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Microbial Community Profiling in *cis*- and *trans*-Dichloroethene Enrichment Systems Using Denaturing Gradient Gel Electrophoresis

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Abstract The effective and accurate assessment of the total microbial community diversity is one of the primary challenges in modern microbial ecology, especially for the detection and characterization of unculturable populations and populations with a low abundance. Accordingly, this study was undertaken to investigate the diversity of the microbial community during the biodegradation of *cis*- and *trans*-dichloroethenes in soil and wastewater enrichment cultures. Community profiling using PCR targeting the 16S rRNA gene and denaturing gradient gel electrophoresis (PCR-DGGE) revealed an alteration in the bacterial community profiles with time. Exposure to *cis*- and *trans*-dichloroethenes led to the disappearance of certain genospecies that were initially observed in the untreated samples. A cluster analysis of the bacterial DGGE community profiles at various sampling times during the degradation process indicated that the community profile became stable after day 10 of the enrichment. DNA sequencing and phylogenetic analysis of selected DGGE bands revealed that the genera *Acinetobacter*, *Pseudomonas*, *Bacillus*, *Comamonas*, and *Arthrobacter*, plus several other important uncultured bacterial phylotypes, dominated the enrichment cultures. Thus, the identified dominant phylotypes may play an important role in the degradation of *cis*- and *trans*-dichloroethenes.

Keywords: Dichloroethene, PCR-DGGE, microbial diversity, 16S rDNA, bacterial community profiling

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It is an established dictum of microbial ecology that the majority of microbial species in nearly all microbial communities are unculturable [4], mainly because selective enrichment cultures fail to mimic the conditions that particular microorganisms require for proliferation in their natural habitat [35]. Environmental microbiologists have therefore begun to explore the use of molecular methods to circumvent the bias of culture-dependent techniques. In particular, the sequence variation of the rRNA gene has been exploited for inferring phylogenetic relationships among microorganisms [55] and designing specific nucleotide probes to determine the genetic diversity of microbial communities and identify several uncultured microorganisms [19, 53]. The sequence variation of the 16S rRNA genes in community DNA can be revealed by the PCR amplification, construction, and sequencing of a clone library, or by techniques such as t-RFLP, SSCP, DGGE, and TGGE [2, 11, 19, 31, 53].

Denaturing gradient gel electrophoresis is an electrophoretic separation method based on the differences in melting behavior of double-stranded DNA fragments [15]. When separated by electrophoresis through a polyacrylamide gel with an increasing gradient of a chemical denaturant (usually formamide and urea), the mobility of the molecule is retarded at the denaturant concentration where the DNA melts [35, 36]. DNA sequence variations cause the melting temperatures to differ and the method is sensitive enough to detect a single base change [15, 35]. The sequence variation of PCR-amplified 16S rRNA fragments from mixed microbial populations produces unique DGGE

banding patterns: a DNA community fingerprint [30], where the number, position, and intensity of the bands reflect the number and relative abundance of dominant rRNA gene types in the sample, thereby facilitating a comparison of different microbial communities [9, 10, 36, 40, 44]. Although a number of methodological limitations have been identified, PCR-DGGE is generally accepted to provide a fingerprint of the dominant phylotypes in natural habitats [24, 34, 49, 51], as well as in enrichment and mixed culture systems [25, 29, 36], and has been successfully applied to monitor spatial and temporal differences in bacterial communities [9, 10, 36, 40, 44].

The frequently observed accumulation of dichloroethenes (DCEs) as the dominant products of microbial reductive dechlorination of higher chloroethenes (tetrachloroethene and trichloroethene) in soil and water continues to be of significant concern worldwide. The innate toxicity of *cis*- and *trans*-DCEs and their tendency to be reduced to vinyl chloride, a known human carcinogen, has warranted investigations into their fate in contaminated environments, resulting in several reports on the biotransformation of these compounds in both enrichment and microcosm settings [7, 8, 27, 41, 42]. Yet, there is little information on the effect of these compounds on the diversity of microorganisms. Accordingly, in this study, DGGE was used to assess the effect of *cis*- and *trans*-DCEs on the microbial population and biodiversity in soil and wastewater enrichments. Sequencing of the dominant DGGE bands revealed an important group of organisms, which could play a dominant role in the natural attenuation of these compounds in polluted soil and groundwater systems.

MATERIALS AND METHODS

Enrichment Cultures

The soil samples used in this study were collected from different locales to represent two soil types, whereas the wastewater samples were collected from the Northern and New Germany wastewater treatment plants in Durban, South Africa. The samples of soil (10%, w/v) or wastewater (10%, v/v) were mixed with a minimal salt medium (MSM) to give a total volume of 150 ml in 250-ml serum bottles (headspace, 100 ml of air), which were crimp-sealed with Teflon-faced butyl rubber stoppers (Wheaton), and amended with either *cis*- or *trans*-DCEs as the sole carbon source up to a final substrate concentration of 0.5 mM. The MSM used was that of Hartmans *et al.* [21], which was modified by reducing the phosphate, ammonium, and chloride concentrations to 20 mM, 10 mM, and 0.02 mM, respectively [13]. The bottles were incubated aerobically at 26±2°C with shaking at 150 rpm, and the total heterotrophic bacterial population analyzed at different sampling times by plating 100 µl of appropriate culture dilutions on a minimal salt agar medium with DCE as the carbon source, using a standard spread plate technique [18, 46]. The plates were incubated at 28°C for 48 h before estimating the bacterial population. The compound degradation was monitored using gas chromatography, as described below.

Analytical Methods

The amount of *cis*- and *trans*-DCEs degraded in the different enrichments was monitored based on a headspace sample analysis using a gas chromatograph (Varian model 3700), as described previously [13, 47]. As such, 100 µl headspace samples were injected using a gas-tight syringe (Hamilton) into a gas chromatograph equipped with a flame ionization detector. The samples were analyzed with the injector and detector at 200°C and the column at 100°C. The *cis*- and *trans*-DCE concentrations were quantified by comparison with a standard curve derived from known quantities of the compounds in serum bottles with the same gas and liquid volumes as the experimental bottles. To allow for an equilibration of the compounds between the gas and aqueous phases, the bottles were incubated at 30°C on a rotary shaker at 150 rpm for at least 2 h before determining the initial concentrations of the compounds. Uninoculated MSM containing *cis*- or *trans*-DCEs was used as the negative control to reveal compound concentrations above the losses of the negative control. The degradation rate constant in each enrichment was estimated according to LaGrega *et al.* [28].

DNA Extraction, Purification, and PCR

Total DNA was isolated and purified from the soil and wastewater samples at different sampling times using a QIAquick DNA Purification Kit (QIAGEN, Germany), following the manufacturer's instructions, and used directly for the PCR. The PCR amplification reaction mixture contained 20 ng of purified DNA (or 1 µl of the first-round PCR), 200 µM dNTPs, 0.5 µM of each primer, and 1.5 U *Taq* polymerase in a 1×PCR buffer (20 mM Tris-HCl, pH 8.8; 10 mM KCl; 10 mM $(NH_4)_2SO_4$; 0.1% w/v Triton X100) with 2 mM $MgSO_4$. The universal DGGE primer set 341F (CCTACGGGAGGCAGCAG) with a 5' GC clamp: CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCA CGGGGGG and 534R (ATTACCGCGGCTGCTGG) were used in the PCR. A modified form [35] of the touchdown thermal profile technique [54] was used: an initial denaturation (94°C, 5 min), followed by 20 cycles of 94°C, 45 s; 65°C, 45 s; and 72°C, 2 min with a decrease in the annealing temperature of 0.5°C per cycle. This was followed by 20 cycles of 94°C, 30 s; 55°C, 30 s; and 72°C, 2 min; plus a final 10 min extension step at 72°C [54]. The amplification of the PCR products to the correct size was confirmed by electrophoresis in a 1.5% (w/v) agarose gel in a 1×TAE buffer, followed by staining with ethidium bromide.

Table 1. Total heterotrophic bacterial population (CFU/ml×10^5) in *cis*-DCE enrichment cultures.

Sampling time (d)	Soil AE	Soil BF	Wastewater CG	Wastewater DH
0	2.99±0.23	7.15±1.131	18.99±1.794	0.203±0.045
3	71.5±1.20	108.25±3.08	229.17±0.99	25.75±1.17
5	221.5±7.57	52.5±1.77	344.0±0.85	53.25±0.95
7	170.0±2.0	44.0±0.85	53.5±1.63	37.0±0.35
10	171.25±3.64	9.35±0.16	29.25±0.67	14.25±0.035
15	213.1±7.14	14.75±0.32	33.4±0.34	13.3±0.81

AE, Clay soil; BF, Humus soil; CG, Northern wastewater; DH, New Germany wastewater.
Values are means of triplicate data±standard deviation.

Denaturing Gradient Gel Electrophoresis (DGGE)

The denaturing gradient gel electrophoresis (DGGE) was performed according to the method of Muyzer *et al.* [35] and Muyzer [37]. The PCR amplicons (10 µl) were separated on 16.5×16.5 cm, 1-mm-thick 9% polyacrylamide (37.5:1 [w/v] Acrylamide:Bisacrylamide, Fluka) gels with urea/formamide denaturing gradient (BioRad 475 gradient former and Sciplas V20-HCDC) apparatus. The polyacrylamide gel was made with a denaturing gradient ranging from 40 to 60% (where 100% denaturant contained 7 M urea and 40% [v/v] formamide), and the electrophoresis carried out at 100 V for 16 h at 60°C in a 1×TAE buffer (20 mM Tris, 10 mM acetate, 0.5 mM EDTA [pH 7.4]). The gels were stained for 10 min in ethidium bromide (0.5 µg/ml in 1×TAE buffer), destained in 1×TAE for 15 min, and visualized with a 312 nm wavelength transilluminator (Spectroline).

Analysis of DGGE Banding Patterns

The statistical comparison of the different DGGE patterns was carried out using GelCompar software 4.1, the similarity among the band patterns calculated using Dice's similarity coefficient, as described elsewhere [14, 48], and the clustering algorithm of Ward [52] used to construct the dendograms.

Sequencing and Analysis of DGGE Fragments

The DNA fragments to be sequenced were excised from the gel with sterile razors and placed into sterile Eppendorf tubes containing 1 ml of sterile deionized water. The gels were briefly washed by centrifuging at 16,000 ×*g* for 1 min and the supernatant aspirated. Next, 50 µl of a TE buffer (10 mM Tris-HCl; 1 mM EDTA, pH 8.0) was added and the microfuge tubes left overnight to elute the DNA. The eluted DNA then served as the template for PCR amplification under the same PCR conditions described above. Thereafter, the amplified products were subjected to a new DGGE step to confirm their electrophoretic mobility, and then the re-amplified bands were gel-purified (GFX gel band kit, Amersham) and cloned into pTZ57R/T (InsT/Aclone, Fermentas), according to the manufacturer's instructions, while using 1/3 ligation reaction volumes. The amplicons were compared again with the original sample by DGGE before sequencing. The automated sequencing of the cloned genes was performed by a Spectrumedix SCE2410 genetic analysis system with 24 capillaries using the universal M13 forward primer. For the sequencing reactions, a Big Dye version 3.1 dye terminator cycle sequencing kit (Applied Biosystems) was used. All the reactions were performed according to the manufacturer's instructions. The raw sequencing data were manually edited using DNAMAN software to remove the vector sequences. The 16S rRNA gene sequences of the bacterial isolates were then compared with those in the GenBank database using BLAST [5] to determine the most similar sequences.

RESULTS

Microbial Diversity in the Enrichment Cultures

The total heterotrophic bacterial population density of the different enrichments is shown in Tables 1 and 2, which

Table 2. Total heterotrophic bacterial population (CFU/ml×10^5) in *trans*-DCE enrichment cultures.

Sampling time (d)	Soil AE	Soil BF	Wastewater CG	Wastewater DH
0	3.12±0.535	4.232±0.908	17.217±2.146	0.330±0.048
3	142.5±2.47	98.75±1.24	192.0±2.83	23.67±0.96
5	139.5±2.90	68.0±0.99	284.0±2.536	38.0±0.28
7	33.0±0.28	31.0±0.14	49.0±0.14	25.5±0.071
10	95.5±2.05	27.4±0.34	66.15±2.17	5.80±0.18
15	164.75±3.29	15.6±0.34	25.25±0.53	10.35±0.21

AE, Clay soil; BF, Humus soil; CG, Northern wastewater; DH, New Germany wastewater.
Values are means of triplicate data±standard deviation.

revealed an alteration in the population of the microbial communities subsequent to the addition of *cis*- and *trans*-DCEs. The peak bacterial cell density ranged from 108.25 to 221.5×10^5 CFU/ml (Table 1) and 98.75 to 164.75×10^5 CFU/ml (Table 2) in the soil enriched with *cis*- and *trans*-DCEs, respectively. Similarly, the bacterial population peaked between 53.25 and 344.0×10^5 CFU/ml in the *cis*-DCE-enriched wastewater samples (Table 1), and between 38.0 and 284.0×10^5 CFU/ml in the *trans*-DCE-enriched wastewater samples (Table 2). However, the population densities based on the total heterotrophic count did not seem to follow any regular pattern, although the growth appeared biphasic with an initial increase in bacterial density, followed by a decrease with time.

PCR-DGGE Analysis of Bacterial Community Structure

In parallel with the culture-dependent approaches, DGGE analysis of PCR-amplified 16S rDNA fragments was also used to investigate the effect of *cis*- and *trans*-DCEs on the soil and wastewater microbial communities. The banding patterns for the different enrichments, as shown in Figs. 1A–8A, did not demonstrate much difference in bacterial

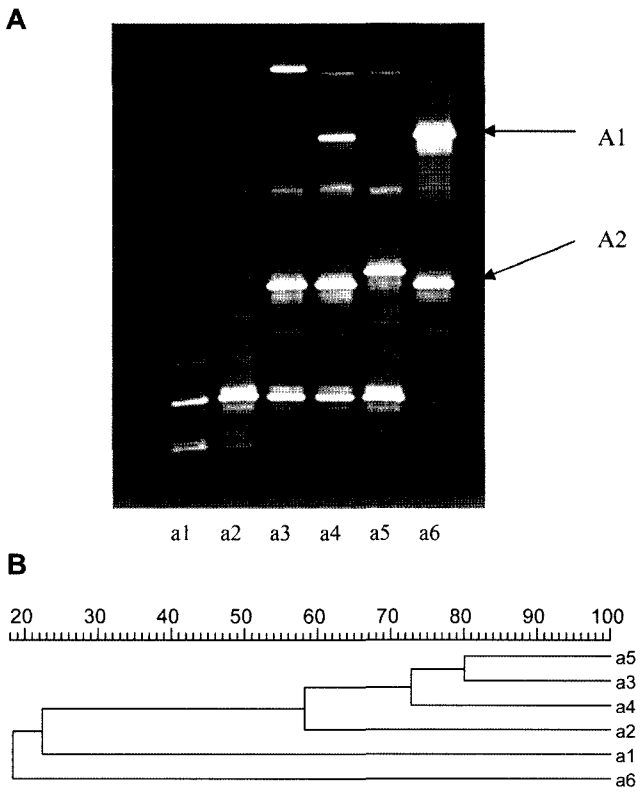

Fig. 1. A. DGGE profiles of 16S rDNA gene fragments of soil sample AE enriched with *cis*-DCE. Lanes: a1, a2, a3, a4, a5, and a6 represent days 0, 3, 5, 7, 10, and 15 of the enrichment, respectively. **B.** Dendogram revealing relatedness of PCR-DGGE fingerprints from the gel in Fig. 1A, using Dice's similarity coefficient.

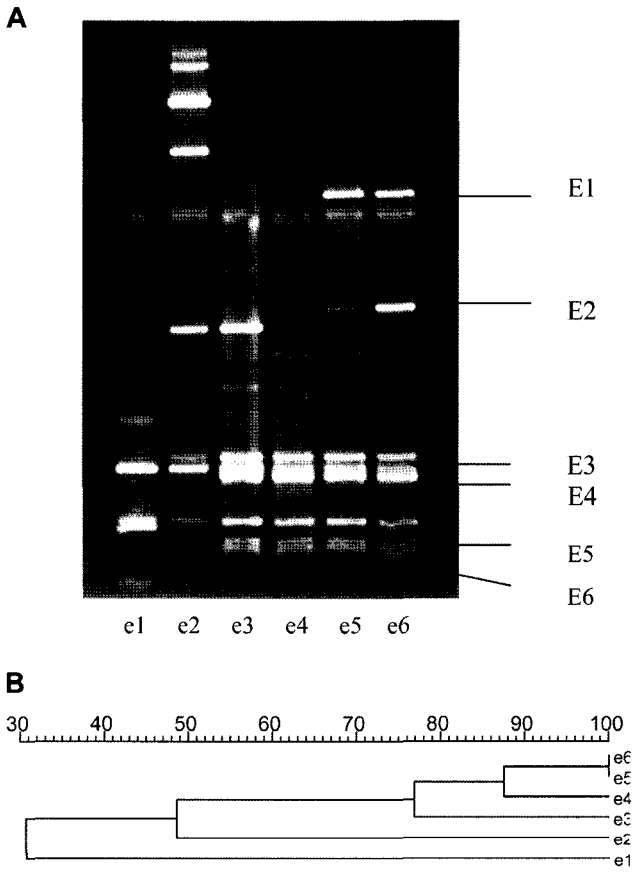

Fig. 2. A. DGGE profiles of 16S rDNA gene fragments of soil sample AE enriched with *trans*-DCE. Lanes: e1, e2, e3, e4, e5, and e6 represent days 0, 3, 5, 7, 10, and 15 of the enrichment, respectively. **B.** Dendogram revealing relatedness of PCR-DGGE fingerprints from the gel in Fig. 2A, using Dice's similarity coefficient.

diversity between the *cis*- and *trans*-DCE enrichments of the same sample (soil or wastewater). The analysis also indicated the appearance and disappearance of certain genospecies during the enrichment culture. In soil sample AE (clay soil) enriched with *cis*-DCE (Fig. 1A), the presence of two sharp bands (A1 and A2), representing the dominant degrading population, was observed on day 15 of the degradation process. These bands were absent on day 0. Band A1 first appeared on day 7, and became sharper by day 15, indicating that the organism had been enriched. Meanwhile, band A2 was first noticed on day 5 of the degradation process and maintained its dominance (indicated by the brightness of the band) until the last day of the degradation process. In the *trans*-DCE enrichment of the same soil type, six new bands (E1, E2, E3, E4, E5, and E6) emerged on day 15, which were either completely absent, or present at very low concentrations on day 0 (Fig. 2A). In soil sample BF (humus soil), the banding patterns observed for the *cis*- and *trans*-DCE enrichments seemingly exhibited a great similarity (Figs. 3A and 4A). In both cases, two

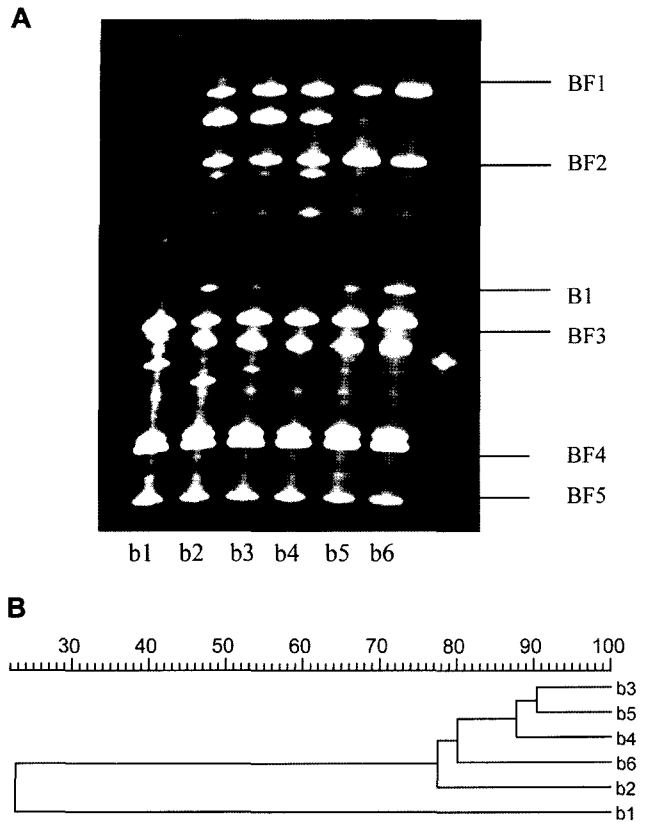

Fig. 3. A. DGGE profiles of 16S rDNA gene fragments of soil sample BF enriched with *cis*-DCE. Lanes: b1, b2, b3, b4, b5, and b6 represent days 0, 3, 5, 7, 10, and 15 of the enrichment, respectively. **B.** Dendogram revealing relatedness of PCR-DGGE fingerprints from the gel in Fig. 3A, using Dice's similarity coefficient.

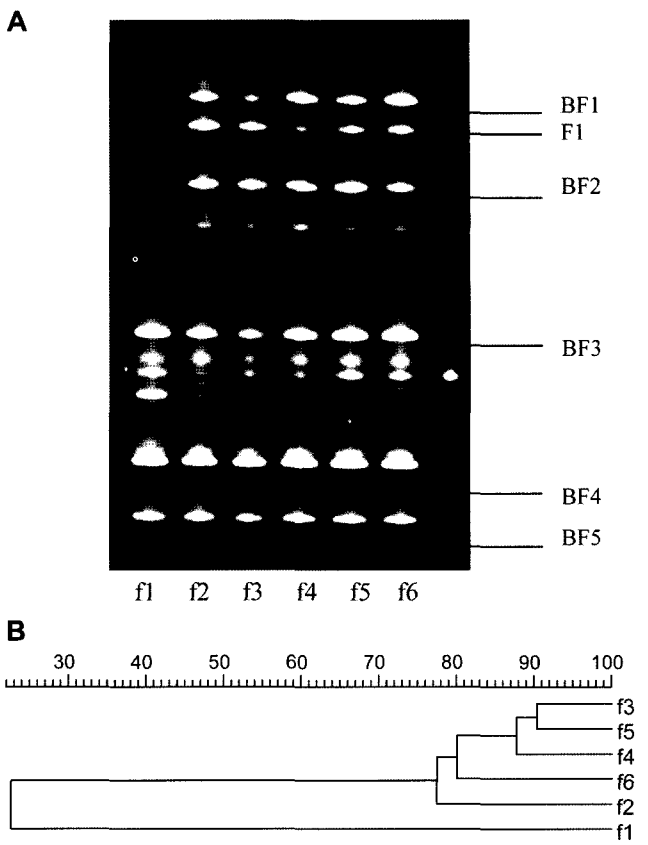

Fig. 4. A. DGGE profiles of 16S rDNA gene fragments of soil sample BF enriched with *trans*-DCE. Lanes: f1, f2, f3, f4, f5, and f6 represent days 0, 3, 5, 7, 10, and 15 of the enrichment, respectively. **B.** Dendogram revealing relatedness of PCR-DGGE fingerprints from the gel in Fig. 4A, using Dice's similarity coefficient.

sharp bands (BF1 and BF2) appeared from day 3 and maintained a high band intensity until day 15. Three additional bands (BF3, BF4, and BF5) were also present from the beginning to the end of the degradation process. In additon, band B1 was only observed in the *cis*-DCE enrichment (Fig. 3A), whereas band F1 was only observed in the *trans*-DCE enrichment (Fig. 4A).

In the wastewater (CG) enriched with *cis*- and *trans*-DCEs (Figs. 5A and 6A), four dominant bands (CG1, CG2, CG3, and CG4) were observed to be common to both enrichments and absent on day 0 of the reaction process, except for band CG3 that appeared faintly on day 0. Three additional bands (G1, G2, and G3) were also noted in just the *trans*-DCE enrichment (Fig 6A), with only band G1 noted on day 0, while the other two bands (G2 and G3) were most prominent towards the end of the enrichment period. In wastewater DH, the addition of *cis*- and *trans*-DCEs resulted in the enrichment of seven organisms, represented by bands DH1, DH2, DH3, DH4, DH5, DH6, and DH7, all of which were common to both enrichments (Figs. 7A and 8A). However, enrichment with *cis*-DCE also resulted in two additional bands [D1 and D2] (Fig. 7A), whereas three extra bands (H1, H2, and H3) were identified in the *trans*-DCE enrichment DGGE gel (Fig. 8A).

Cluster Analysis of DGGE Banding Patterns

The cluster analysis of the DNA banding patterns at various sampling times during the degradation process in the enrichment cultures is represented in Figs. 1B–8B. The banding patterns of the gel with soil type AE enriched with *trans*-DCE (Fig. 2A) revealed a 100% similarity between days 10 and 15 of the enrichment (Fig. 2B). Similarly, lanes 5 and 6, representing days 10 and 15 of the enrichment, respectively, clustered with a very high similarity matrix in most cases. For example, similarities of up to 74.5% were observed when lane c5 was compared with lane c6 (Fig. 5B), up to 91.5% between lanes g5 and g6 (Fig. 6B), and up to 75.5% between lanes d5 and d6 (Fig. 7B) and lanes h5 and h6 (Fig. 8B), indicating the importance of the 10^{th} day of enrichment as regards the stability of the dominant microbial populations.

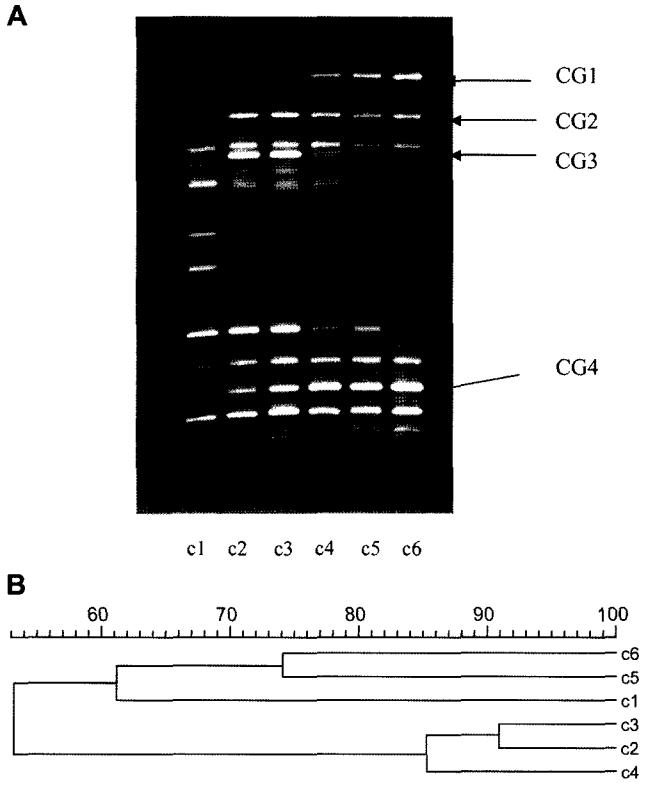

Fig. 5. A. DGGE profiles of 16S rDNA gene fragments of wastewater sample CG enriched with *cis*-DCE. Lanes: c1, c2, c3, c4, c5, and c6 represent days 0, 3, 5, 7, 10, and 15 of the enrichment, respectively. **B.** Dendogram revealing relatedness of PCR-DGGE fingerprints from the gel in Fig. 5A, using Dice's similarity coefficient.

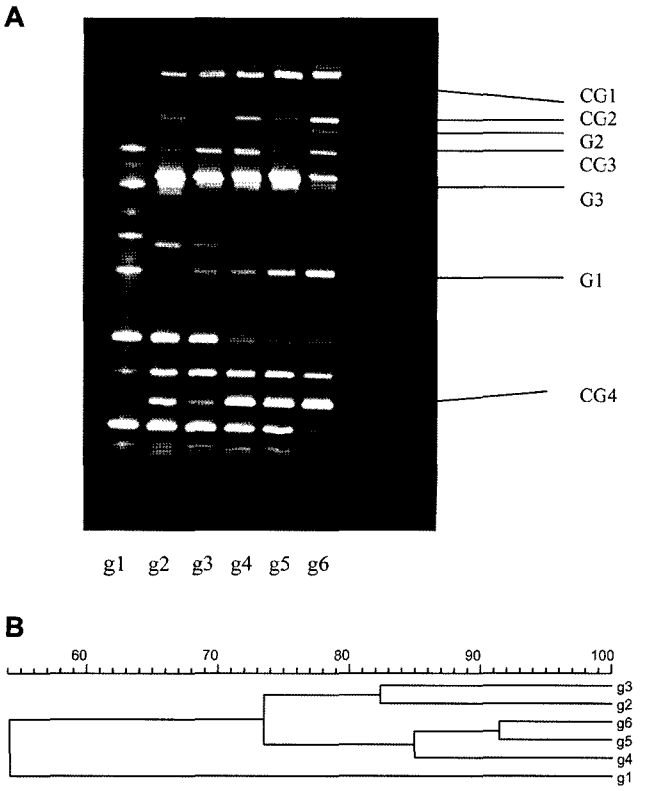

Fig. 6. A. DGGE profiles of 16S rDNA gene fragments of wastewater sample CG enriched with *trans*-DCE. Lanes: g1, g2, g3, g4, g5, and g6 represent days 0, 3, 5, 7, 10, and 15 of the enrichment, respectively. **B.** Dendogram revealing relatedness of PCR-DGGE fingerprints from the gel in Fig. 6A, using Dice's similarity coefficient.

Phylogenetic Analysis of Sequenced 16S rDNA Genes from Representative DGGE Clones

The DNA sequencing of selected DGGE bands and BLAST similarity searches against a nucleic acid database enabled the identification of the dominant genospecies, where bands A1 (Fig. 1A), BF2 (Figs. 3A and 4A), CG2 (Figs. 5A and 6A), and DH2 (Figs. 7A and 8A) exhibited a 100% similarity to *Pseudomonas putida* KT 2440 (Accession No. AE016778) and *Pseudomonas* sp. AN 30 (Accession No. AY734880), recently characterized for aniline degradation. The sequences also showed a 100% similarity to the uncultured soil bacterium clone SAL 2d22 (Accession Number AY699600) found in a bacterial mixture degrading salicylate, naphthalene, and phenanthrene in a bioreactor treating contaminated soil. Similarly, the sequences of bands E1 (Fig. 2A) and DH3 (Figs. 7A and 8A) were found to be 98% similar to both *Pseudomonas* sp. k2 (Accession No. AF532866), a novel N-acylhomoserine lactone-degrading bacterium [50], and uncultured gamma-proteobacterium clone DGGE band 1B found among the microbial community in a MTBE-contaminated shallow aquifer [22]. The organisms represented by bands A2 (Fig. 1A) and BF3 (Figs. 3A and 4A) were found to be *Bacillus licheniformis*, whereas the sequence for BF1 (Figs. 3A and 4A) was 99% similar to that for *Bacillus fusiformis* (Accession No. AY803975) and *Bacillus fusiformis* strain HPC 709, isolated from activated sludge from an effluent treatment plant that was treating nitroaromatic wastewater and pharmaceutical industrial wastewater. However, band E2 (Fig. 2A) observed in soil type AE enriched with *trans*-DCE was found to be 98% similar to *Acinetobacter calcoaceticus* (Accession No. AB046736), previously identified in an organic wastewater treatment process [23] and *Acinetobacter* sp. w-17 (Accession No. AF390089), a phenol-degrading bacterium [3].

Furthermore, analysis of the 16S rDNA gene sequences of the DGGE bands revealed that the organism represented by band CG1 (Figs. 5A and 6A) was a member of the *Arthrobacter* family, with a 100% similarity to *Arthrobacter nicotinovorans* strain ph 49 (Accession No. AY833102) and *Arthrobacter histidinolovorans* strain HAMB12383 (Accession No. AF501360), both isolated from an oil-contaminated site. The sequences for CG2 (Figs. 5A and 5B), H1 (Fig. 8A), and G2 (Fig. 6A) all showed a 100% similarity to *Acinetobacter calcoaceticus* subsp. *anitratus*

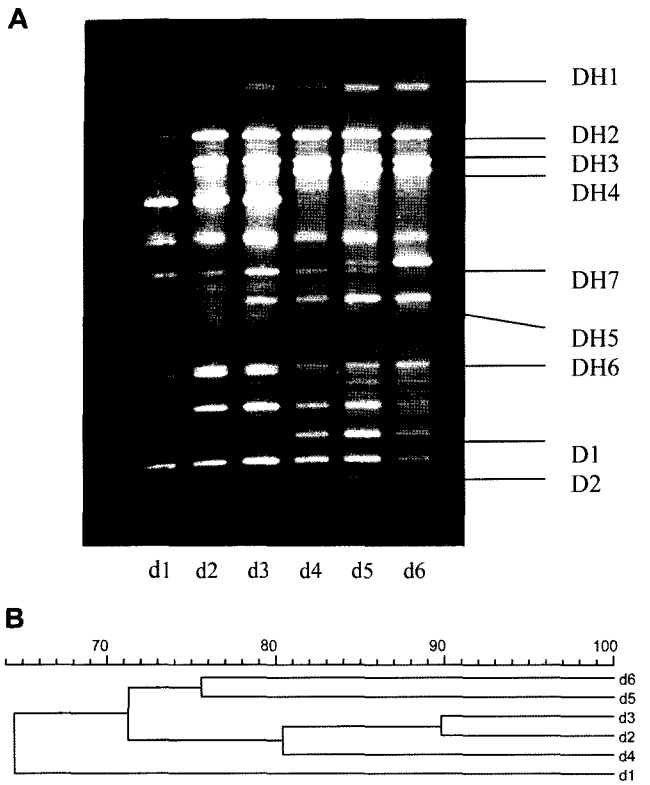

Fig. 7. A. DGGE profiles of 16S rDNA gene fragments of wastewater sample DH enriched with *cis*-DCE. Lanes: d1, d2, d3, d4, d5, and d6 represent days 0, 3, 5, 7, 10, and 15 of the enrichment, respectively. **B.** Dendogram revealing relatedness of PCR-DGGE fingerprints from the gel in Fig. 7A, using Dice's similarity coefficient.

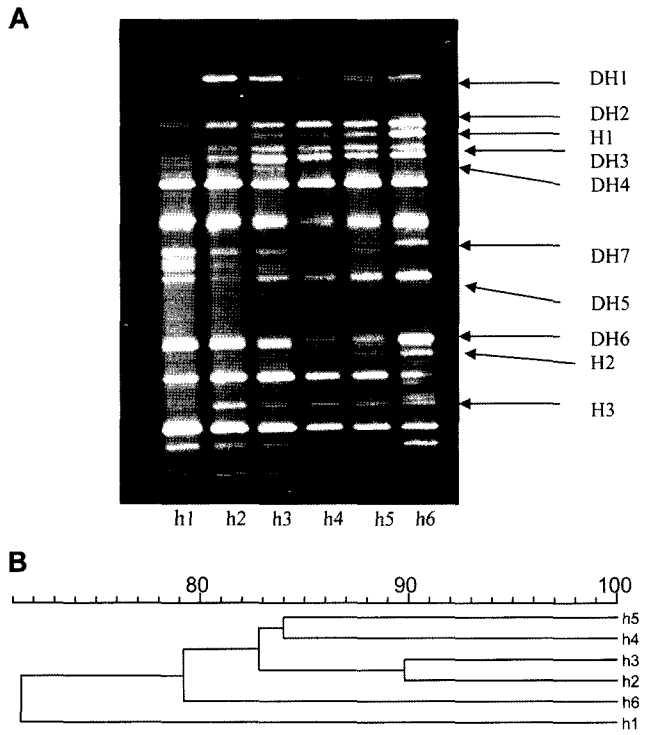

Fig. 8. A. DGGE profiles of 16S rDNA gene fragments of wastewater sample DH enriched with *trans*-DCE. Lanes: h1, h2, h3, h4, h5, and h6 represent days 0, 3, 5, 7, 10, and 15 of the enrichment, respectively. **B.** Dendogram revealing relatedness of PCR-DGGE fingerprints from the gel in Fig. 8A, using Dice's similarity coefficient.

strain 2910 (Accession No. AY 738399). The DGGE bands CG3 (Figs. 5A and 6A) and DH4 (Figs. 7A and 8A) exhibited sequences that were 100% similar to those for *Pseudomonas putida* strains, DSM 3601 (Accession No, AF509330) and CR7 (Accession No. AY785244), whereas the sequence for DH5 (Figs. 7A and 8A) was 99% similar to that for both *Bacillus cereus* strain ATCC 25621 (Accession No. AY795568) and the uncultured soil bacterium clone TCA1 (Accession No. AY 242738) isolated from metabolically active bacteria in heavy metal-contaminated bulk and rhizosphere soil [20]. However, the sequence for band CG4 (Figs. 5A and 6A) revealed a 98% similarity to *Comamonas testosteroni* strain CNB1 (Accession No. AY 291591), recently found to carry a novel 2-aminophenol 1,6-dioxygenase involved in the degradation of *p*-chloronitrobenzene [56].

Biodegradation of *cis*- and *trans*-Dichloroethenes in Culture Enrichment

The biodegradation patterns of *cis*- and *trans*-DCEs in the different enrichment cultures are shown in Figs. 9 and 10. Both compounds were degraded to varying degrees with the degradation rate constants ranging between 0.0609–0.0828 d^{-1} and 0.0827–0.165 d^{-1} for *cis*- and *trans*-DCEs, respectively (Table 3), representing 10–20 times more than that in the control. *Trans*-DCE was found to disappear faster from both the soil and wastewater samples, with up to 91.45% and 78.35% being degraded, respectively, after 15 days (Fig. 10). In contrast, up to 66.38% and 71.10% of *cis*-DCE was degraded in the soil and wastewater samples, respectively (Fig. 9).

Discussion

Various morphological, biochemical, and genetic characteristics have been exploited to identify constituents in a complex population of microorganisms. In particular, the 16S rDNA sequence divergence of different bacterial species has been utilized as an indicator of diversity. To assess this diversity, PCR amplification of the 16S rDNA [33], along with cloning and sequence analysis of selected clones, has been previously applied [19]. However, these approaches only provide qualitative information about the population composition and only after an extensive analysis of large numbers of clones. Thus, species that constitute a low percentage of the population are not readily detectable in

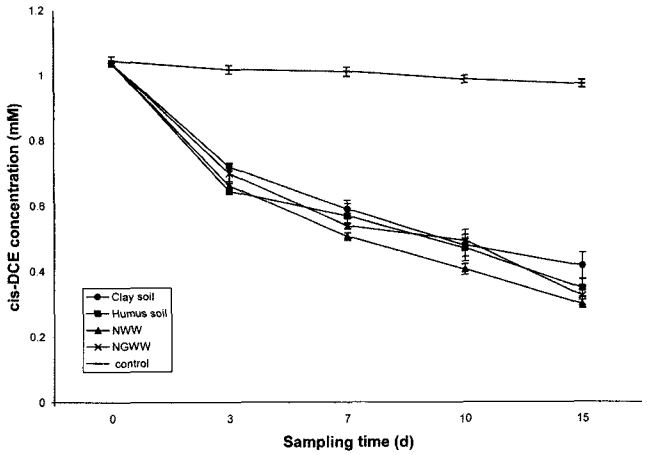

Fig. 9. Biotransformation of *cis*-DCE in soil and wastewater enrichment cultures.

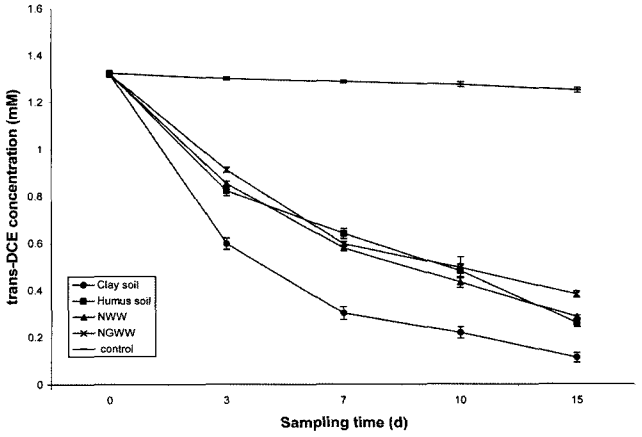

Fig. 10. Biotransformation of *trans*-DCE in soil and wastewater enrichment cultures.

this way. Despite typically underestimating the number of species present because of a failure to detect minor genospecies in highly diverse populations, the bias of DNA extraction and PCR amplification, and an inherent limited resolving power [17, 32, 35, 37], the DGGE technique has proven invaluable for comparative community profiling [16, 35]. DGGE banding patterns provide population profiles based on the intensities of each band, which also represent the relative abundance of a particular species in the population [35]. Thus, DGGE can offer a reasonable picture of the differences between microbial communities, and has already been successfully used in a variety of settings [9, 10, 12, 36, 40, 44].

In this study, a combination of culture-dependent methods and the DGGE technique was used to gain a better understanding of the impact of *cis*- and *trans*-DCEs on soil and wastewater microbial communities. The results showed an alteration in the microbial communities with time after the addition of *cis*- and *trans*-DCEs. The banding patterns for the different enrichments, as determined by a PCR-DGGE analysis of the bacterial community structure, revealed few differences in the bacterial community profiles for the *cis*- and *trans*-DCE enrichment cultures of the same soil or wastewater samples, suggesting that the same organisms were able to utilize both isomeric forms of the compound.

Table 3. Degradation rate (d^{-1}) of *cis*- and *trans*-DCEs in different enrichments.

Sample	*cis*-DCE	*trans*-DCE
AE	0.0609±0.00717	0.165±0.0115
BF	0.0728±0.00511	0.109±0.00473
CG	0.0828±0.00282	0.102±0.00115
DH	0.0773±0.00649	0.0827±0.00271
Control	0.00421±0.0024	0.00383±0.000912

Values are means of triplicate data±standard deviation.

However, the more rapid degradation of *trans*-DCE compared with *cis*-DCE suggested that *trans*-DCE was the preferred substrate and that isomerization may have been required before *cis*-DCE was effectively utilized. The community profiling showed a general decrease in the number of DGGE bands during the time course of the enrichment, including the disappearance of some genospecies and the appearance of others, which would seem to indicate the selection of bacteria capable of utilizing and/or surviving the toxicity of the DCE. Similarly, the changes of DGGE patterns observed in forest soils contaminated with diesel also indicated a significant effect of diesel contamination on the indigenous microbial communities and, subsequently, leading to the selection of several microbial populations well-adapted to diesel contamination [1]. The bacterial community profile appeared stable after day 10 of enrichment, as revealed by a cluster analysis of the DGGE profiles, suggesting the establishment of a defined community for the degradation of DCE. The initial stimulation of growth probably reflected this selection of DCE-degrading bacteria, whereas the subsequent decrease in the rate of DCE degradation may have been due to substrate limitation, product inhibition, or the death of the DCE-degrading microbes caused by toxic metabolic products [7, 8, 27, 41].

To identify the bacterial taxa that were enriched with the addition of *cis*- and *trans*-DCEs, selected DGGE bands were sequenced and a database search conducted. The results revealed that the enrichment cultures were dominated by the genera *Acinetobacter*, *Pseudomonas*, *Bacillus*, *Comamonas*, and *Arthrobacter*, together with some other important uncultured bacterial phylotypes. Thus, since these bacterial groups were dominant in the enrichments, it is likely that they were involved in the observed transformation of DCE, although a definitive link between phylogeny and function could not be established from this

study. Therefore, future work will include the use of catabolic gene-specific primers to confirm the roles of these organisms in DCE degradation. Several previous studies have already shown the involvement of these bacterial groups in the degradation of various other xenobiotics [6, 13, 23, 26, 38, 39, 43, 45, 47], suggesting that they may play an important general role in xenobiotic degradation.

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References

1. Ahn, J., M. C. Kim, H. C. Shin, M. K. Choi, S. S. Yoon, T. Kim, H. G. Song, G. H. Lee, and J. O. Ka. 2006. Analysis of bacterial diversity and community structure in forest soils contaminated with fuel hydrocarbon. *J. Microbiol. Biotechnol.* **16:** 704–715.
2. Amann, R. L., W. Ludwig, and K. H. Schleifer. 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59:** 143–169.
3. Abd-El-Haleem, D., H. Moawad, E. A. Zaki, and S. Zaki. 2002. Molecular characterization of phenol-degrading bacteria isolated from different Egyptian ecosystems. *Microb. Ecol.* **43:** 217–224.
4. Atlas, R. M. and R. Bartha. 1987. *Microbial Ecology*, 2nd Ed. The Benjamin Cummings Publishing Co., Inc., Reading, Mass.
5. Benson, D. A., M. S. Boguski, D. J. Lipman, and J. Ostell. 1997. GeneBank. *Nucleic Acid Res.* **25:** 1–6.
6. Boon, N., J. Goris, P. De Vos, W. Verstraete, and E. M. Top. 2000. Bioaugmentation of activated sludge by an indigenous 3-chloroaniline degrading *Comamonas testosteroni* strain, 12*gfp*. *Appl. Environ. Microbiol.* **66:** 2906–2913.
7. Bradley, P. M. and F. H. Chapelle. 1998. Effect of contaminant concentration on aerobic microbial mineralization of DCE and VC in stream-bed sediments. *Environ. Sci. Technol.* **32:** 553–557.
8. Bradley, P. M., F. H. Chapelle, and D. R. Lovley. 1998. Humic acids as electron acceptors for anaerobic microbial oxidation of vinyl chloride and dichloroethene. *Appl. Environ. Microbiol.* **64:** 3102–3105.
9. Casamayor, E. O., H. Schafer, and G. Muyzer. 2000. Identification of and spatio-temporal differences between microbial assemblages from two neighboring sulfurous lakes: Comparison by microscopy and denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* **66:** 499–508.
10. Casamayor, E. O., C. Pedros-Alio, G. Muyzer, and R. Amann. 2002. Microheterogeneity in 16S ribosomal DNA-defined bacterial populations from a stratified planktonic environment is related to temporal changes and to ecological adaptations. *Appl. Environ. Microbiol.* **68:** 1706–1714.
11. Chang, Y.-J., J. R. Stephen, A. P. Richter, A. D. Venosa, J. Bruggemann, S. J. Macnaughton, G. A. Kowalchuk, and J. R. Haines. 2000. Phylogenetic analysis of aerobic freshwater and marine enrichment cultures efficient in hydrocarbon degradation: Effect of profiling method. *J. Microbiol. Methods* **40:** 19–31.
12. Choo, Y. F., J. Lee, I. S. Chang, and B. Y. Kim. 2006. Bacterial communities in microbial fuel cells enriched with high concentrations of glucose and glutamate. *J. Microbiol. Biotechnol.* **16:** 1481–1484.
13. Coleman, N. V., T. E. Mattes, J. M. Gossett, and J. C. Spain. 2002. Biodegradation of *cis*-dichloroethene as the sole carbon source by a β-proteobacterium. *Appl. Environ. Microbiol.* **68:** 2726–2730.
14. El Fantroussi, S., L. Verschuere, W. Verstraete, and E. M. Top. 1999. Effect of phenylurea herbicides on soil microbial communities estimated by analysis of 16S rRNA gene fingerprints and community-level physiological profiles. *Appl. Environ. Microbiol.* **65:** 982–988.
15. Fisher, S. G. and L. S. Lerman. 1979. Length-independent separation of DNA restriction fragments in two-dimensional gel electrophoresis. *Cell* **16:** 191–200.
16. Fromin, N., J. Hamelin, S. Tarnawski, D. Roesti, K. Jourdain-Miserez, N. Forestier, S. Teyssier-Cuvelle, F. Gillet, M. Arangno, and P. Rossi. 2002. Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. *Environ. Microbiol.* **4:** 634–643.
17. Gelsomino, A., A. C. Keijzer-Wolters, G. Cacco, and J. D. van Elsas. 1999. Assessment of bacterial community structure in soil by polymerase chain reaction and denaturing gradient gel electrophoresis. *J. Microbiol. Methods* **38:** 1–15.
18. Gerhardt, P., R. Murray, R. Costilow, E. Nester, W. Wood, N. Krieg, and G. B. Phillips. 1991. *Manual of Methods for General Bacteriology*. American Society for Microbiology, Washington, D.C.
19. Giovannoni, S. J., T. B. Britschgi, C. L. Moyer, and K. G. Field. 1990. Genetic diversity in Sargasso Sea bacterioplankton. *Nature* **345:** 60–63.
20. Gremion, F., A. Chatzinotas, and H. Harms. 2003. Comparative 16S rDNA and 16S rRNA sequence analysis indicates that *Actinobacteria* might be a dominant part of the metabolically active bacteria in heavy metal-contaminated bulk and rhizosphere soil. *Environ. Microbiol.* **5:** 896–907.
21. Hartmans, S., A. Kaptein, J. Tramper, and J. A. M. de Bont. 1992. Characterization of a *Mycobacterium* sp. and a *Xanthobacter* sp. for the removal of vinyl chloride and 1,2-dichloroethane from waste gases. *Appl. Microbiol. Biotechnol.* **37:** 796–801.
22. Hristova, K., B. Gebreyesus, D. Mackay, and K. M. Scow. 2003. Naturally occurring bacteria similar to the methyl *tert*-butyl ether (MTBE)-degrading strain PM1 are present in

MTBE-contaminated groundwater. *Appl. Environ. Microbiol.* **69:** 2616–2623.
23. Izu, K., F. Nakajima, K. Yamamoto, and F. Kurisu. 2001. Aeration conditions affecting growth of purple nonsulfur bacteria in an organic wastewater treatment process. *Syst. Appl. Microbiol.* **24:** 294–302.
24. Jensen, S., L. Øvreas, F. L. Daae, and V. Torsvik. 1998. Diversity in methane enrichments from an agricultural soil revealed by DGGE separation of PCR amplified 16S rDNA fragments. *FEMS Microbiol. Ecol.* **26:** 17–26.
25. Kaewpipat, K. and C. P. L. Grady Jr. 2002. Microbial population dynamics in laboratory-scale activated sludge reactors. *Water Sci. Technol.* **46:** 19–27.
26. Kaplan, C. W. and C. L. Kitts. 2004. Bacterial succession in a petroleum land treatment unit. *Appl. Environ. Microbiol.* **70:** 1777–1786.
27. Klier, N. J., R. J. West, and P. A. Donberg. 1999. Aerobic biodegradation of dichloroethylenes in surface and subsurface soils. *Chemosphere* **38:** 1175–1188.
28. LaGrega, M. D., P. L. Buckingham, and J. C. Evans. 1994. Growth kinetics, pp. 581. *In* Clark, B. J. and Morris, J. M. (eds.), *Hazardous Waste Management.* McGraw-Hill Inc. Publishers.
29. LaPara, T. M., C. H. Nakatsu, L. M. Pantea, and J. E. Alleman. 2000. Phylogenetic analysis of bacterial communities in mesophilic and thermophilic bioreactors treating pharmaceutical wastewater. *Appl. Environ. Microbiol.* **66:** 3951–3959.
30. Li, C. and W. M. Moe. 2004. Assessment of microbial populations in methyl ethyl ketone degrading biofilters by denaturing gradient gel electrophoresis. *Appl. Microbiol. Biotechnol.* **64:** 568–575.
31. Liu, W., T. L. Marsh, H. Cheng, and L. J. Forney. 1997. Characterization of microbial diversity by determining terminal restriction fragment polymorphisms of genes encoding 16S rRNA. *Appl. Environ. Microbiol.* **63:** 4517–4522.
32. McCaig, A. E., L. A. Glover, and J. I. Prosser. 2001. Numerical analysis of grassland bacterial community structure under different land management regimens by using 16S ribosomal DNA sequence data and denaturing gradient gel electrophoresis banding patterns. *Appl. Environ. Microbiol.* **67:** 4554–4559.
33. Medlin, L., H. J. Elwood, S. Stickel, and M. L. Sogin. 1988. The characterization of enzymatically amplified eukaryotic 16S-like rRNA coding regions. *Gene* **71:** 491–499.
34. Murray, A. E., J. T. Hollibaugh, and C. Orrego. 1996. Phylogenetic compositions of bacterioplankton from two California estuaries compared by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Appl. Environ. Microbiol.* **62:** 2676–2680.
35. Muyzer, G., E. C. de Waal, and A. Uitterlinden. 1993. Profiling of complex microbial populations using denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **59:** 695–700.
36. Muyzer, G. and K. Smalla. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Van Leeuwenhoek* **73:** 127–141.
37. Muyzer, G. 1999. DGGE/TGGE: A method for identifying genes from natural ecosystems. *Curr. Opin. Microbiol.* **2:** 317–322.
38. Nakatsu, C. H., M. Providenti, and R. C. Wyndham. 1997. The *cis*-diol dehydrogenase *cbaC* gene of *Tn5271* is required for growth on 3-chlorobenzoate. *Gene* **196:** 209–218.
39. Nogales, B., E. R. B. Moore, E. Llobet-Brossa, R. Rossello-Mora, R. Amann, and K. N. Timmis. 2001. Combined use of 16S ribosomal DNA and 16S rRNA to study the bacterial community of polychlorinated biphenyl-polluted soil. *Appl. Environ. Microbiol.* **67:** 1874–1884.
40. Nübel, U., F. Garcia-Pichel, M. Kühl, and G. Muyzer. 1999. Quantifying microbial diversity: Morphotypes, 16S rRNA genes, and carotenoids of oxygenic phototrophs in microbial mats. *Appl. Environ. Microbiol.* **65:** 422–430.
41. Olaniran, A. O., D. Pillay, and B. Pillay. 2004. Aerobic dechlorination of *cis*- and *trans*-dichloroethenes by some indigenous bacteria isolated from contaminated sites in Africa. *J. Environ. Sci.* **16:** 968–972.
42. Olaniran, A. O., D. Pillay, and B. Pillay. 2005. Characterization of two bacteria isolated from a wastewater treatment plant in South Africa for aerobic dehalogenation of some aliphatic chlorinated compounds. *Int. J. Environ. Stud.* **62:** 59–68.
43. Pepper, I. L., T. J. Gentry, D. T. Newby, T. M. Roane, and K. L. Josephson. 2002. The role of cell bioaugmentation and gene bioaugmentation in the remediation of co-contaminated soils. *Environ. Health Perspec.* **110:** 943–946.
44. Riemann, L., G. F. Steward, L. B. Fandino, L. Campbell, M. R. Landry, and F. Azam. 1999. Bacterial community composition during two consecutive NE Monsoon periods in the Arabian Sea studied by denaturing gradient gel electrophoresis (DGGE) of rRNA genes. *Deep Sea Res. A* **46:** 1791–1811.
45. Roane, T. M., K. L. Josephson, and I. L. Pepper. 2001. Dual-bioaugmentation strategy to enhance remediation of co-contaminated soil. *Appl. Environ. Microbiol.* **67:** 3208–3215.
46. Seeley, H. W. and P. J. Vandemark. 1981. *Microbes in Action. A Laboratory Manual of Microbiology*, 3rd Ed. Freeman WH and Company, U.S.A.
47. Shim, H., D. Ryoo, P. Barbieri, and T. K. Wood. 2001. Aerobic degradation of mixtures of tetrachloroethylene, trichloroethylene, dichloroethylenes and vinyl chloride by toluene-o-xylene monooxygenase of *Pseudomonas stutzeri* OX1. *Appl. Microbiol. Biotechnol.* **56:** 265–269.
48. Simpson, J. M., V. J. McCracken, H. R. Gaskins, and R. I. Mackie. 2000. Denaturing gradient gel electrophoresis analysis of 16S ribosomal DNA amplicons to monitor changes in fecal bacterial populations of weaning pigs after introduction of *Lactobacillus reuteri* strain MM53. *Appl. Environ. Microbiol.* **66:** 4705–4714.
49. Teske, A., C. Wawer, G. Muyzer, and N. B. Ramsing. 1996. Distribution of sulfate-reducing bacteria in a stratified fjord (Mariager fjord, Denmark) as evaluated by most-probable-number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments. *Appl. Environ. Microbiol.* **62:** 1405–1415.

50. Uroz, S., C. D. Angelo-Picard, A. Carlier, M. Elasri, C. Sicot, A. Petit, P. Oger, D. Faure, and Y. Dessaux. 2003. Novel bacteria degrading N-acylhomoserine lactones and their use as quenchers of quorum-sensing-regulated functions of plant-pathogenic bacteria. *Microbiology* **149:** 1981–1989.
51. Vallaeys, T., E. Topp, G. Muyzer, V. Macheret, G. Laguerre, A. Rigaud, and G. Soulas. 1997. Evaluation of denaturing gradient gel electrophoresis in the detection of 16S rDNA sequence variation in rhizobia and methanotrophs. *FEMS Microbiol. Ecol.* **24:** 279–285.
52. Ward, J. H. 1963. Hierarchial grouping to optimize an objective function. *J. Am. Stat. Assoc.* **58:** 236–244.
53. Ward, D. M., R. Weller, and M. M. Bateson. 1990. 16S rRNA sequences reveal numerous uncultured inhabitants in a natural community. *Nature* **345:** 63–65.
54. Watanabe, K., M. Teramoto, H. Futamata, and S. Harayama. 1998. Molecular detection, isolation, and physiological characterization of functionally dominant phenol-degrading bacteria in activated sludge. *Appl. Environ. Microbiol.* **64:** 4396–4402.
55. Woese, C. R. 1987. Bacterial evolution. *Microbiol. Rev.* **51:** 221–271.
56. Wu, J. F., C. W. Sun, C. Y. Jiang, Z. P. Liu, and S. J. Liu. 2005. A novel 2-aminophenol 1,6-dioxygenase involved in the degradation of p-chloronitrobenzene by *Comamonas* strain CNB-1: Purification, properties, genetic cloning and expression in *Escherichia coli*. *Arch. Microbiol.* **183:** 1–8.