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FATE OF A METAL-RESISTANT INOCULUM IN CONTAMINATED AND PRISTINE SOILS ASSESSED BY DENATURING GRADIENT GEL ELECTROPHORESIS

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Abstract—Cesium, cadmium, cobalt, and strontium are four contaminants frequently found in soils at biotoxic levels. Introduction of certain nongenetically modified bacteria has been frequently suggested as a method for the immobilization of heavy metal contaminants in soil, thereby reducing runoff and bioavailability. In this study, we have used the polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) to track the survival of five bacterial species added to soil microcosms with and without the addition of a mixture of these metals. The PCR primers targeted conserved regions of the 16S rDNA molecule present in all bacteria. The reaction products were shown to reflect the relative abundance of the bacteria both in mixtures of pure cultures and against a background of all the eubacterial species present in the soil following inoculation. Three of the species (*Pseudomonas aeruginosa* FRD-1, *Shewanella putrifaciens* 200, and *Desulfovibrio vulgaris* Hildenborough) decreased rapidly following inoculation into both soils. The proportion of *Alcaligenes eutrophus* CH34 remained at a constant level throughout the 8-week experiment in both soil treatments. *Sphingomonas aromaticivorans* B0695 showed toxic metal-dependent survival in that its relative abundance dropped rapidly in pristine soil but remained at approximately inoculation levels throughout the experiment in contaminated microcosms.

Keywords—Microbial ecology Toxic metals Denaturing gradient gel electrophoresis Polymerase chain reaction

INTRODUCTION

Radioactive and other toxic metal wastes from defense-related activities, industry, and municipal sources have routinely entered the environment through disposal in landfill sites or by accidental release in accidents such as that that occurred at Chernobyl, Russia. These practices have resulted in surface contamination problems, transport to groundwater, and/or bioaccumulation of radionuclides and toxic metals [e.g., 1–4]. Radionuclides and toxic metals were the most frequently reported classes of pollutants at U.S. Department of Energy waste sites [4]. Metals such as Cs, Sr, Cd, and, to a lesser extent, Co are prevalent in soils near industrial centers [e.g., 4,5] at concentrations up to 50 μg Cs/g, 350 μg Cd/g, and 500 μg Sr/g [4]. As cocontaminants, toxic metals are often inhibitory to other bioremediative processes, for example, hydrocarbon degradation [6]. Therefore, the characterization of metal-resistant bacterial species useful in the practice of soil remediation is important.

Mobilization of toxic metals from these sites leads to spread of the contamination via ground and surface water, posing health and environmental problems. Engineering [6], phytoremediative [7], and microbial bioremediative [8] solutions have recently been employed to control the dissemination of toxic metals from heavily contaminated soils. In this study, we have assessed the suitability of the polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE) [9] to monitor the fate of a consortium of bacteria introduced to pristine and metal-contaminated soil microcosms. The PCR targeted highly conserved regions of the eubacterial 16S rDNA gene. Direct nucleic acid-based analyses of microbial communities have increased in popularity due to the difficulties, inaccuracies, and time consumption of traditional culture-based methods [10–11]. The PCR-DGGE approach is fundamentally different from standard PCR detection of inoculated bacteria and carries certain advantages and disadvantages. The primary advantage is that, in conjunction with assessment of cell lysis and amplification efficiency, the relative abundance of all inoculated bacteria can be measured simultaneously by analysis of a single PCR reaction with a single set of primers. Products of near-identical length are generated from the inoculated and indigenous bacteria present in the sample. The products are separated on the basis of their melting behavior in an acrylamide gel matrix. The intensities of the recovered bands provide a semiquantitative measure of the relative abundance of each inoculated species as a proportion of the exogenously added cells and of the total eubacterial population carried by the sample, including both inoculated and indigenous species. A further advantage to this approach is that enzyme inhibitors derived from the soil samples do not affect the results. The presence of these inhibitors may lead to weaker amplification products but should not affect the banding pattern recovered. The chief disadvantage is low sensitivity. Although it has not been exhaustively tested,

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it is likely that a given species must compose approximately 1% of the total target organisms in a sample to remain above the background level of indigenous bacterial amplification products [9].

In this study, we have applied PCR-DGGE to monitor the survival of an inoculum of *Pseudomonas aeruginosa* FRD-1, *Shewanella putrifaciens* 200, *Sphingomonas aromaticivorans* B0695, *Alcaligenes eutrophus* CH34, and *Desulfovibrio vulgaris* Hildenborough in soil microcosms with and without the addition of high levels of nonradioactive toxic metal isotopes. The cell lysis and rDNA amplification efficiencies for these strains were compared in mixtures of laboratory-grown cultures. These bacteria were chosen as candidate organisms for microbially mediated immobilization of toxic metals in soils because of their ability to sequester or alter the chemical properties of metal ions.

METHODS AND MATERIALS

Inoculum culture conditions

The soil inoculum consisted of a five-strain mixture, each strain grown separately to stationary phase in batch culture. *Shewanella putrifaciens* 200 [12], *P. aeruginosa* FRD-1 (cystic fibrosis patient isolate [13]), and *A. eutrophus* CH34 (metal resistant strain; ATCC 43123) were grown ~26 h at 23°C in nutrient broth with shaking. *Sphingomonas aromaticivorans* B0695 (subsurface soil isolate [14]) was grown as above for 48 h. *Desulfovibrio vulgaris* (ATCC 29579) was grown anaerobically for 48 h in an acetate/lactate medium at pH 7.2 containing 2.8 g/L sodium acetate, 2.26 g/L sodium lactate, 0.5 g/L yeast extract, 0.1 g/L ascorbic acid, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L Na_2SO_4, 0.5 g/L K_2HPO_4, 0.5 g/L NH_4Cl, 0.1 g/L $FeSO_4 \cdot 7H_2O$, 7.0 g/L NaCl, and 0.1 g/L sodium thioglycolate. Strains were then washed twice in 0.85% (w/v) NaCl by centrifugation and resuspended together in distilled water to a density of $\sim 2.5 \times 10^9$ cells/ml each for delivery of 3 ml cell suspension per 75-g microcosm, providing $\sim 2 \times 10^7$ cells of each species per gram dry weight of soil.

Comparison of lysis/amplification efficiency of inocula

Stationary phase cultures of each of the inocula were enumerated by acridine orange direct counts of cells grown and washed as above. Mixtures of cells containing equal proportions of each species (10^6 cells each) or equal proportions of four species (24.5%, 10^6 cells each) and 2% of the remaining species (8×10^4 cells) were generated in a total volume of 500 µl. These mixtures were then lysed by bead beating and the DNA extracted as described above. A proportion (3 ng) of the recovered DNA was used in a PCR amplification and the products separated by DGGE as described above.

Lipid analysis

All solvents used were of GC grade and were obtained from Fisher Scientific (Pittsburgh, PA, USA). Triplicate soil samples (metal treated, with and without inocula, 35 g wet weight) from week 0 were extracted for phospholipid fatty acids. Phospholipid fatty acids (PLFA) were extracted using the modified Bligh and Dyer extraction [15]. The organic layer was fractionated into glyco-, neutral, and polar lipids and the latter then transesterified into methyl esters [16]. The methyl esters were then separated, quantified, and identified by gas chromatography-mass spectrometry [16] (GC/MS; Hewlett-Packard HP5890 series II gas chromatograph) interfaced with a HP5972 series mass selective detector (Hewlett Packard, Avondale, PA, USA). Fatty acids were designated as described in [17].

DNA extraction

The direct nucleic acid extraction was performed using a bead-beating system adapted from [18] with modifications. Soil (0.5 g), sodium phosphate buffer (425 µl, 0.12 M, pH 8.0), chaotropic reagent (175 µl; BIO-101, Vista, CA, USA), and 0.17-mm glass beads (0.5 g) were agitated in a 1.5-ml microcentrifuge tube using a high speed Crescent WIG-L-BUG™ bead beater (Crescent Dental, Lyons, IL, USA) for 1.5 min. The sample mixture was centrifuged at 13,000 *g* for 5 min and the supernatant was collected. Chloroform (300 µl) was added to the soil pellet, mixed thoroughly, and centrifuged at 13,000 *g* for 5 min. The aqueous supernatant was collected and combined with the first supernatant fraction. DNA was precipitated from the aqueous phase with an equal volume of 100% isopropanol in an ice bath for 30 min. The DNA was pelleted by centrifugation at 13,000 *g* at 4°C for 15 min, washed with 80% ethanol (1 ml) twice, air dried, and redissolved in TE buffer (10 mM Tris, 1 mM EDTA, 200 µl; pH 8.0). The DNA extract was purified by extraction twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) followed by a glass-milk DNA purification protocol using a Gene Clean™ kit (BIO-101) as described by the manufacturer.

PCR-DGGE

A PCR amplification of 16S rDNA gene fragments was performed as described in [9] with modifications. Thermocycling consisted of 35 cycles of 92°C, 45 s; 55°C, 30 s; and 68°C, 45 s using 1.25 units of Expand polymerase (Boehringer Mannheim, Mannheim, Germany) and 10 pmol each of the forward primer 341f-GC and reverse primer 534r in a total volume of 25 µl performed on a Robocycler™ PCR block (Stratagene, La Jolla, CA, USA). The final extension period was performed for 7 min at 68°C. Hot-start and touchdown procedures were found to be unnecessary.

DGGE employed a D-Code 16/16 cm gel system (BioRad, Richmond, CA, USA) maintained at a constant temperature of 60°C in 0.5 TAE buffer (20 mM tris-acetate, 0.5 mM EDTA, pH 8.0). Gradients were formed between 15 and 55% denaturant (100% denaturant was defined as 7 M urea, 40% v/v formamide) and the gels run at 35 V for 16 h. Gels were stained in purified water (Milli-Ro™, Millipore, Bedford, MA, USA) containing ethidium bromide at 0.5 mg/L and destained twice in 0.5 TAE. Images were captured by use of an Alpha Imager™ system (Alpha Innotech, San Leandro, CA, USA). Quantification of ethidium bromide fluorescence of DGGE lanes and individual bands was carried out using the 1D Multi function of the accompanying image software.

Band excision, reamplification, and sequence analysis

The central portion of bands of interest were excised using a razor blade (American Safety Razor Company, Verona, VA, USA) and soaked in 50 µl of purified water (Milli-Ro, Millipore) overnight. A portion (5 µl) was removed and used as the template in a PCR reaction as above except that the forward primer was 341f [9]. The products were gel purified by electrophoresis through 1.2% agarose TAE and isolated by use of a Gene-Clean™ kit (BIO-101). Purified DNA was sequenced using the primer 516r [19] and an ABI-Prism automatic se-

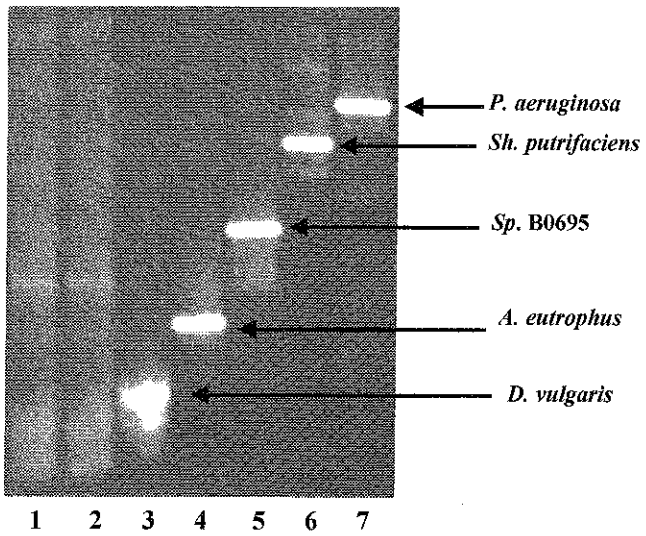

Fig. 1. Analysis of the indigenous eubacterial community by DGGE on a 15 to 55% denaturant gel. Lanes 1 and 2: Analysis of soil microcosm DNA at time zero. Lanes 3 through 7: Separation of 16S rDNA PCR fragments from the individual members of the inoculum. Lane 3: *Pseudomonas aeruginosa* FRD-1. Lane 4: *Shewanella putrifaciens* 200. Lane 5: *Sphingomonas aromaticivorans* B0695. Lane 6: *Alcaligenes eutrophus* CH34. Lane 7: *Desulfovibrio vulgaris* Hildenborough.

quencer (Perkin-Elmer, Foster City, CA, USA). Analysis was by use of the BLASTN facility of the National Center for Biotechnology Information.

RESULTS AND DISCUSSION

Evaluation of indigenous bacterial biomass

To quantify only the bacterial-type PLFA, polyenoic fatty acids, characteristic of eukaryote PLFA [20], were subtracted from the total PLFA yield. Prior to inoculation, total soil bacterial-type PLFA was quantified at 31 ± 2.5 nmol/g dry weight soil. Following inoculation, total bacterial-type PLFA was 34 ± 1.8 nmol/g dry weight soil. Based on bacterial PLFA content, therefore, the inoculum comprised ~10% of the entire soil biomass and each inoculated species comprised ~2% total bacterial biomass.

Separation of ribosomal fragments from the consortium by DGGE

The ~200 bp ribosomal fragments generated from each of the consortium members separated well by DGGE on a 15 to 55% denaturant gel. The *P. aeruginosa* fragment denatured first (28.3%), followed by *S. putrifaciens* (29.5%), *S. aromaticivorans* B0295 (33.3%), *A. eutrophus* (39%), and *D. vulgaris* (two bands at 43.2 and 44.8% denaturant), as can be seen in lanes 9 through 13 of Figure 1 and 11 through 15 of Figures 2 and 3. The two bands generated from *D. vulgaris* were initially interpreted as evidence of multiple and different 16S rDNA genes in this organism as has been demonstrated for other organisms by a similar technique [21]. However, we demonstrated that this pattern was due to a gel artifact by DGGE analysis of a single cloned 16S rDNA fragment from *D. vulgaris* that also generated a double band (PCR Topo2.1 cloning system; Invitrogen, Carlsbad, CA, USA; data not shown).

DGGE analysis of native microcosm DNA

DNA extracted from the soil microcosms prior to inoculation or metal addition are shown in Figure 1 (lanes 1 and 2). PCR products of 16S rDNA from "healthy" soils typically represent many thousands of bacterial species, the individual products of which generate a smear of DNA with a few visible bands, representing the most abundant organisms. The single strong band seen in Figure 1 was excised, sequenced, and shown to have 100% sequence homology to a cloned DNA fragment from an unidentified α-proteobacterium closely related to *Caulobacter subvibroides, Rhizomonas suberifacians,* and *Sphingomonas* sp. [22]. This band was visible in all treatments throughout the course of the study (Figs. 1 to 3). The sequence has been submitted to GenBank as Acc. AF065627. The patterns shown in Figure 1 are typical of such a complex bacterial community. No strong bands precisely

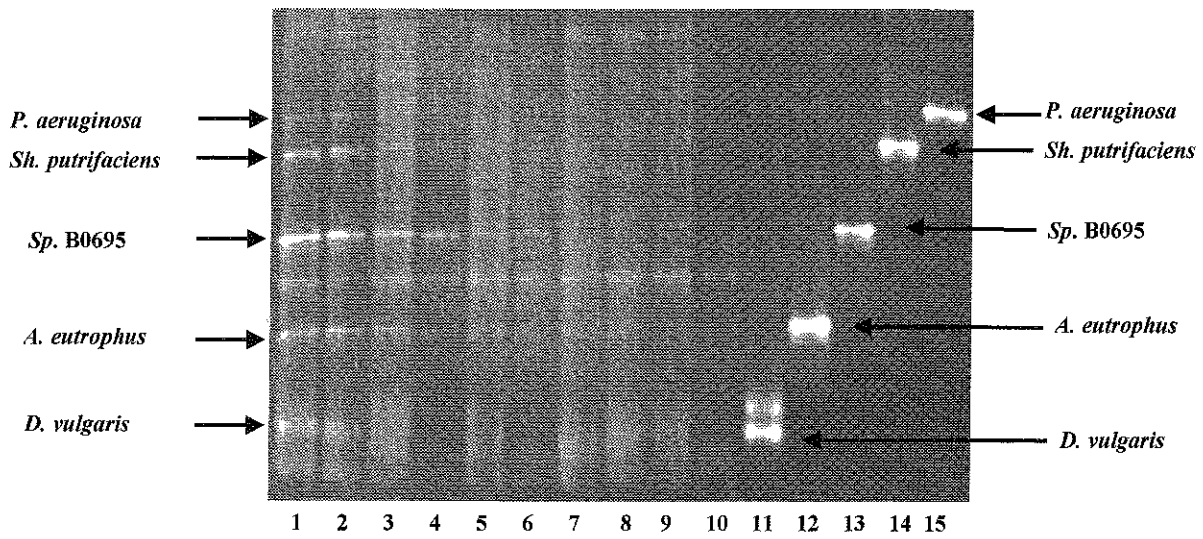

Fig. 2. Detection of community members by PCR-DGGE over an 8-week incubation in pristine soil. Lanes 1 through 10: PCR amplification products from DNA extracted from duplicate soil microcosms over the period of the experiment. Lanes 1 and 2: Time zero. Lanes 3 and 4: Week 1. Lanes 5 and 6: Week 2. Lanes 7 and 8: Week 4. Lanes 9 and 10: Week 8. Lanes 11 through 15: Separation of 16S rDNA PCR fragments from the individual members of the inoculum. Lane 11: *Pseudomonas aeruginosa* FRD-1. Lane 12: *Shewanella putrifaciens* 200. Lane 13: *Sphingomonas aromaticivorans* B0695. Lane 14: *Alcaligenes eutrophus* CH34. Lane 15: *Desulfovibrio vulgaris* Hildenborough.

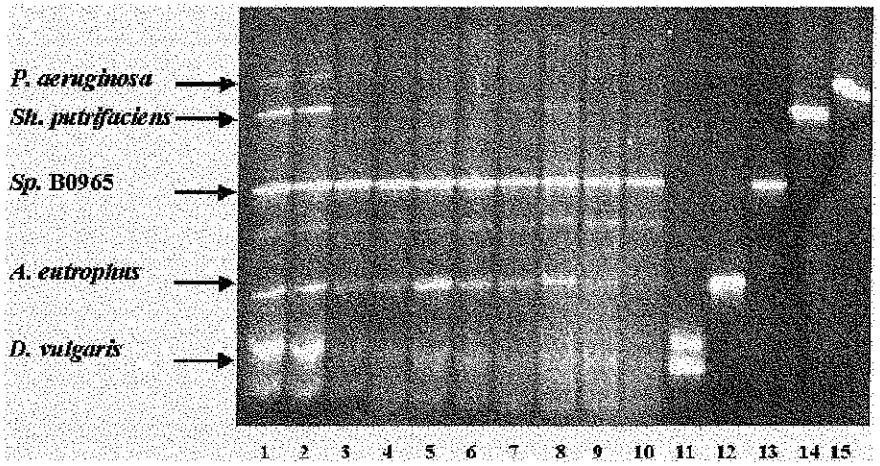

Fig. 3. Detection of community members by PCR-DGGE over an 8-week incubation in toxic metal contaminated soil. Lanes 1 through 10: PCR amplification products from DNA extracted from duplicate metal-contaminated soil microcosms over the period of the experiment. Lanes 1 and 2: Time zero. Lanes 3 and 4: Week 1. Lanes 5 and 6: Week 2. Lanes 7 and 8: Week 4. Lanes 9 and 10: Week 8. Lanes 11 through 15. Separation of 16S rDNA PCR fragments from the individual members of the inoculum. Lane 11: *Pseudomonas aeruginosa* FRD-1. Lane 12: *Shewanella putrifaciens* 200. Lane 13: *Sphingomonas aromaticivorans* B0695. Lane 14: *Alcaligenes eutrophus* CH34. Lane 15: *Desulfovibrio vulgaris* Hildenborough.

comigrated with products derived from any of the inoculum members.

Detection of consortium members within soil microcosms

The PCR-DGGE of DNA extracted immediately following inoculation generated similar patterns in both pristine and metal-contaminated soils. Bands comigrating with each of the standards were clearly visible in both treatments, as was a single strong band at 35.6% denaturant, which was generated from a bacterial species indigenous to the soil used (Fig. 2 and 3, lanes 1 and 2). These signals were superimposed on a background of DNA-ethidium bromide fluorescence generated from the complex indigenous eubacterial community. Although each strain was inoculated at approximately equal levels ($\sim 2 \times 10^7$ organisms per gram soil), the signals from each were quite different, which may have reflected differences in lysis efficiency, rDNA copy number per chromosome, or chromosome copy number per cell [23; see below]. However, signals of sufficient strength to allow sample comparison were recovered from all samples.

Detection of consortium members in pristine soils

In pristine microcosms, little or no trace of signal from *P. aeruginosa* FRD-1, *S. putrifaciens* 200, or *D. vulgaris* could be detected after 1 week of incubation (Fig. 2, lanes 3 and 4). The signal corresponding to *S. aromaticivorans* B0695 dropped over the first 2 weeks of incubation and was undetectable by week 4 (Fig. 2, lanes 1–8). After 8 weeks of incubation, only *A. eutrophus* CH34 remained detectable in this soil at approximately inoculation levels.

Detection of consortium members in toxic metal-contaminated soils

The pattern of consortium survival in soil microcosms contaminated with toxic metals was strikingly different from the pristine controls. The levels of *P. aeruginosa* FRD-1 and *A. eutrophus* CH34 appeared similar in both environments, the first becoming undetectable after 1 week, the latter surviving at inoculation levels throughout the study. The signals corresponding to *S. putrifaciens* 200 and *D. vulgaris* Hildenborough fell over the first week but remained constant at detectable levels throughout the remaining period of the experiment. The most dramatic difference between survival rates in the two treatments was seen with *S. aromaticivorans* B0695. The signal from this organism persisted at approximately inoculation level throughout the 8 weeks of this experiment in toxic metal-contaminated microcosms. The identities of the bands that comigrated with the *S. aromaticivorans* B0695 and *A. eutrophus* CH43 amplification products were confirmed by band excision, reamplification, and sequence analysis. The sequences generated (172 bp of unambiguous sequence) were 100% identical to the published sequences of the parental organisms (GenBank accession numbers U20755 and Y10824, respectively).

Relative lysis and amplification efficiency of inoculum members

A major concern in the quantitative assessment of DGGE banding patterns lies in differential cell lysis, rDNA copy number, and amplification efficiency of different rDNA sequences [23]. In addition, certain PCR primer pairs and templates have been shown to preferentially amplify rarer sequences in a mixed template reaction due to inhibition of amplification of abundant templates through product reannealing during the PCR [24]. In order to address these concerns and strengthen the quantitative interpretation of the data presented here, we carried out PCR-DGGE analysis on mixtures of pure cultures of the five inoculated strains. The mixtures were constructed with known proportions of each of the five strains in stationary phase. These mixtures were made with equal proportions of each strain, or mixtures containing 2% of each strain in turn, and 24.5% each of the remaining four strains. Results of these analyses are shown in Figure 4. Using equal starting proportions of each strain, recovery of DGGE bands demonstrated that *A. eutrophus* was underrepresented (E = 20%, O = 7%), whereas *S. putrifaciens* was overrepresented (E = 20%, O = 27%). The observed proportions of the remaining three species were not significantly different from expected values. When the proportion of each of the five species was reduced to 2% of total cell number in turn, the recovery of *P. aeruginosa*,

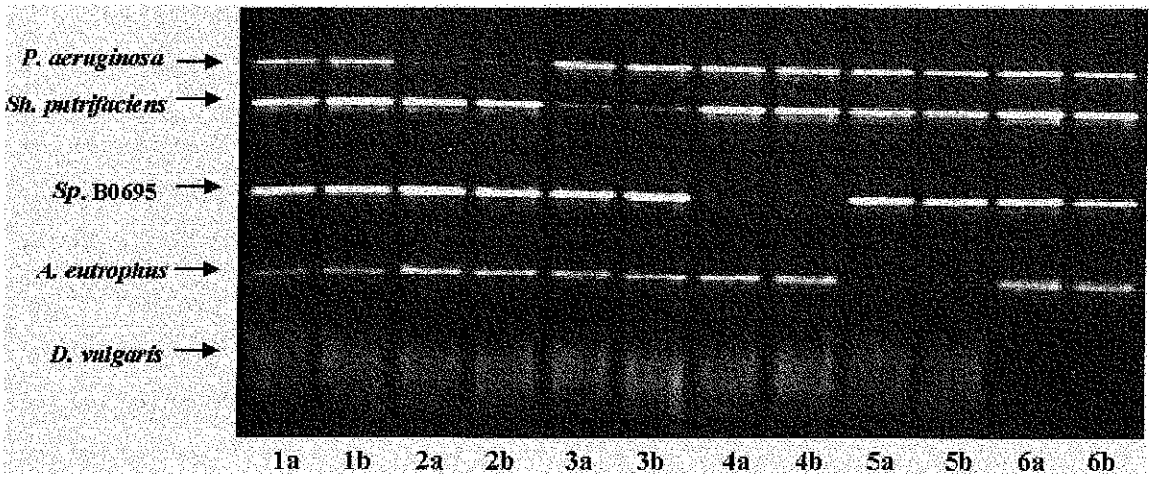

Fig. 4. Recovery of PCR-DGGE amplification products from defined mixtures of inoculum members in pure culture. Starting proportions (judged by averaged AODC of cells by three researchers): lanes 1a and b, and equal proportions of each of the five strains; lanes 2 through 6, 2% *Pseudomonas aeruginosa, Shewanella putrifaciens, Sphingomonas aromaticivorans, Alcaligenes eutrophus,* and *Desulfovibrio vulgaris,* respectively. a/b refer to duplicate DNA extraction/PCR-DGGE process.

S. putrifaciens, S. aromaticivorans, and *A. eutrophus* sequences were not significantly different from expected (E = 2%, O = 1.3–2.7%). *Desulfovibrio vulgaris* was not detected at this proportion, probably because sequences from this species formed two rather diffuse bands rather than a single sharp band under the gel conditions used. In this latter set of experiments, recovery of *S. putrifaciens* rDNA was again overrepresented when it was included at a starting proportion of 24.5%, providing ~30% of the recovered amplification products. *Alcaligenes eutrophus* sequences were proportionately underrepresented.

CONCLUSIONS

This study has demonstrated the use of PCR-DGGE as an effective method to monitor the fate of bacteria introduced into soils. Of the five species used in this inoculum, only *A. eutrophus* CH34 appeared to compete successfully with the indigenous microbial population in the unperturbed systems. However, perturbation of the soil community by contamination with toxic metals provided a strong selective advantage to an introduced inoculum of *S. aromaticivorans* B0695. This suggested that *S. aromaticivorans* B0695 is highly resistant to metal toxicity in soil. *Sphingomonas* sp., which are generally regarded as important organisms in the remediation of pentachlorophenol (PCP) and hydrocarbon contamination [25], have been isolated from several toxic metal contaminated soils [26] and are known to bind high concentrations of metal ions in their cell walls [27]. Unlike *A. eutrophus* CH34, this species appeared unable to compete with the indigenous microbiota in uncontaminated conditions. These conclusions were strongly supported by similar analyses on the inoculated bacteria in pure culture. While some bias in recovery of signal from the inocula was apparent, this tended to underrepresent *A. eutrophus* (which was recovered from both soil treatments at all time points) and overrepresent *S. putrifaciens* (recovery of which dropped rapidly under both soil conditions). These biases were not detected when *A. eutrophus* and *S. putrifaciens* were minor components of the starting mixture, although we suggest that this was due to the lysis/amplification bias being cancelled out by the inhibitory effects of abundant template self-annealing [24]. Therefore, this study has demonstrated that DGGE analysis of amplified 16S rDNA fragments from soil is a useful method of tracking the survival of introduced bacteria while they provide ~2% of the viable biomass. These results also suggest that, if used at a contaminated site, *S. aromaticivorans* B0695 would not persist beyond the contaminated area. Thus, *S. aromaticivorans* B0695 may have potential as a self-containing inoculum to assist in the degradation of organic xenobiotics in soils containing high levels of toxic metals as cocontaminants and to assist in immobilization of toxic metal contaminants at the impacted site.

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