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Prevalence of monochloroacetate degrading genotypes among soil isolates of *Pseudomonas* sp.

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Abstract

This study reports the isolation of *Pseudomonas* sp. strains with monochloroacetate (MCA) degradation function, from uncontaminated soil, and the use of Southern blot hybridization technique to detect MCA degrading catabolic genes and their divergence. Based on their capacity to remove Cl^- from MCA in a minimal medium containing 185 ppm Cl^-, the strains were classified into three groups: poor degraders (Cl^- release between 0–15 ppm), medium degraders (Cl^- release between 16–30 ppm), and high degraders (Cl^- release between 31–45 ppm). We have applied a gene probe assay for determining the diversity of MCA degradative genotypes of 61 strains. Two different gene probes, *dehCI* and *dehCII* were used in Southern blot hybridization assays. Majority of the DNA samples that produced signals on the membrane blots (18 out of 24) hybridized with only *dehCI* DNA probe, while 6 strains hybridized with only *dehCII* probe. On the other hand, 37 isolates did not hybridize to either of the gene probes used. The results indicated the high specificity of the DNA hybridization method and the divergence of metabolic functions and/or genotypes among the native MCA-degrading *Pseudomonas* sp. populations in the soil.

Introduction

Chlorinated aliphatic and aromatic compounds form one of the most important groups of industrially produced chemicals and their emissions and spills have frequently led to the pollution of terrestrial and aquatic ecosystems. This is of particular concern, since these pollutants are highly toxic and may be rather long-lived in the environment. Removal of halogens, particularly flourine and chlorine, from organic molecules by microorganisms is the basis of the recycling of recalcitrant organic matter in the biosphere (Hardman 1991; Chaudry and Chapalamadgu 1991; Janssen et al. 1994; Slater et al. 1995). Microbial growth on halogenated substrates requires the production of catabolic enzymes that cleave carbon-halogen bonds. Such enzymes are commonly called 'dehalogenases'. Recently, nucleic acid sequence analysis of haloalkanoic acid dehalogenases suggested that these enzymes form a class of closely related enzymes (Kawasaki et al. 1994; Nardi-Dai et al. 1994). On the other hand, microorganisms from various environmental samples that possessed dehalogenation activities are of biotechnological interest, since they could be used for potential treatment of halogenated wastes.

This study includes defining and synthesizing information about the diversity of microbial populations with the biodegradation function, with specific reference to monochloroacetate (MCA) degradation. We have used MCA degradation as a model because of the substantial background information available on the biochemistry and genetics of its biodegradability. On the other hand, MCA is a common environmental pollutant in agricultural soils and feasible dehalogenation takes place in a single step.

We have isolated *Pseudomonas* sp. strains capable of growing on MCA as the sole source of carbon and energy, from uncontaminated soils in the Middle East Technical University Campus' forest. DNA hybridization technique in conjunction with the Southern blot transfer was employed for the analysis of MCA degrading genotypes of various *Pseudomonas*

sp., isolates. We have used two gene probes (*dehCI* and *dehCII*)) originally cloned from *Pseudomonas* sp. CBS3 which is an haloalkanoic acid metabolizing strain (Schneider et al. 1991) in Southern blot hybridizations to explore the genetic diversity among the *Pseudomonas* sp. soil isolates known to be capable of degrading MCA to different extents.

Materials and methods

Isolation of monochloroacetate degrading bacteria

The bacterial isolates used in this study were isolated from uncontaminated soils through MCA enrichment, as described before (Kocabıyık and Türkoğlu 1989). Soil samples (10 g dry weight) were added to 500 ml shake flasks containing 200 ml minimal salts medium which contained (per liter) 0.2 g $MgSO_4.7H_2O$, 0.5 g $(NH_4)_2SO_4$, 0.5 g KH_2PO_4, 1.5 g K_2HPO_4 and 10 ml trace element solution, and supplemented with MCA (0.1% w/v) as the sole carbon source. The trace element solution consisted of (per liter) 12 g Na_2EDTA, 2 g NaOH, 1 g $MgSO_4$. $7H_2O$, 0.4 g $ZnSO_4.7H_2O$, 0.4 g $MnSO_4.4H_2O$, 0.1 g $CuSO_4.5H_2O$, 0.5 ml conc. H_2SO_4, 10 g Na_2SO_4, 0.1 g $Na_2MoO_4.2H_2O$, 2.0 g $Fe_3SO_4.7H_2O$. The stock solution of MCA (10% w/v, neutralized with NaOH) was filtered through 0.2 μm membrane filter (Millipore), while minimal salts medium and trace element solutions were autoclaved for sterilization. The enrichment culture flasks were incubated at 30 °C on an orbital shaker (New Brunswick Scientific Co Inc., NJ, USA) through shaking at 250 rpm. During a total of 7-day incubation period MCA concentration was increased from 10 mM to 20 mM and 40 mM by two supplementations with 2-day intervals. Then, the samples (1 ml) were removed, appropriate dilutions were made in 0.1 M phosphate buffer (pH 7.0), and 0.1 ml portions were plated on the solidified minimal medium, containing the same chlorinated compound as the liquid enrichment. Single colonies were picked up and their stock cultures were maintained in LB broth (per litre: 10 g tryptone, 10 g NaCl, 5 g yeast extract) containing 0.1% (w/v) MCA and 50% (w/v) glycerol, at −80 °C.

A total of 112 colonies were collected and 86 of which produced green flourescence. We have randomly selected 61 isolates among those that formed green colour on agar plate, and characterized them by morphological and biochemical tests as described in Bergey's Manual of Systematic Bacteriology (Holt 1989).

Measurement of free chloride ion release

The bacterial cultures were prepared in 10 ml liquid minimal medium supplemented with MCA solution to maintain a total Cl^- concentration at 185 ppm. After incubation at 30 °C for 3 days through vigorous shaking at 300rpm, the cultures were centrifuged at 3500g for 15 min and 0.5 ml supernatants were used in the assays. The chloride ions liberated from MCA due to putative dehalogenase activity were measured spectrophotometrically (Iwasaki et al. 1956). Controls lacking MCA or inoculum were included in each set of experiments. Standard curve was prepared using a series of KCl solutions with chloride concentrations ranging from 2 to 12.5 ppm.

Also, chloride release was monitored using a qualitative assay, by adding 50 μl of 1 M $AgNO_3$ solution to 100 μl culture. The formation of a white precipitate (AgCl) was used as an indicator of Cl^- release.

Total DNA isolation from soil Pseudomonas *sp. strains*

Total cellular DNAs from bacterial isolates were extracted using a modification of the method described by Sermswan et al. (1994). The bacteria were grown overnight at 30 °C in 1.5 ml LB medium. The cells were harvested at 3,500 g for 5 min. The cell pellet was suspended in 200 μl of extraction buffer (25 mM Tris-HCl, 50 mM glucose, 10 mM NaCl, 10 mM EDTA, pH 8.0) and treated with lysozyme (Sigma) at a final concentration of 3 mg/ml for 30min on ice. The suspension was mixed with equal volume of proteinase K (Sigma) solution (0.1 mg/ml proteinase K in 2% SDS solution). The mixture was incubated at 56 °C until digestion was completed which took about 2 h and 1/3 volume of saturated NaCl was then added. The suspension was precipitated with 2 volumes of absolute ethanol. The DNA was spooled onto a glass rod and dissolved in TE buffer (33 mM Tris, pH 8.0; 1 mM EDTA).

Preparation of dehCI *and* dehCII *gene probes*

The DNA samples from 61 selected *Pseudomonas* sp. strains were analyzed on Southern blots using a two gene probes, *dehCI* and *dehCII* which were derived from *Pseudomonas* sp. Strain CBS3 (Schneider et al. 1991). The genes code for two 2-haloalkanic acid dehalogenases which catalyze hydrolytic dehalogenation of MCA and 2-monochloropropionate. Both enzymes dehalogenate only L-2-monochloropropionate

to D-lactate, but neither enzyme show activity with D-2-monochloropropionate. Comparison of their nucleic acid sequences revealed 45% homology on the DNA level.

The first probe, *dehCI* (1.1 kb) from *Pseudomonas* sp. strain CBS3 was obtained by digesting the pUKS 202 recombinant plasmid with *Eco*RI and *Bam*HI, simultaneously. The second probe, *dehCII* gene (1.0 kb) from *Pseudomonas* sp. strain CBS3, was prepared by digesting pUKS plasmid with *Bam*HI. The excised inserts were isolated from low melting agarose gel using the Gene Clean Kit (BIO 101 Inc., La Jolla, CA). The two probes were labelled by using the non-radioactive DIG-DNA Labeling and Detection Kit (Boehringer Mannheim Biochemica GmbH, Germany).

Southern blot hybridization

For Southern blot experiments, purified chromosomal DNAs (1–5 μg) of soil bacteria were digested with *Bam*HI according to the manufacturer's specifications. The restriction fragments were size fractionated by electrophoresis through horizontal 0.8% agarose gels (6.3 cm $\times$ 10 cm) in 1 $\times$ TAE buffer (0.04 M Tris-Acetate, 0.001 M EDTA) for 4 h at 60 V. The DNA from gels was transferred to nylon hybridization membranes (Boehringer Mannheim Biochemica GmbH, Germany), by the capillary transfer technique, using 0.4 N NaOH as transfer buffer (Sambrook et al. 1989). The DNA was then fixed to the membranes by baking at 80 °C (with vacuum) for 15–30 min. Prehybridization, hybridization and post hybridization washes were performed as described in the manufacturer's protocol (DIG-Nucleic Acid Labeling and Detection Kit, Boehringer Mannheim Biochemica GmbH, Germany). The membrane blots were prehybridized in a sealed plastic bag with at least 20 ml hybridization buffer (5 $\times$ SSC$-$1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate; blocking reagent-supplemented with the Kit, 1% w/v; N-lauroyl sarcosine, 0.1% w/v; SDS, 0.02% w/v) per 100 cm^2 of filter at 50 °C for 2 h. For hybridization, the hybridizaton buffer was replaced with fresh hybridization solution containing denaturated labelled probe DNA. Hybridization was performed at 45 °C for 24 h by using 2.5 ml of hybridization solution per 100 cm^2 filter. After hybridization, the membranes were washed 2 $\times$ 5 min at room temperature with at least 50 ml of 2 $\times$ SSC; SDS 0.1% w/v, per 100 cm^2 filter, and 2 $\times$ 15 min with 0.1 $\times$ SSC; SDS, 0.1% w/v.

The filters were used directly after the washing steps for enzyme-linked immunoassay using a DIG-Nucleic Acid Labeling/Detection Kit (Boehringer Mannheim Biochemica GmbH, Germany). The labelled probes were detected after hybridization to target nucleic acids using an antibody conjugate (anti-digoxigenine alkaline phosphatase conjugate). A subsequent enzyme catalyzed color reaction with 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) and nitroblue tetrazolium (NBT) salt was used to visualize hybrid molecules.

Results and discussion

Isolation of soil Pseudomonas *sp. through MCA selective enrichment*

A total of 112 bacterial strains were isolated from soil through MCA enrichment in minimal medium supplemented with MCA. Among these, 61 strains which produced green flourescence were selected from MCA minimal agar medium and all identified by morphological and biochemical tests as *Pseudomonas* sp. Taxonomic characteristics which were taken into consideration are shown in Table 1. By using a qualitative $AgNO_3$ test, about 25% of the strains were found to be better MCA degraders than the others, as deduced by the relatively higher amounts of white precipitate formation with $AgNO_3$ test, after a 3-day incubation. These strains also grew better than the others when cultured in liquid minimal medium containing 10 mM MCA which were able to reach the stationary phase of growth within 3-4 days.

Measurement of Cl^- release from MCA

Free chloride release was measured colorimetrically, after a 3-day incubation period in a 10 ml liquid minimal medium supplemented with MCA (total Cl^- concentraion was 185 ppm). MCA degradative capacities of the 61 test strains varied over a wide range (Figure 1). According to the amount of free Cl^- released from MCA, the strains were classified into 3 groups: poor degraders (62%, Cl^- release between 0–15 ppm), moderate degraders (11%, Cl^- release between 16–30 ppm), and high degraders (12%, Cl^- release between 31–45 ppm). About 15% of the strains were identified as inefficient MCA degraders, since Cl^- release could not be detected by the colorimetric method used. The results of the spectrophotometric

Table 1. Identification characteristics of the 61 bacterial isolates from soil that degrade MCA

Shape	Rod
Motility	Motile by polar flagella
Gram reaction	Negative
Oxidase test	Positive
Catalase test	Positive
Oxidation/fermentation	Aerobic acid production
Indole production	Negative
Hydrogen sulfide (H_2S) production	Negative
Nitrate reduction to nitrates	Positive
Arginine dihydrolase test	Positive
Urease test	Negative
Gelatine hydrolysis	Negative
Fixation of atmospheric nitrogen	Negative
Methyl red test	Negative
Voges-Proskauer test	Negative
Lipase	Negative
Lecitinase	Negative

Carbon sources utilized: D-glucose, L-arabinose, D-xylose, D-mannose, D-fructose, D-galactose, lactose, D-sorbitol, D-mannitol, acetate, citrate, propionate, inositol, 2-ketoglutarate, DL- β -hydroxybutyrate, L-alanine, L-valine, DL-arginine, histidine, L-proline.
Not utilized: D-sucrose, maltose, starch, glycogen, oxalate, 3-hydroxybenzoate.

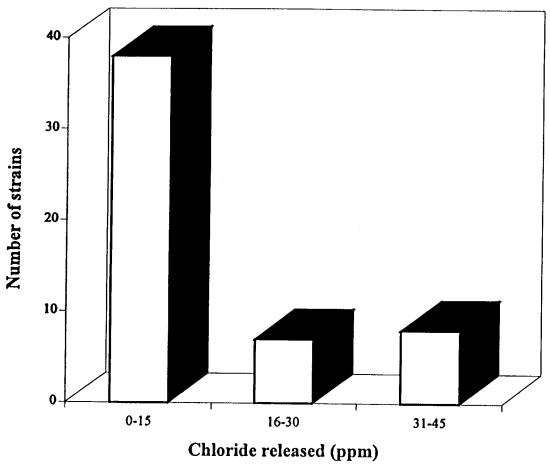

Figure 1. The frequency distribution of 61 *Pseudomonas* sp. strains according to level of chloride that they released from MCA supplemented minimal medium. The bacterial cultures were prepared in 10 ml liquid minimal medium containing 185 ppm Cl^- in the form of MCA. Free chloride release was determined colorimetrically in the culture supernatants as described in Materials and Methods.

measurment were in good agreement with those of qualitative assay.

Most of the MCA degraders were identified as poor Cl^- releasing strains. High degrading strains could remove, at most, about 25% of the Cl^- from MCA under test conditions. This may be due to the absence of a selective pressure for the specific selection of the strains with high dehalogenation capacity. This notion is supported by the fact that test soils from which the *Pseudomonas* sp. were isolated, were not contaminated by xenobiotic compounds.

Southern blotting and hybridization

DNA hybridization techniques can be powerful tools in environmental analysis because they offer high sensitivity and specificity. For the general detection of a genotype in a microbial community colony hybridization, or direct DNA extraction from soils and sediments followed by slot blot or dot blot analysis is probably sufficient (Sayler et al. 1985; Steffan et al. 1989). Such approaches can be used to monitor specific gene function on a presence and absence basis. However, DNA extraction followed by Southern hybridization can be used for finer detailed analysis of specific catabolic genes in the environmental samples, such as variability of catabolic genotypes due to gene rearrangements, deletions and possibly catabolic gene transfer among the bacterial community (Holben et al. 1988; Walia et al. 1990).

In this study, a primary consideration in the use of DNA probes is to test the fidelity of *dehCI* and *dehCII* gene probes for detecting homologous or related catabolic genes, and also to monitor the genotypic diversity among the *Pseudomonas* sp. soil isolates known to be capable of degrading MCA. To this end, we have used gene probe methodology in conjunction with Southern blotting. The hybridization experiments with the gene probes of two specific haloalkanoic acid dehalogenases were designed as described in the Materials and Methods. Chromosomal DNA samples were digested with *Bam*HI and electrophoretically separated restriction fragments were blotted onto nylon membranes. DIG labelled *dehCI* and *dehCII* genes were used as probes in Southern blot analysis of the total DNA.

Hybridizations with each probe were carried out separately at different temperatures ranging from 30 °C to 60 °C, and at two different concentrations of the probe DNAs (500 ng and 1 μg). In the majority of the blots, the detectable signals with no background hybridization (when the control lanes were considered), were obtained when excess amounts of

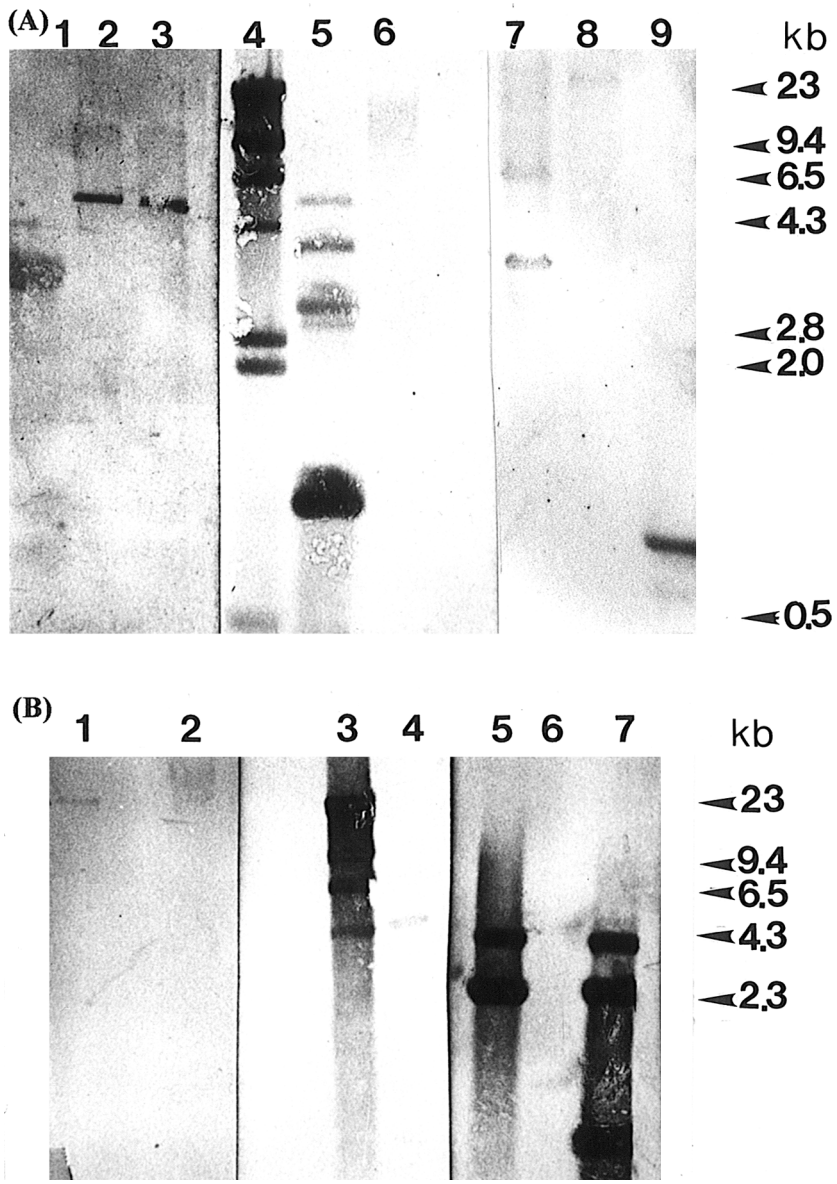

Figure 2. Detection of MCA degrading *Pseudomonas* soil isolates by Southern blot analysis. DNA from soil *Pseudomonas* sp. was digested with *Bam*HI, size fractioned by electrophoresis, and hybridized with two gene probes as described in the Materials and Methods.
Panel A. Hybridization bands obtained with *deh*CI probe. Lanes 2,3,7 and 8: template DNAs from isolates No.23, 42, 52 and 15, respectively; lanes 1,5 and 9: linearized pUKS 202 plasmid, undigested pUKS 202 plasmid and *deh*CI gene isolated from gel, respectively, as positive controls; lane 6: template DNA from *E. coli* TG1, as negative control. Lane 4: *Hind* III digested DNA from phage lambda, as size standard.
Panel B. Hybridization bands obtained with *deh*CII gene probe, lane 1 and 4, template DNA from isolates No. 29 and 34, respectively; lane 2: template DNA from *E. coli* TG1, as negative control; lanes 5 and 7: *Bam*HI digested pUKS107 plasmid and undigested pUKS plasmid, respectively, as positive controls; lane 6: *deh*CI gene probe isolated from gel. Lane 3: *Hind*III digested DNA from phage lambda, as size standard.

the probes (1 μg) were used in the hybridizations at 45 °C. From 61 DNA samples from the test isolates, 24 yielded bands on their membrane blots with either of the *dehCI* or *dehCII* probes. Some representative Southern hybridization pictures are presented in Figure 2. The DNA samples from a majority of test isolates that produced a signal, hybridized with only *dehCI* probe, while a small number of the target DNAs hybridized with only *dehCII* probe (Table 2). On the basis of their hybridization patterns, the 61 *Pseudomonas* sp. isolates were placed into 3 groups: Hybridization group I, which included 18 isolates that exhibited sequence homology only with *dehCI* probe; Hybridization group II, which included 6 isolates that exhibited homology only with *dehCII* probe; and Hybridization group III which included 37 isolates that did not hybridize to any of the probes used. Most of the DNA samples gave single bands rather than multiple bands with either of the probes used. Multiple hybridization signals in the blots of DNAs from some strains might indicate the presence of more than one copies of haloacid dehalogenase genes which are homologous to *dehCI* or *dehCII*, in the same organism or different organizations of the MCA degrading genes in the genomes of different strains.

The frequency of the *dehCI* genotype among the MCA degrading *Pseudomonas* sp. were 3 times higher than that of the *dehCII* genotype. DNA from some bacterial strains did not hybridize with either of the gene probes. In the later group, organisms with different DNA sequences for MCA degradation than those detected by the gene probes we have used, should exist. On the other hand, some of the *dehCI* hybridized genotypes had shared common bands (eg., 25.1 kb and 23 kb fragments) in their hybridization blots, suggesting that these organisms carry the genes encoding putative dehalogenase enzymes with a similar organization.

With *dehCII* gene probe only low degraders (Cl^-release ranged between 0.6 and 10.2 ppm) were detected while *dehCI* gene probe produced hybridization signals with DNA samples from poor, medium and high degraders (Table 2). No correlation exists between the specific hybridization pattern obtained with *dehCI* probe and degradative capacities of the strains as revealed by the amount of Cl^- liberated.

These observations may indicate the high specificity of the DNA hybridization method since *dehCI* and *dehCII* homologous sequences could only be detected with their specific DNA probes, but not with both. Our results also implied an abundance of metabolic and/or genotypic diversity among the MCA degrading *Pseudomonas* sp. in the test soils. Similar genotypic heterogeneity has been shown in DNA homology studies with PCB degrading bacteria, when several gene probes for PCB degrading genotypes were used in the analysis of purified DNA from PCB contaminated soil bacterial community (Taira et al. 1988; Mondello 1989; Walia et al. 1990). Ka et al. (1994) analyzed DNA samples from soil bacterial community after 2,4-D treatment on slot blots and Southern blots by using two gene probes, each

Table 2. Hybridization patterns obtained with *dehCI* and *dehCII* gene probes and the amounts of free Cl^- released by *Pseudomonas* sp. soil isolates after a 3 day incubation in liquid minimal medium supplemented with 185 ppm MCA

Pseudomonas sp. Strain Number	Free $C1^-$ release* (ppm)	DNA fragments (MW kb) hybridized with *dehCI* probe
4	0.3	9.4; 6.5
8	16.5	21.5
9	6.02	50; 23; 10.7
11	3.9	17.1
13	8.0	23.0
14	4.5	25.1
15	20.9	23.0
16	6.2	25.1
21	4.4	25.1
22	20.1	15.8
23	44.5	4.5
40	40.4	25.1; 18.5
42	7.1	5.3
51	1.2	3.2
52	1.5	6.5; 3.2
53	38.5	9.4
58	0.5	13.5
60	1.7	5.2; 3.1; 2.2
		with *dehCII* probe
3	0.6	6.5
29	0.6	23.1
34	4.9	5.4
39	9.6	23.1; 12.5; 6.8; 1.9
45	2.6	3.6
48	5.4	14.6; 6.8; 2.9

* Cl^- release was measured spectrophotometrically as described in the Materials and Methods and strains were classified as poor degraders (Cl^- release: 0–15 ppm), medium degraders (Cl^- release: 16–30 ppm) and high degraders (Cl^- release: 31–45).

being isolated from a different 2,4-D degrading isolate. Their results indicated a diversity among the 2,4-D degrading natural populations, since a dominant 2,4-D degrading population that does not exhibit sequence homology with the *tfd* probe could be detected by *spa* probe, or *vice-versa*. Therefore, a single probe may not be sufficient to monitor native bacterial populations with a specific degradative function, unless the target genes have highly conserved sequences. There are several haloalkanoic acid dehalogenases isolated from a wide variety of microorganisms degrading various halogenated compounds. To date, in addition to *dehCI* and *dehCII*, seven members of the 2-haloacid dehalogenases from various microorganisms were identified and their genes (*had L, dehH2, dehIB, deh*H109, *hdlIVA*, *deh*19S and *L-DEX*) have been cloned (Kawasaki et al. 1992; Barth et al. 1992; Ploeg et al. 1991; Murdiyatmo et al. 1992; Kawasaki et al. 1994; Nardi-Dei et al. 1994; Kocabıyık et al. 1995). To maximize the successful application of the Southern blot analysis, for tracking and identifying MCA degrading genotypes in soil microbial community, the use of alternative gene probes from different dehalogenases may be helpful.

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