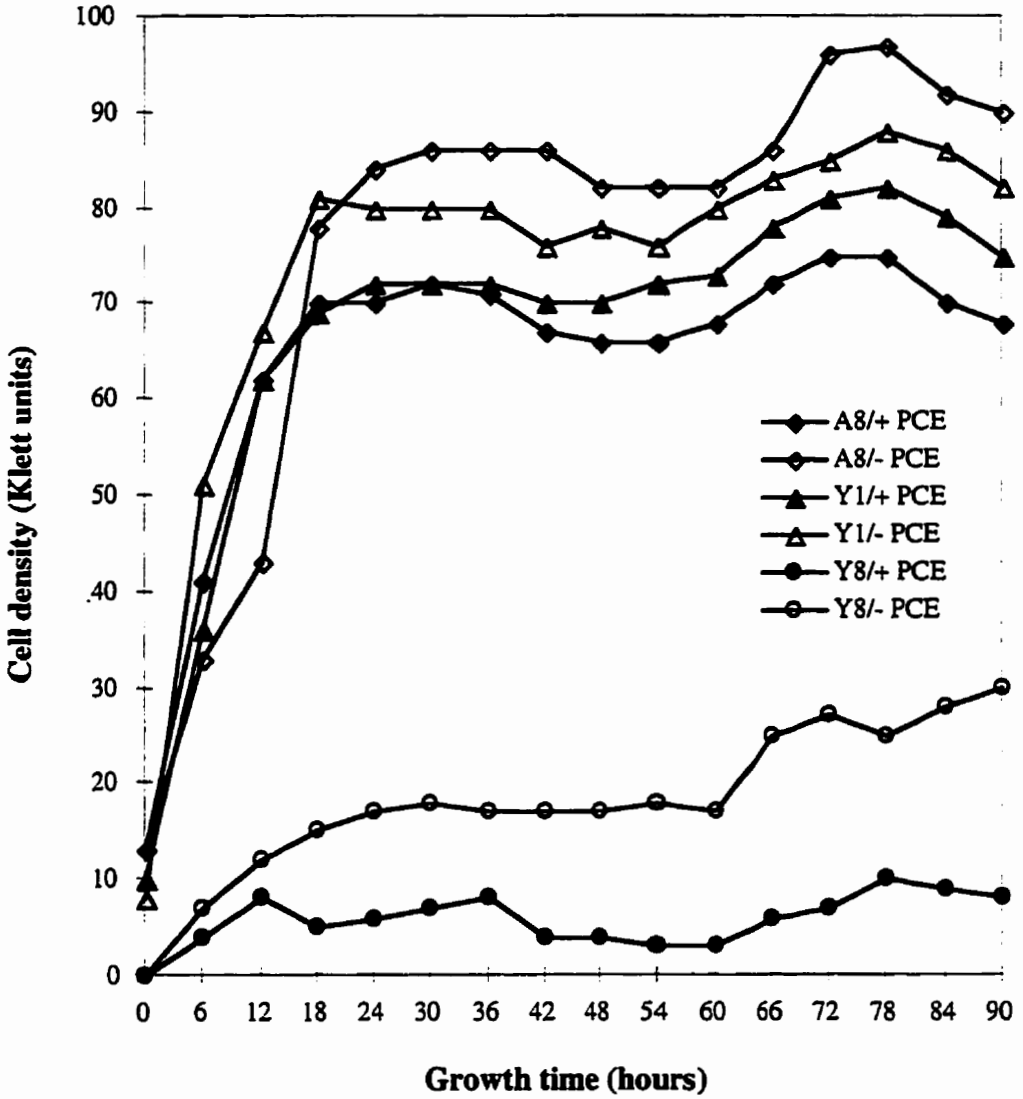


Figure 5-3. Growth of strain A8, Y1, and Y8 in MY medium with or without PCE. 100 µl of saturated bacterial cultures were inoculated into 5 ml MY medium with and without 20 mM PCE (3316 ppm), and grown in screw capped glass tubes containing a mixed gas (5% H_2, 10% CO_2 and 85% N_2) atmosphere (A8 and Y1), or a 100% nitrogen atmosphere (Y8). The cell densities were measured with a Klett meter and expressed as Klett units versus incubation time in hours.

master filter (see Figure 5-1). Therefore, strains A8, Y1, and Y8 are candidates for dechlorination of PCE under anaerobic conditions and strain Y6 is a candidate for dechlorination of DCE under aerobic conditions. The growth of strains A8, Y1, and Y8 in MY medium with 20 mM PCE (3316 ppm) is shown in Figure 5-3. Strains A8 and Y1 were able to grow significantly in MY medium with PCE, whereas strain Y8 grew only a little. MY medium contains a large amount of yeast extract, and the growth of A8, Y1 and Y8 without PCE was better than the growth of these strains with PCE. This result does not prove that these strains have the capacity of dechlorination, but means that these strains were able to tolerate large amounts of PCE in the medium, with only minor effects on growth.

5.3 Dechlorination by selected strains and an enrichment culture

To determine whether strains A8 and Y1 have PCE dechlorination activity, individual strains and a combination of both strains were incubated with PCE and the appearance of completely (ETH) or partially (TCE, DCE, and VC) dechlorinated products was monitored as a function of time. The dechlorination activity of an enrichment culture was examined in a similar way

5.3.1 Dechlorination of PCE

100 µl of saturated cultures of A8 and Y1 in MY medium were inoculated into 50 ml MY medium with 100 ppm PCE. The accumulation of ethene over a 63 day period is shown in Figure 5-4A. Both strains A8 and Y1 started to gradually dechlorinate PCE to ethene, especially after incubation for 42 days. However, the ethene concentrations produced were very small. No partially dechlorinated intermediates were detected in the headspace. The PCE contaminated soil sample (1 g) was incubated in the same way, and the ethene concentration accumulated over 28 days of incubation is shown in Figure 5-4B. This soil community was more efficient in the dechlorination of PCE to ethene, and

approximately 27% of the PCE added was reductively dechlorinated to ethene after 28 days of incubation, assuming that ethene is completely insoluble in the medium. The latter experiment was monitored also for 65 days. However, the ethene peaks recorded on the gas chromatograms increased rapidly, and were out of the range of the ethene standard curve used after 28 days of incubation. The data in Figure 5-4B are therefore plotted for a shorter time interval than in Figure 5-4A. No partially dechlorinated intermediates were detected in the headspace in the incubation with the soil sample.

5.3.2 Nutritional requirements

The initial enrichment of the PCE contaminated soil sample was conducted in MY medium, which contains 10 g of yeast extract per liter. To determine the potential requirement for yeast extract, basal medium (BM) containing 250 mg of yeast extract per liter was used in experiments as replacement of MY medium. 500 µl of a culture in MY medium with 100 ppm PCE was inoculated into 50 ml of BM medium containing 2 mM PCE (332 ppm). Duplicate incubations were performed and the dechlorination of PCE by this enrichment culture was monitored by measuring ethene concentration accumulated in the headspace of the incubation bottles. Figure 5-5 shows that addition of yeast extract (1 ml of 10% yeast extract) greatly stimulated dechlorination of PCE to ethene by the enrichment culture. The dechlorination was significantly limited without yeast extract addition to the culture (Figure 5-5). If yeast extract was depleted from BM medium, the growth of the culture was decreased gradually (data not shown). No growth was observed without yeast extract. Similar results were observed in the incubation of a combination of Y1 and Y8 in 50 ml BM medium containing 2 mM *cis*-DCE (194 ppm) (see Figure 5-6B). These results suggested that yeast extract contained the essential nutrients required for PCE dechlorination by the soil microbial community and by individual strains. BY medium containing 5 g of yeast extract per liter was used in the latter experiments instead of MY medium.

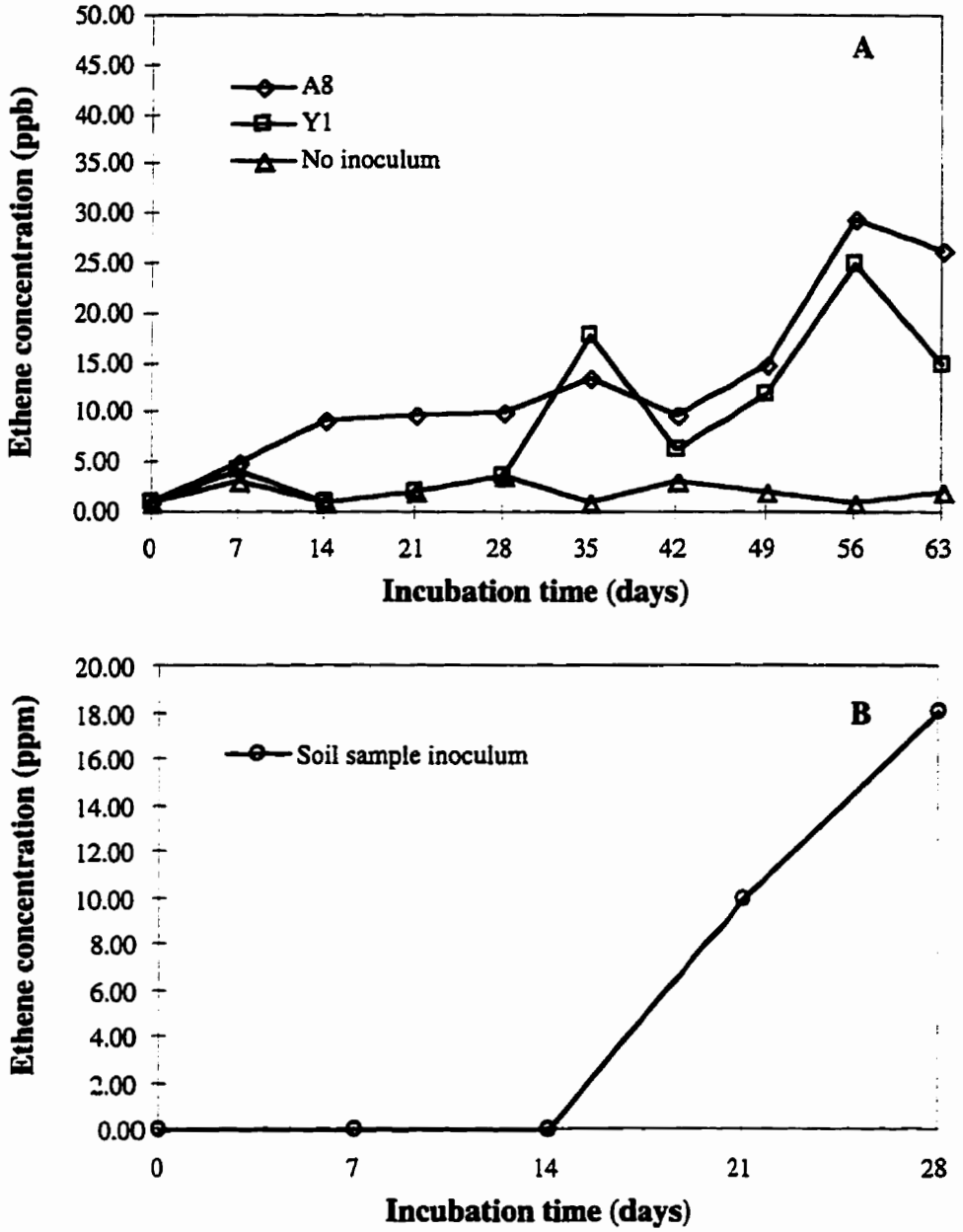


Figure 5-4. Reductive dechlorination of PCE to ethene by strain A8, Y1 and the soil sample under anaerobic conditions in a H_2 (5%), CO_2 (10%), and N_2 (85%) atmosphere. 100 µl of each saturated culture of A8 and Y1, and 1 g of soil sample were incubated in 125 ml serum bottles with 50 ml of MY medium containing 100 ppm of PCE. The accumulation of ethene in the headspace is plotted vs. incubation time (days).

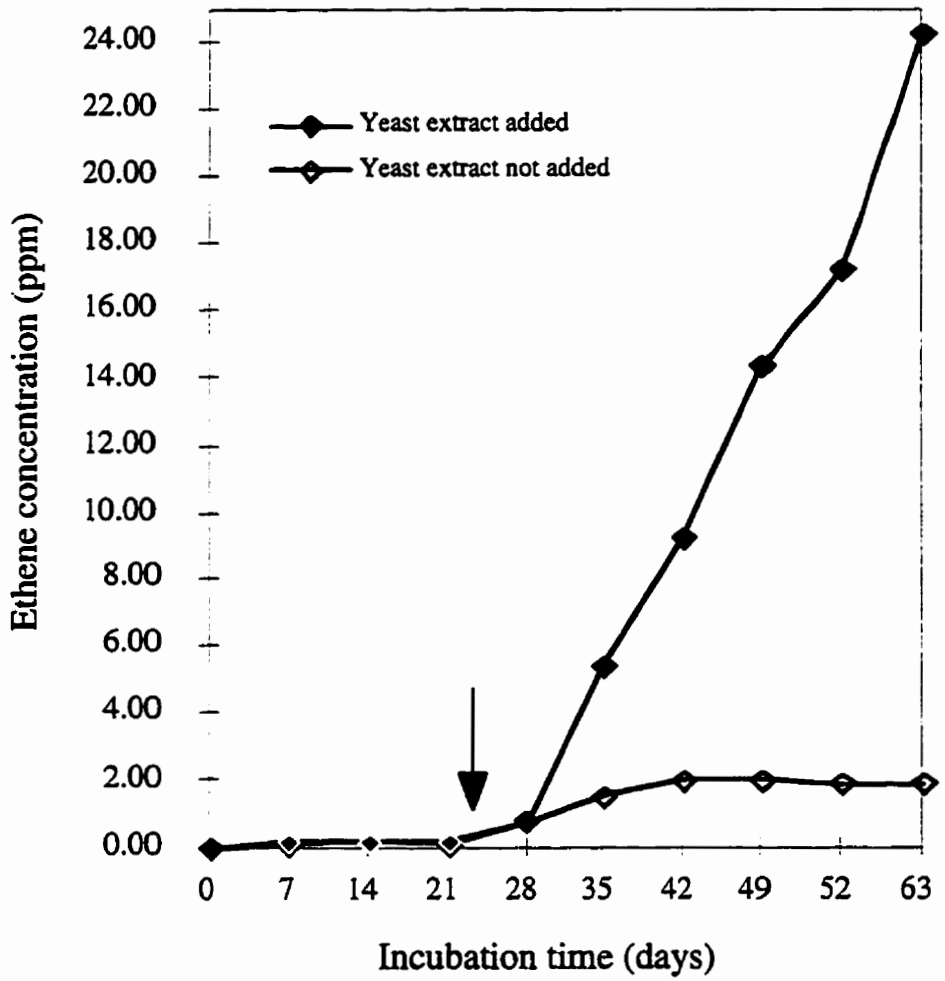


Figure 5-5. Effect of yeast extract on the rate of PCE dechlorination to ethene by an enrichment culture under anaerobic conditions. 500 µl of each enrichment culture was incubated in duplicate 125 ml serum bottles with 50 ml of BM medium containing 2 mM PCE (332 ppm). 1 ml of 10% yeast extract solution was added into one of the incubation bottles after 22 days. The amount of ethene (ppm) accumulated in the headspace was plotted vs. incubation time (days).

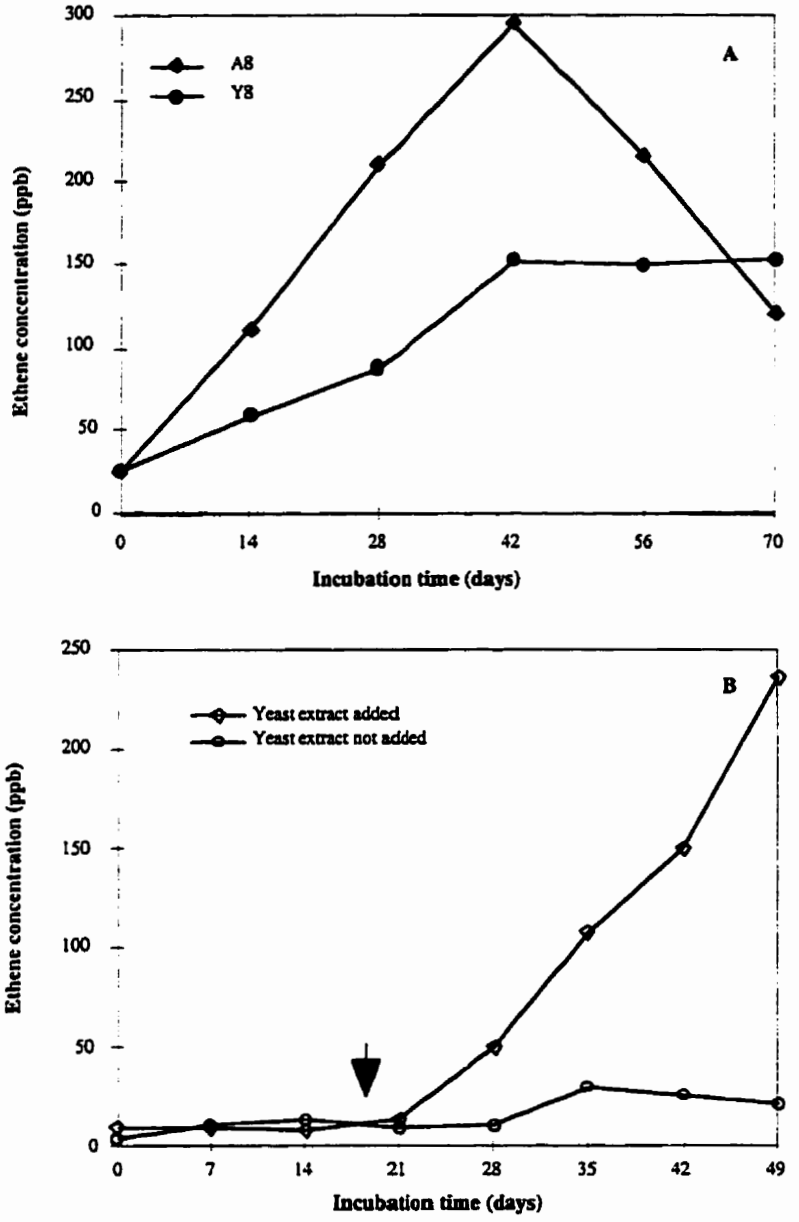


Figure 5-6. (A) Dechlorination of *cis*-DCE to ethene by strains A8 and Y8 under aerobic conditions. 500 µl of saturated cultures of strains A8 and Y8 in MY medium were incubated with 50 ml of BY medium containing 2 mM *cis*-DCE. (B) Dechlorination of *cis*-DCE to ethene by a combination of strains A8 and Y8. 500 µl of each saturated culture of A8 and Y8 in MY medium were inoculated into 50 ml BM medium containing 2 mM *cis*-DCE, and 1 ml of 10% (w/v) yeast extract was added to the culture after 14 days as indicated by the arrow. The accumulation of ethene (ppb) in the headspace of each incubation bottle was measured by GC and is plotted vs. incubation time (days).

5.3.3 Dechlorination of TCE and DCE

Dechlorination of PCE by strains A8 and Y1 alone was very limited, during anaerobic incubation in MY medium with PCE under a mixed gas atmosphere, although both were greatly enriched in the incubation with PCE. The ability to dechlorinate the less chlorinated ethene compound, *cis*-DCE, was investigated for the facultative bacterium A8 and the aerobe Y8 under aerobic conditions. Figure 5-6A shows ethene accumulation in the headspace over a 70 day period. Strain A8 was able to dechlorinate *cis*-DCE to ethene for 42 days, after which its dechlorinating activity decreased. Strain Y8 had a more or less constant dechlorinating activity during the 70 day incubation. However, the amount of ethene produced was also very small. The combination of A8 and Y8 in BM medium showed good dechlorination activity of *cis*-DCE to ethene after yeast extract was added to the culture (Figure 5-6B). We may not be able to equate ethene production with *cis*-DCE removal under these conditions, because *cis*-DCE may also be removed oxidatively, which would not result in ethene production.

Dechlorination of TCE by an enrichment culture was also examined. The enrichment culture (500 µl) was incubated with 50 ml BY medium containing 100 ppm TCE under anaerobic conditions (mixed gas atmosphere). After incubation for 14 days, the end product ethene rapidly increased (Figure 5-7), and 19 ppm of ethene had accumulated in the headspace on day 28. Approximately 28.5% of the TCE added was dechlorinated to ethene after 28 days of incubation. Unlike dechlorination of PCE, a partially dechlorinated intermediate (*cis*-DCE) accumulated in the headspace of the incubation. The concentration of *cis*-DCE was gradually increased during the first 21 days of incubation, and then decreased as ethene was formed (Figure 5-7).

Reductive dechlorination of *cis*-DCE was also determined under aerobic conditions. Rapid dechlorination of *cis*-DCE to ethene was observed after 7 days of incubation, and the concentration of ethene detected on day 14 was already out of the standard curve range. The partially dechlorinated intermediate VC was not detected in the headspace of

the bottles. The anaerobic enrichment culture in MY medium in the presence of PCE (Figure 5-4B) was also able to dechlorinate TCE and *cis*-DCE. Its rate of dechlorination of *cis*-DCE to ethene under aerobic conditions was larger than its rate of dechlorination of PCE to ethene under anaerobic conditions.

5.3.4 New bacterial isolates

The profiles of the soil community incubated in 500 ml MY with 100 ppm PCE in mixed gas atmosphere were analyzed again by RSGP, after 16 weeks of incubation. Figure 5-8 shows the RSGP patterns of after 4 weeks (A) and after 16 weeks (B) of incubations in the presence of 100 ppm PCE in MY medium. Only standards 8 and 16 (A8 and Y1) were significantly enriched after 4 weeks of incubation (Figure 5-8A). More standards, e.g. 10, 17, 19, and 23 (A10, Y2, Y4, and Y8), were enriched after 16 weeks (Figure 5-8B). Thus more standards were enriched after longer incubation time. It is unlikely that these organisms are the most prominent dechlorinators in the community. Incubation with single strains gave ppb amounts of ethene (Figure 5-4A and Figure 5-6A), whereas incubation with the consortium gave ppm amounts of ethene (Figure 5-4B, Figure 5-5, and Figure 5-7). Another attempt was therefore made to isolate bacteria more active in dechlorination.

A 100 µl sample of the 16 week enrichment culture was diluted with BY medium and spread on BY agar plates. These were incubated inverted with a drop of PCE on the lids in a closed container under anaerobic mixed gas conditions. Six bacterial isolates (Y9 to Y14) with different colony morphologies were selected from these plates, and inoculated in BY liquid medium. To test if these were better able to dechlorinate PCE, 200 µl of each saturated culture were incubated in 50 ml BY medium with 162 ppm PCE under anaerobic mixed gas conditions. Accumulation of ethene in the headspace of each bottle was monitored by GC. Five of these isolates (Y9, Y10, Y11, Y12, and Y13) dechlorinated PCE to ethene at a moderate rate (Figure 5-9) without accumulation of

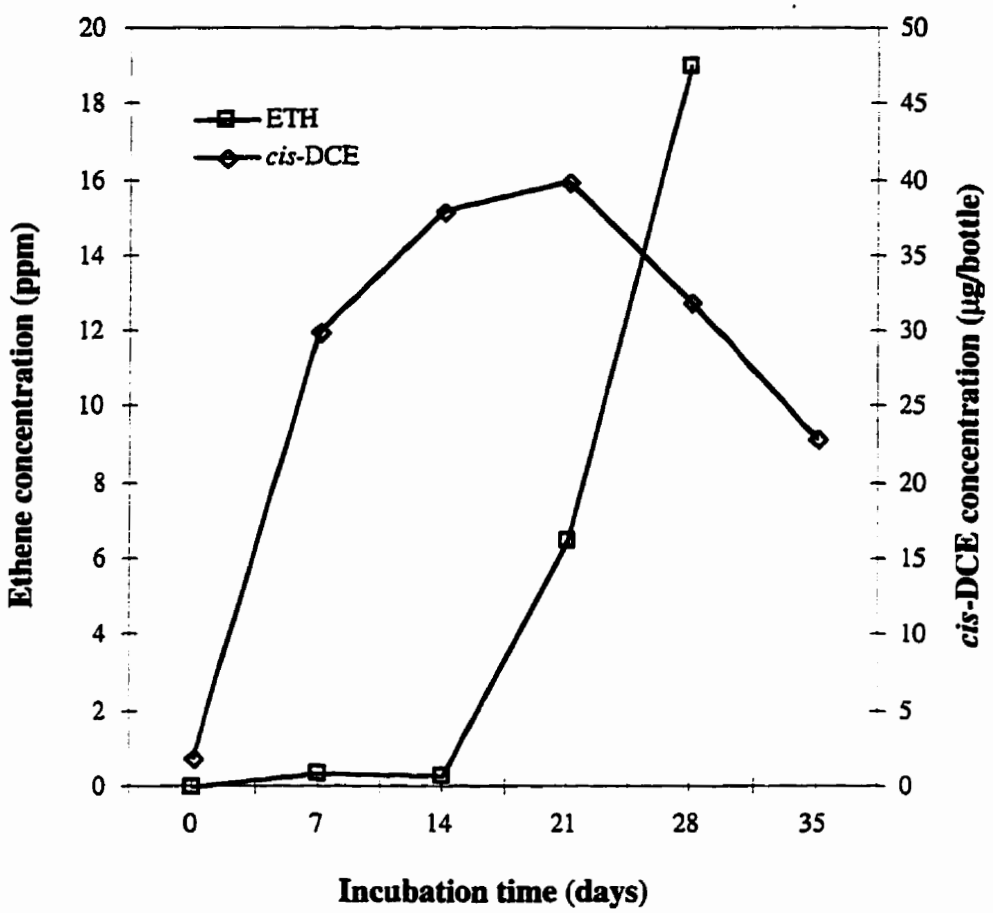


Figure 5-7. Dechlorination of TCE by an enrichment culture under anaerobic conditions. 500 µl of enrichment culture was incubated with 50 ml BY medium containing 100 ppm TCE in a 125 ml sealed serum bottle. The end product ethene (ppm) and dechlorination intermediate *cis*-DCE (µg/bottle) in the headspace were determined by GC and plotted against incubation time (days). The results are the average for duplicate bottles.

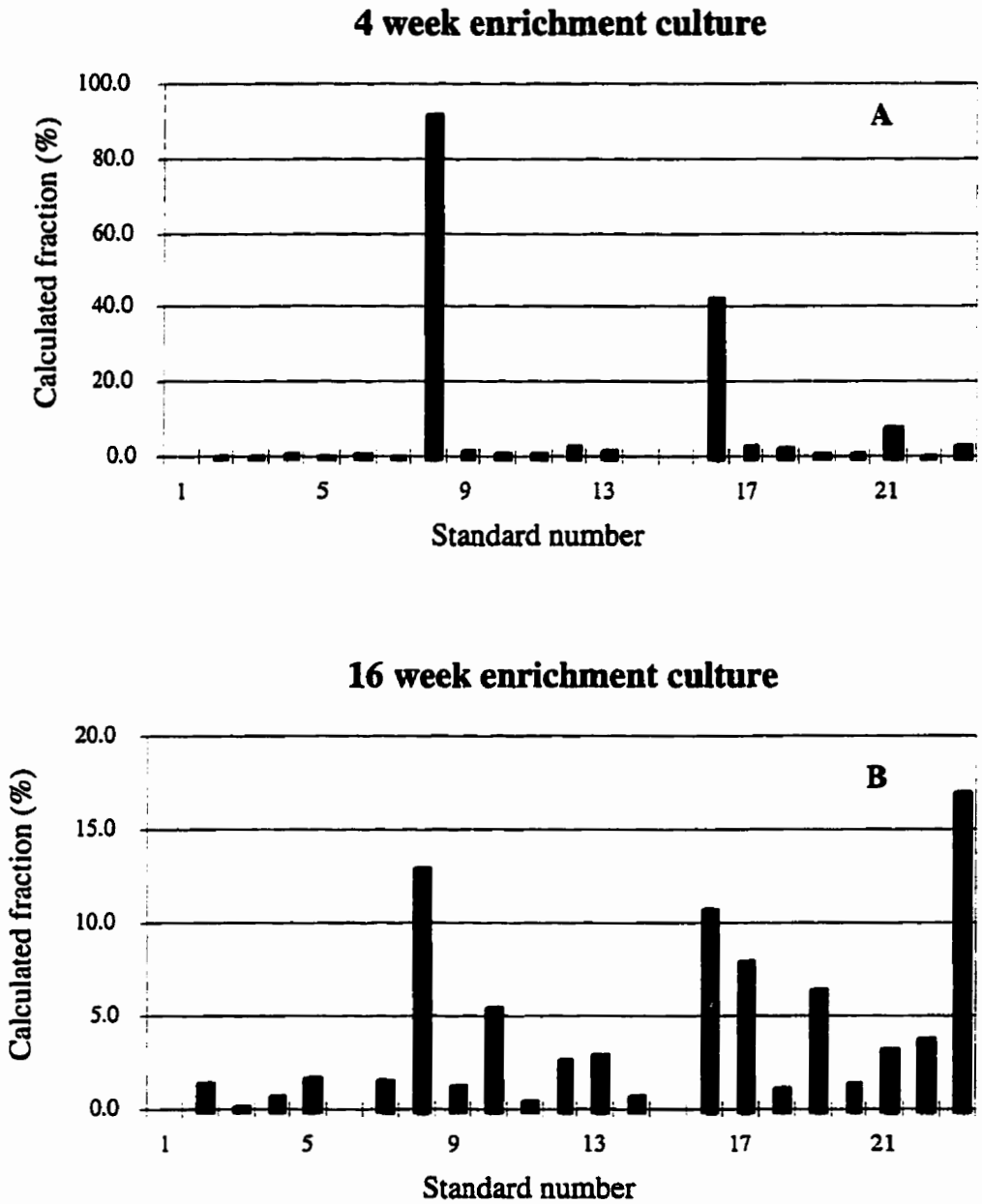


Figure 5-8. RSGP patterns of the soil microbial community enriched by incubation of 1 g soil with 50 ppm PCE in 500 ml MY medium for 4 weeks (A) and 16 weeks (B) under anaerobic conditions in a mixed gas atmosphere. The calculated fraction f_x of each standard DNA in the total community DNAs extracted from the incubated enrichment culture is plotted vs. standard number.

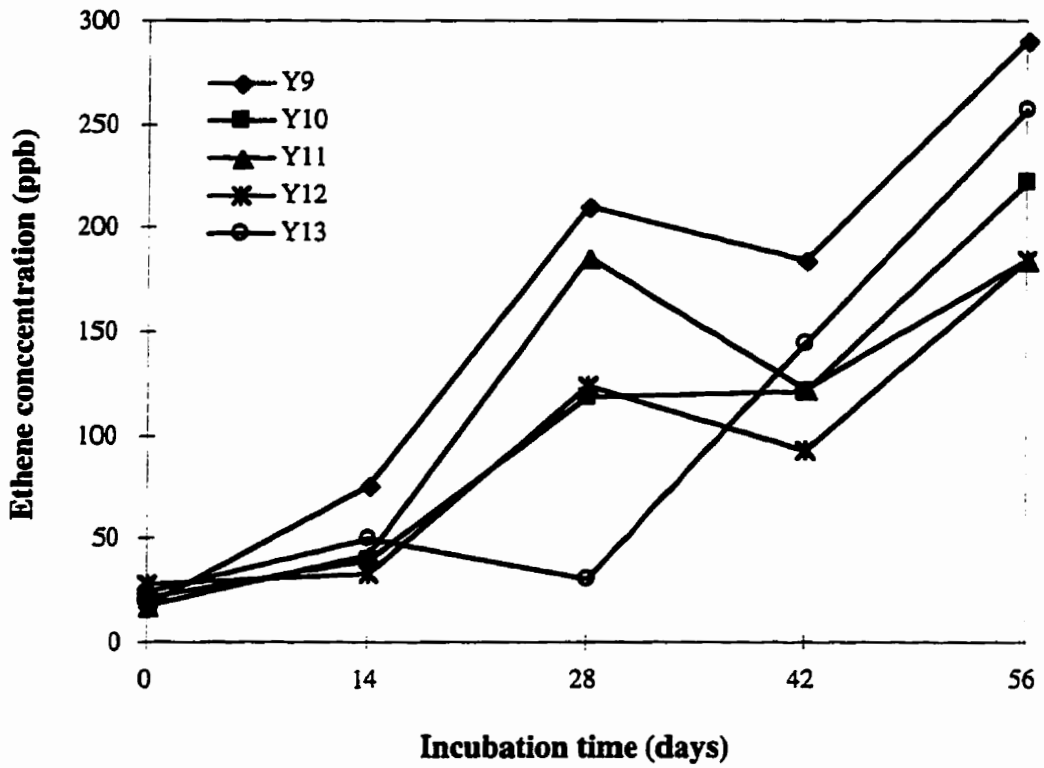


Figure 5-9. Dechlorination of PCE to ethene by five new bacterial isolates (Y9 to Y13). These were obtained by plating the soil enrichment culture on MY medium plates and incubation in a mixed gas atmosphere, saturated with PCE. Dechlorination of PCE was tested after growth of liquid cultures in BY medium containing 162 ppm PCE.

Table 5-3. Cross-hybridization of new isolates with the standards on the master filter[a]

Isolates	Standard 8	Standard 16	Standard 22	Standard 23
Y9	63.0	46.8	48.5	16.9
Y10	48.3	27.4	19.1	7.8
Y11	74.0	38.8	45.7	16.2
Y12	79.8	80.2	0.4	3.4
Y13	56.2	55.0	52.4	21.2

[a]Percentage of cross-hybridization of new isolates (Y9 to Y13) with the standards 8, 16, 22, and 23 present on the master filter, 100% was taken for self-hybridization.

partial dechlorinated intermediates. The sixth isolate (Y14) did not grow at all. Production of ethene by these isolates was somewhat higher than by standards tested previously (e.g., compare scales of Figure 5-9 to Figure 5-4A). The chromosomal DNAs of these isolates were labeled and hybridized with the master filter. The percentage of cross-hybridization of isolates Y9 to Y13 with standards present on the master filter are listed in Table 5-3.

5.5 Discussion

The results presented are a first step in the characterization of a soil microbial community capable of dechlorinating PCE to ethene by RSGP. Further work would require isolation of more bacterial species from PCE contaminated soil to generate a master filter containing most standards, including those that are more active. The number of bacterial standards isolated in this study is small. Fifteen of 23 standards were obtained by plating on rich medium plates. Of these only 8 were isolated from enrichment cultures in the presence of PCE or DCE. The MY medium used for enrichment contains 1% (w/v) yeast extract and mineral salts. This medium does not select for dechlorinating activity. The standards obtained may only survive in the presence of PCE or DCE, while utilizing the carbon sources provided by yeast extract for their growth. RSGP allowed a rapid screen to indicate the standards enhanced in the presence of PCE or DCE. The enriched bacterial standards were found to have dechlorinating activity.

The values of k_λ/k_χ were determined for all standards, and this allowed more precise calculation of the fraction (f_χ) in the analysis of the soil microbial community response to chlorinated ethenes. The different k_λ/k_χ values reflect the range of genome complexities of the bacterial standards. Because measurement of k_λ/k_χ values is not precise, duplicate measurements are usually required to obtain average k_λ/k_χ values, which are close to the actual values. A too high k_λ/k_χ value may result if the genomic DNA of the standard is impure (Telang et al., 1997). Duplicate measurements of k_λ/k_χ were not conducted in this

study. The k_λ/k_χ values listed in Table 5-2 are therefore less reliable than those listed in Table 4-2. The high value (k_λ/k_χ = 26401) for standard 1 (A1) is likely due to an impure chromosomal DNA, causing preferential labeling of λ DNA. This high k_λ/k_χ value causes overestimation of the f_χ value of A1. To avoid this, we excluded the f_χ values of A1 in all bar diagrams shown in Figures 5-2 and Figures 5-8.

The cross-hybridization data shown in Figure 5-1 were helpful to interpret community profiles obtained by RSGP. For example, the genome of standard 8 (A8) cross-hybridized 60% with that of standard 16 (Y1), while Y1 cross-hybridized 17% with A8. The f_χ values of both A8 and Y1 were significantly increased during growth on MY medium in the presence of PCE in a mixed gas atmosphere compared to growth in medium without PCE (Figure 5-2 A and B). Y1 had a calculated f_χ = 13%. However, this value drops to 5.2% if cross-hybridization with A8 is considered. Thus part of the f_χ value calculated for Y1 was contributed by cross-hybridization with A8. Standard 10 showed 208% cross-hybridization with standard 7. The reason for this is unclear.

Identification of the standards present on the master filter has not yet been completed. Only 3 of them (A8, Y3, and Y8) were identified by comparing their partial 16S rRNA gene sequences with those in the RDP database. Five standards (A1, A9, A12, Y1, and Y2) were identified based on their genomic DNA similarity (> 80%) with known standards present on another master filter. This study focused on identification of standards with dechlorinating activity. More 16S rRNA sequencing is required for complete characterization of the isolated standards.

Figure 5-2 shows that strains A8, Y1, and Y8 could be significantly enriched after incubation in MY medium with PCE for 4 weeks, and that strain Y6 was greatly enhanced in the presence of DCE. To assay the dechlorinating activity of these standards, the end product (ethene) and partially dechlorinated intermediates were measured by GC. Ideally, these measurements should be complemented with measurement of the disappearance of the chlorinated substrates. Plots of substrate versus

incubation time were provided in this study. The chlorinated substrate disappearance was monitored by GC, but it was not possible to convert peak areas into concentrations because a good standard curve was not available. Calculations should take the distribution of PCE, TCE, and DCE over the gas and liquid phases into account. Since ethene is a gaseous compound with little solubility in water, measuring the headspace of incubation bottles gives a good measurement of ethene concentrations. This easy assay was therefore used to monitor dechlorination.

Reductive dechlorination of PCE to ethene by strains A8, Y1, and the soil community is shown in Figure 5-4 A and B. Compared with the soil microbial community, strains A8 and Y1 produced little ethene. Strain Y8 was also incubated, but did not produce more ethene than the no inoculum control (data not shown). None of these standards were able to dechlorinate PCE at high rates, and they may only be effective in combinations. The dechlorination of PCE by combinations of strains has not been tested. Another possibility is that the active species have not been isolated from this soil community or that longer time of incubation is required for bacterial strains to start dechlorination. The community profiles of a 16 week incubation in the presence of PCE (Figure 5-3B) indicated that more bacterial strains were enriched if incubation time was extended. Five of six bacterial isolates obtained from this 16 week enrichment culture showed dechlorinating activity of PCE (Figure 5-9). Although the rate of dechlorination by individual strains (Y9 to Y13) was much lower than that of the entire soil community, it was improved over that of A8 and Y1. The colony morphologies of these isolates were different, but their chromosomal DNAs highly cross-hybridized with standards on the master filter (Table 5-1). More research is required to identify these isolates and define their dechlorinating activity. In this study, only one PCE-contaminated soil sample was available, and that may limit the opportunity to obtain more bacterial isolates. Enrichment cultures of this soil sample can possibly provide more bacterial isolates with

dechlorinating activity. Enrichment conducted in media with different electron donors may also help to analyze this soil community in more detail.

The complete dechlorination of PCE to ethene with mixed cultures has been reported before by Freedman & Gossett (1989), de Bruin et. al. (1992), DiStephano et al. (1992), and Maymo-Gatell et al. (1995). The final dechlorination step (VC to ethene) usually was rate limiting for pure cultures or mixed cultures which can carry out dechlorination of PCE. Significant amounts of VC persisted. The enrichment culture obtained in this study was able to completely dechlorinate PCE to ethene without any detectable chlorinated intermediates accumulated in the headspace.

Hydrogen served as the electron donor for reductive dechlorination of PCE to ethene by a mixed culture as reported by DiStefano et al. (1992). The enrichment of soil sample was performed in MY medium in a mixed gas atmosphere (5% H_2:10% CO_2:85% N_2). The electron donor utilized by this enrichment culture has not been investigated. Growth in 100% N_2 as the gas phase was slower than in a mixed gas phase. Complete dechlorination of *cis*-DCE to ethene was also observed under aerobic conditions. In addition to yeast extract, H_2 may serve as electron donor and PCE or *cis*-DCE as electron acceptor in the reductive dechlorination of PCE or *cis*-DCE to ethene. Yeast extract is high in amino acids, peptides, water-soluble vitamins, and carbohydrates, and yeast extract at a concentration of 0.1 to 0.5% can provide all vitamin requirements for bacterial growth (Cote & Ghema, 1994). The potential requirement of yeast extract for dechlorination has been examined for the soil enrichment culture. The rate of dechlorination of PCE to ethene in BM medium with 250 mg/liter of yeast extract (Figure 5-5) was much lower than in MY medium with 1% (w/v) of yeast extract (Figure 5-4B). The rate of dechlorination of PCE could be rapidly increased by addition of 1 ml of 10% (w/v) yeast extract to the culture (Figure 5-5). The rate of *cis*-DCE dechlorination to ethene by strain A8 plus Y8 was also greatly increased by addition of 1 ml of 10% (w/v) yeast extract to the culture in BM medium. Therefore, yeast extract is an essential

facultative bacterium (MS-1) has been reported by Sharma & McCarty (1996). Both strain A8 and Y8 dominating in the 16 week enrichment culture (Figure 5-8B), are facultative bacteria. They, as well as other facultative bacteria not yet identified, may play important roles in the complete dechlorination of PCE to ethene.

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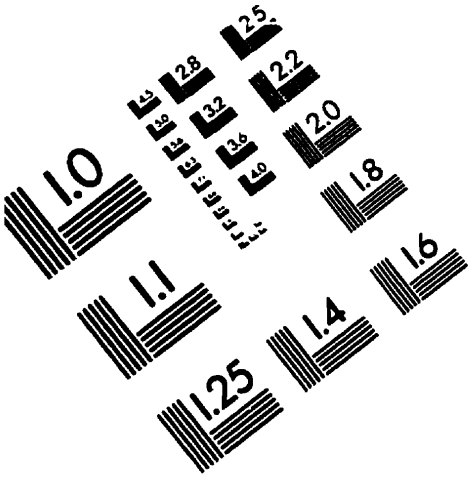
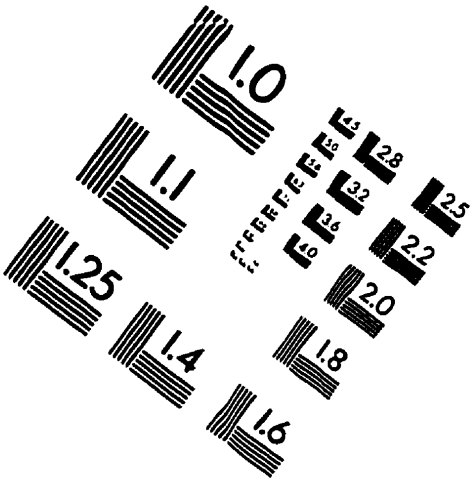
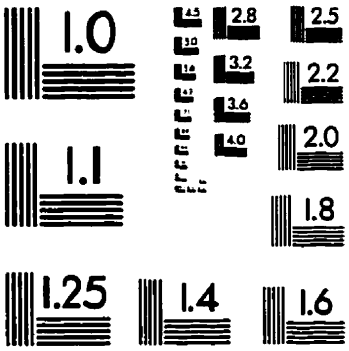
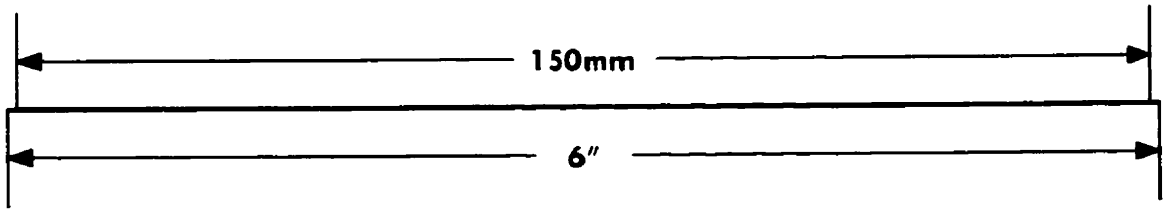
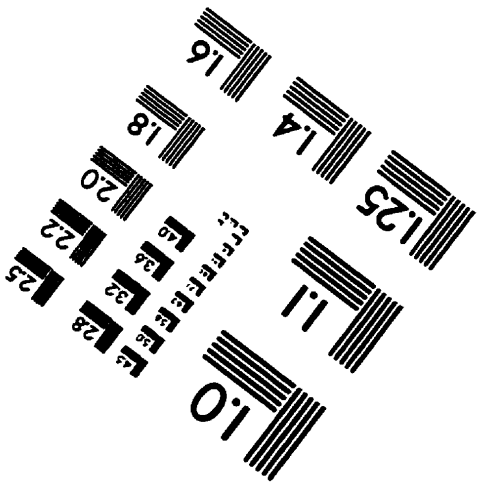
IMAGE EVALUATION TEST TARGET (QA-3)

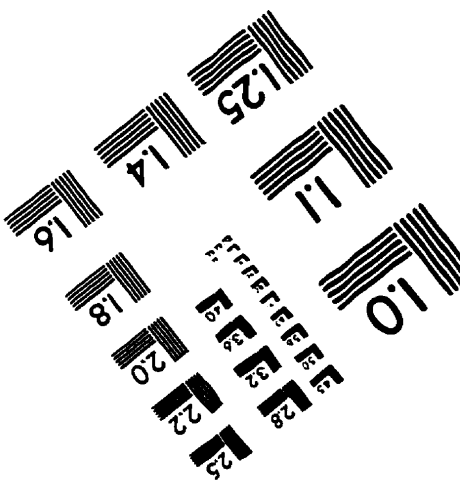












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