Characterization of metal-resistant soil eubacteria by polymerase chain reaction – denaturing gradient gel electrophoresis with isolation of resistant strains

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Abstract: Contamination of soils with heavy metal ions is a major problem on industrial and defense-related sites worldwide. The bioavailability and mobility of these contaminants is partially determined by the microbial biomass present at these sites. In this study, we have assessed the effect of the addition of a mixture of toxic metal salts on the prokaryotic community of microcosms consisting of sandy-loam soil using direct molecular analysis of the recoverable eubacterial 16S rDNA molecules by polymerase chain reaction – denaturing gradient gel electrophoresis (PCR–DGGE) and limited phospholipid fatty acid analysis (PLFA). Addition of toxic metals (nonradioactive surrogates of Sr, Co, Cs, Cd) resulted in rapid (ca. 1 week) changes in the DGGE profile of the indigenous eubacterial community when compared with pristine controls. These changes were stable over the course of the experiment (8 weeks). No changes in the eubacterial population of control microcosms were detected. The major changes in community structure in metal-contaminated microcosms consisted of the appearance of four novel bands not detected in controls. Sequence analysis of these bands suggested that two organisms related to the genus *Acinetobacter* and two related to the genus *Burkholderia* carried a selective advantage over other indigenous eubacteria under heavy metal induced stress. The *Burkholderia* spp. were then cultured and further characterized using lipid analysis.

Key words: metals, PCR–DGGE, microbial community response.

Résumé : La contamination des sols par les ions métalliques lourds est un problème d'importance mondiale au niveau des terrains industriels et des sites militaires. La biodisponibilité et la mobilité de ces contaminants peuvent être partiellement mesurées par la biomasse microbienne présente dans ces sites. La présente étude a évalué l'effet de l'addition d'un mélange de sels de métaux toxiques sur la population de procaryotes dans des microcosmes représentatifs d'un loam sablonneux. Cet effet a été mesuré par une analyse moléculaire directe PCR–DGGE de l'ADNr 16S des eubactéries qui peuvent être retrouvées et par une analyse limitée PLFA. L'addition de métaux toxiques (substituts non radioactifs de Sr, Co, Cs, Cd) a rapidement provoqué (environ 1 semaine) des changements dans le profil DGGE de la population bactérienne indigène comparativement aux contrôles de départ. Ces changements ont été stables durant toute la durée des expériences (8 semaines). Aucun changement n'a été détecté dans la population d'eubactéries des microcosmes de contrôle. Le principal changement dans l'organisation de la population des microcosmes contaminés avec des métaux a été, selon le profil DGGE, l'apparition de quatre nouvelles bandes non rencontrées chez les contrôles. L'analyse de séquence de ces bandes a suggéré que deux organismes apparentés au genre *Acinetobacter* et deux apparentés au genre *Burkholderia* étaient avantagés par rapport à d'autres eubactéries indigènes lors du stress induit par les métaux lourds. Les *Burkholderia* spp. ont été cultivées et caractérisées par une analyse des lipides.

Mots clés : métaux, PCR–DGGE, réponse d'une population microbienne.

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Introduction

Radionuclides and toxic metals are among the most problematic wastes at industrial and defense-related sites (Riley and Zachara 1992; Pennanen et al. 1996). Such pollution routinely enters the environment mainly as a result of industrial activities, although large scale release via accidents, such as that at Chernobyl, have also occurred (Gadd 1997). These practices have resulted in surface contamination problems, transport to groundwater, and (or) bioaccumulation of radionuclides and heavy metals (e.g., Cornish et al. 1995; Riley and Zachara 1992) at concentrations up to 50 mg Cs/g, 350 mg Cd/g, and 500 mg Sr/g (Riley and Zachara 1992).

Microbiological activity is of primary importance in the bioremediation of metal-contaminated soils as microbial communities represent substantial biomass and play major roles in virtually all biogeochemical pathways (Gadd 1997). Microorganisms can alter metal chemistry and mobility through reduction, accumulation, and immobilization (Beveridge 1989; Kepkay 1986; Lovley 1994). Furthermore, the structure and diversity of soil microbial communities are known to change in the presence of heavy metals as the communities adapt to pollutant loads (Babich and Stotzky 1985; Pennanen et al. 1996).

A general knowledge of the ecology of degradative microbial populations is essential for the design and assessment of any cost-effective, ecologically safe, and environmentally sound bioremediation plan (Liu and Dulfita 1993). Changes in soil and sediment microbial communities following toxic metal amendment have been measured by various methods. These include viable cell counts, ATP assays (Babich and Stotzky 1985), select enzyme activity assays (Barnhart and Vestal 1983; Montuelle et al. 1994), [^{14}C]acetate incorporation into lipids (Barnhart and Vestal 1983), community respiration (Flemming and Trevors 1988), target toxicant biodegradation rates (Said and Lewis 1991), and phospholipid fatty acid (PLFA) analysis (Bååth et al. 1998; Frostegård et al. 1996). Excepting the lipid analysis, these techniques often require manipulation of soils and (or) culture techniques, rendering them less useful for real-time in situ monitoring of contaminated sites. Moreover, the limitations of culture-based techniques for microbial community assessment are well documented (Bakken and Olsen 1989; White 1983).

Herein we have assessed the use of polymerase chain reaction (PCR), targeting highly conserved regions of the eubacterial 16S rDNA gene, and denaturing gradient gel electrophoresis (DGGE) to monitor in situ changes in the major components of the eubacterial population of soil microcosms following the addition of toxic metals. The primary advantage of this technique over conventional PCR for the detection of bacteria is that the relative abundance of all the numerically dominant bacteria can be assessed simultaneously by analysis of a single PCR reaction with a single set of primers. The PCR reaction products are separated on the basis of their melting behavior in an acrylamide gel matrix, the intensities of the recovered bands providing a measure of the changes in the relative abundance of the major eubacterial species present. It is likely that any given species must compose at least 1% of the total target organisms in a sample to remain above the background level of numerically minor bacterial amplification products (Muyzer et al. 1993; Heuer and Smalla 1997). Therefore, this technique can only detect very pronounced changes in the eubacterial community. Subtle changes in species composition cannot be observed without the use of group-specific PCR primers (e.g., Kowalchuk et al. 1997).

In this study, we have applied PCR–DGGE to characterize the most pronounced indigenous soil microbial response to the addition of caesium, strontium, cadmium, and cobalt. Available metal (i.e., water soluble/extractable metal) was also investigated so that shifts in the indigenous microbial community structure could be related to metal mobilization/immobilization. Concurrently, we used plate culture techniques to isolate the culturable metal-resistant bacteria. Isolated metal-resistant bacteria were identified and characterized via analysis of their 16S rDNA and through lipid biomarker analysis, with the isolates' PLFA also compared with the soil PLFA profiles obtained at the beginning and end of the study. Once validated in laboratory microcosm studies, this technology will be applied to field samples from the Department of Energy and other contaminated sites to enable a comprehensive nonculture-based means for improved microbiological community characterization in metal-contaminated environments.

Methods

Soil microcosms

Microcosms consisted of 150-mL polypropylene beakers (VWR Scientific, Atlanta, Ga.) containing 75 g (dry weight) sieved (2 mm) agricultural loam topsoil from the University of Tennessee Agricultural Experiment Station in Alcoa (Sequatchie series). The soil was slightly acidic (pH 5.5) and contained 0.06% w/w organic carbon and 0.05% w/w nitrogen. Nonradionuclide surrogates were mixed in aqueous solution and added to half the soil microcosms as chlorides: $CoCl_2{\cdot}6H_2O$ (EM Industries, Inc., Gibbstown, N.J.), CsCl (Alfa Aesar, Ward Hill, Mass.), $SrCl_2{\cdot}6H_2O$ (Fisher Scientific, Co., Fair Lawn, N.J.), $CdCl_2{\cdot}2½H_2O$ (J.T. Baker Chem. Co., Phillipsburg, N.J.). Final concentrations of Cd, Co, and Sr in soil were 500 µg/g of dry weight soil with Cs at 1800 µg/g of dry weight soil. After metal additions (final water content 17% w/w), microcosms were thoroughly mixed and soils were compacted to 1.2 g/cm^3 and loosely covered with foil for aerobic incubation in the dark at 23°C and high atmospheric humidity (>70%). Metal-treated and nonmetal-treated microcosms were sacrificed at 0, 1, 2, 4, and 8 weeks for analyses. Moist soil samples (10 g) were frozen at –20°C for DNA extraction, PCR amplification, and subsequent DGGE analysis. Extractable metal concentrations were determined immediately.

Metal extractions

Metal extraction was performed by shaking soil for 1 h in distilled water at 1:10 (w/v, soil dry weight : solute). Filtrates were collected after centrifugation (2500 × *g*) using a 12-sample filtration manifold (Millipore Corp., Bedford, Mass.) with Whatman No. 40 filter paper and 2 drops of 1% w/v sodium pyrophosphate per 15 mL of filtrate for the stabilization of metals (Rhoades 1982). Soluble Sr, Co, and Cd were measured by inductively coupled argon plasma atomic emission; (Plant and Soil Science Dept., Univ. Tennessee, Knoxville, Tenn.). Soluble Cs was determined by flame atomic absorption spectrometry (Galbraith Laboratories Inc., Knoxville, Tenn.).

Isolation of metal-resistant bacterial strains

Viable metal-resistant microorganisms were extracted from soil by shaking at 120 rpm for 10 min in 0.1% w/v sodium pyrophosphate diluent (pH 7.0) at 1:4 (w/v, soil:suspension). Metal-resistant bacteria were isolated by spread plating 0.1 mL of 0-, 1-, and 100-fold dilutions onto tryptic soy agar (40 g tryptic soy agar/L deionized water containing cycloheximide at 50 mg/mL). The media also contained Cs, Cd, Sr, and Co chlorides at 4.0, 1.0, 3.0, and 4.0 mM, respectively. These concentrations were directly related to the soluble metals in the soil microcosms determined above after 8 weeks of incubation and measured following water extraction. Colony forming units were picked from the zero-dilution plates after 2 and 5 days for subsequent PCR–DGGE analysis, lipid analysis, and 16S rDNA sequencing.

DNA extraction and amplification

The direct nucleic acid extraction was performed using a bead-beating system adapted from Borneman et al. (1996) with modifications. Soil (0.5 g), sodium phosphate buffer (425 mL, 0.12 M, pH 8.0), chaotropic reagent (175mL, CRSR, Bio 101, Vista, Calif.), 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 MFG. Co., Lyons, Ill.) for 1.5 min. The sample mixture was centrifuged at 13 000 × *g* for 5 min and the supernatant was collected. Chloroform (300 mL) 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 isopropanol in an ice bath for 30 min. DNA was pelleted by centrifugation at 13 000 × *g* and 4°C for 15 min, washed with 1 mL of 80% ethanol twice, air dried, and redissolved in Tris–EDTA buffer (200 mL; pH 8.0). The DNA extract was purified by extracting twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, by volume), followed by a glass-milk DNA purification protocol using a Gene Clean™ kit (Bio 101) as described by the manufacturer.

PCR–DGGE

PCR amplification of 16S rDNA gene fragments prior to subsequent DGGE was performed on DNA extracted from soil microcosms on weeks 0, 1, 2, 4, and 8 and on selected bacterial isolates and cloned DNA fragments. 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 HF polymerase (Boehringer, Indianapolis, Ind.), 10 pmol each of the primers described in Muyzer et al. 1993, and a Robocycler™ PCR block (Stratagene, La Jolla, Calif.). The sequences of the primers were (forward, Primer 3, G–C clamp underlined) CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG CCTACGGGAGGCAGCAG and (reverse) ATTACCGCGGCTGC TGG (Primer 2) in a total volume of 25 mL. The final extension period was performed for 7 min at 68°C. These reactions did not employ hot-start or touchdown procedures.

DGGE employed a D-Code 16/16 cm gel system (BioRad, Hercules, Calif.) maintained at a constant temperature of 60°C in 6 L of 0.5 M Tris–acetate–EDTA (TAE) buffer (20 mM Tris–acetate, 0.5 mM EDTA, pH 8.0). Gradients were formed between 15 and 55% denaturant (100% denaturant defined as 7 M urea plus 40% v/v formamide) and the gels were run at 35 V for 16 h. Gels were stained in purified water (Milli-Ro™, Millipore) containing ethidium bromide at 0.5 mg/L and destained twice in 0.5 TAE. Images were captured using Alpha-Imager™ software (Alpha-Innotech, San Leandro, Calif.).

Cloning of PCR-amplified products

Amplification products were cloned into the PCR-TOPO 2.1 cloning vector (Invitrogen, Carsbad, Calif.) according to manufacturer's instructions. Recombinant (white) clones were screened by a two-stage procedure to ensure identity with the DGGE band of interest. First, plasmid inserts (*N* = 12 for each band) were reamplified by PCR using vector-specific primers (M13 reverse and T7; Invitrogen Corp.). The products were digested with restriction endonuclease *Msp*I and analysed by agarose gel electrophoresis (2% agarose, 1× TAE buffer). Two products from each digestion pattern group were reamplified using the 16S-specific PCR primers described above (Muyzer et al. 1993) and subjected to DGGE analysis to ensure comigration with the original band of interest. Sequences that were of high frequency in clone libraries (as defined by digestion pattern) and comigrated with the original band of interest were selected for sequence analysis and used in membrane hybridization studies.

Preparation of DNA for sequence analysis

The central 1-mm^2 portions of five bands of interest were excised using a razor blade (American Safety Razor Company, Verona, Va.) and soaked in 50 mL of purified water (Millipore) overnight. A portion (15 mL) was removed and used as the template in a PCR reaction as above, except that the forward primer lacked the GC clamp (primer 1, Muyzer et al. 1993). The products were purified by electrophoresis through 1.2% agarose TAE and glass-milk extraction (Gene-Clean™ kit, Bio 101). 16S rDNA fragments from duplicates of the two morphologically different metal resistant isolates were purified directly from amplification reactions with glass milk without gel purification.

Membrane transfer and hybridization analysis

DNA was electrophoretically transferred from DGGE gels to positively charged nylon-support hybridization membranes (Boehringer Mannheim) using a model SD™ electroblotter (BioRad) at 40 mA for 1 h. The transfer buffer was 0.5 TAE. Probes were labeled with digoxigenin-dUTP during a PCR (PCR DIG probe synthesis kit, Boehringer Mannheim) utilizing the same primers as for DGGE analysis, except that the GC clamp was omitted from the forward primer. Templates were either whole cells or PCR products generated from DNA fragments inserted into the PCR-TOPO 2.1 vector reamplified with vector-specific primers as above (Invitrogen Corp.). Pre-hybridization and hybridization were at 68°C in the standard buffer described by the manufacturer (5× SSC, 0.1% *N*-laurylsacrosine, 0.02% sodium dodecyl sulfate (both w/v)), and 1% blocking reagent (Boehringer-Mannheim) overnight in a rotary oven (Personal Hyb™, Stratagene). Detection employed a DIG nucleic acids detection system (Boehringer Mannheim) used according to manufacturer's instructions without modification.

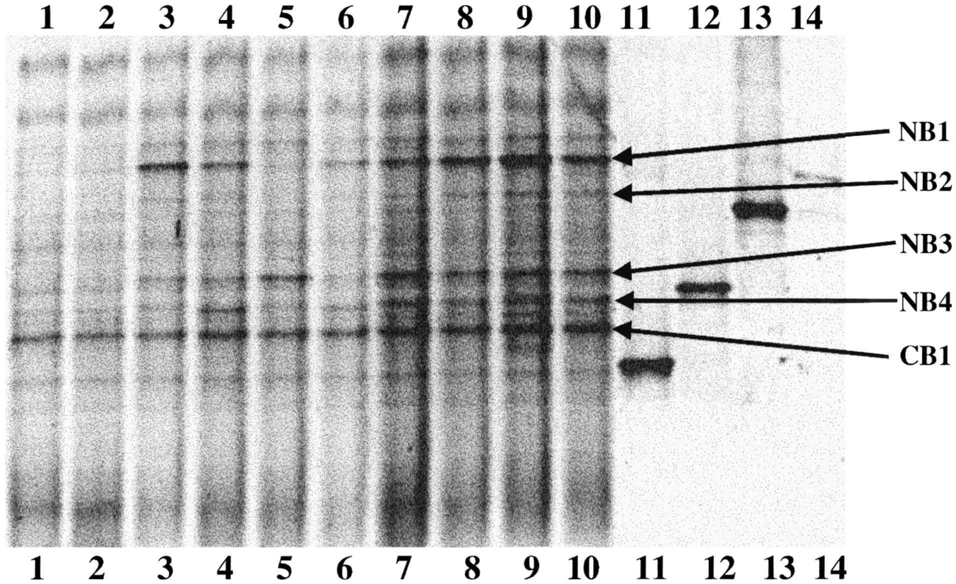
Sequence analysis

PCR products from excised bands and colonies were sequenced using the primer 516r (GWATTACCGCGGCKGCTG; W = A or T, K = G or T: Lane et al. 1985) and an ABI-Prism model 373 automatic sequencer with dye terminators (Perkin-Elmer, Foster City, Calif.). Sequences were compared with the GenBank data base by use of the BLASTN facility of the National Center for Biotechnology Information and with the RDP data base by use of the Similarity Rank Facility (Maidak et al. 1997). Sequences were aligned with reference sequences using Seqpup Version 0.6. (Gilbert, 1996).

PLFA and hydroxy fatty acid analyses

All solvents used were of GC grade and were obtained from Fisher Scientific (Pittsburgh, Pa.). Triplicate soil samples (pristine and metal treated, 35 g wet weight) from weeks 0 and 8 and isolates representative of the two different colony morphologies from the metal-amended media were extracted for PLFA and PLFA – hydroxy fatty acids, respectively. The isolates were grown until

Fig. 1. Analysis of the indigenous eubacterial community by DGGE on a 15–55% denaturant gel following the addition of metals. Lanes 1 and 2, week 0; 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 11–14, migration standards.



early stationary phase in tryptic soy broth, centrifuged at 10 000 × *g* and washed twice in phosphate buffer (0.05 M, pH 7.4) before lipid characterization. Phospholipids (PLFA) were extracted using the modified Bligh and Dyer extraction (White et al. 1979). From both the soil and isolate extracts, the organic layer was fractionated into glyco-, neutral-, and polar-lipids and the latter then transesterified into methyl esters (Guckert et al. 1985). The methyl esters were then separated, quantified, and identified by gas chromatography – mass spectrometry (Guckert et al. 1985; Hewlett-Packard HP5890 series II gas chromatograph) interfaced with a HP5972 series mass selective detector (Hewlett Packard, Wilmington, Del.). Fatty acids were designated as described by Ringelberg et al. (1989). Hydroxy fatty acids (OH-FA) were recovered from the aqueous fraction, obtained following the initial Bligh and Dyer extraction of the isolates, and esterified and derivatized as described by Mayberry and Lane 1993, with slight modifications. Briefly, the aqueous layer and interface from the modified Bligh and Dyer extraction was evaporated under vacuum (AS290 Automatic Speed Vac Concentrator, Savant Instruments Inc., Framingdale, N.Y.). The residue was hydrolysed with 2 M HCl at 100°C overnight and then allowed to cool. Hydrolysates were partitioned using chloroform (5 mL), with the lower layer recovered to a clean tube. The remaining aqueous phase was washed with 2.5 mL chloroform and the two chloroform fractions were combined. Esterification, derivatization with bis(trimethylsilyl) trifluoroacetamide, and gas chromatography – mass spectrometry analysis were performed as described in Mayberry and Lane (1993).

Statistical analysis

Nucleic acid extractions were carried out in duplicate. The Student *t* test was used to determine significant differences between band intensities for constant band, CB1, and novel bands, NB1 through NB4 between weeks 0 through 8.

Nucleotide sequence accession numbers

The nucleotide sequences for NB1 through NB4, MRI1, MRI2, and CB1 were deposited into GenBank as accession numbers AF065621 through AF06527, respectively.

Results

Metal availability

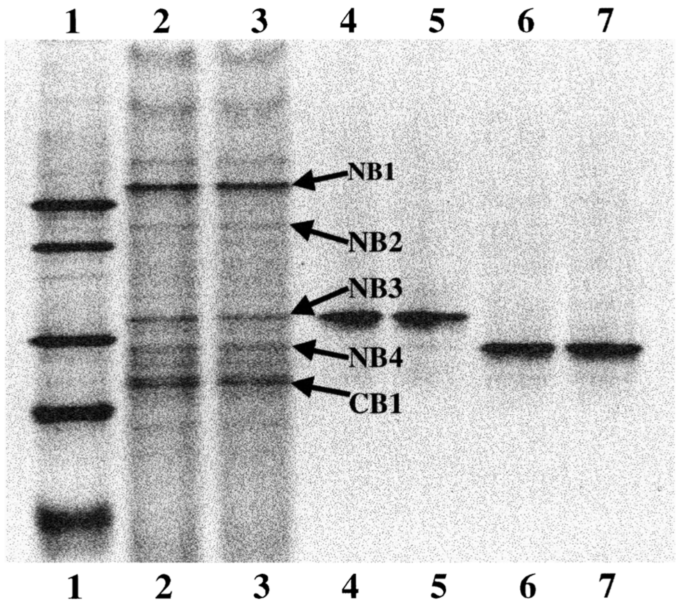
At each time point, metal availability and mobility consistently followed the following order: Sr > Co > Cs > Cd. Percent of Sr and Co extracted was largely invariant with time, dropping slightly (less than 7%) in 8 weeks (data not shown). Of the four metals, 22.8 ± 2% (cadmium), 35 ± 2% (caesium), 54.8 ± 9% (cobalt), and 64.5 ± 5.7 (strontium) were water soluble (i.e., bioavailable and mobile) after 8 weeks.

DGGE analysis

Throughout the 8-week study, DGGE analyses of the 16S rDNA from the pristine nonmetal-treated soils generated smears, reflecting a highly complex community structure. One distinct band was clearly visible over the 8-week period (data not shown). Band intensity did not change significantly over the 8 weeks of the study ($P > 0.05$).

The DGGE analysis of the amplified 16S rDNA from the metal-treated soil from week 0 through week 8 is shown in Fig. 1. At week 0, all DNA samples generated the smear containing the constant band (CB1), which was also present in the pristine soil described above. By week 1, novel bands 1, 2, 3, and 4 (NB1–NB4) had become visible and remained so throughout the 8-week study. Quantification of the total ethidium bromide fluorescence of individual DGGE lanes showed that the DNA loading differed by not more than 33% between lanes. Taking differences in DNA loading into account, the intensity of CB1 did not increase significantly over time ($P > 0.05$). In contrast, the intensities of bands NB1 and NB2 increased significantly between weeks 0 and 1 ($P < 0.05$) and then remained constant through week 8, while NB3 increased significantly over the 8 weeks of the study ($P < 0.05$). A band comigrating with NB4 was visible at time 0; however, this band did not hybridize with a specific probe (see below). The intensity of this band showed a

Fig. 2. Comparison of DGGE profiles from the indigenous eubacterial population following addition of metals at week 8 and the two isolated strains (MRI1 and MRI2). Lane 1, migration standards; lanes 2 and 3, eubacterial population at week 8 following the addition of metals; lanes 4 and 5, MRI1 (duplicate isolates); lanes 6 and 7 (MRI2, duplicate isolates).



significant increase between weeks 1 and 8 of the study ($P < 0.05$).

Isolation of metal-resistant bacteria

Two morphologically distinct isolates were cultured on the metal-supplemented tryptic soy agar. Metal-resistant isolate 1 (MRI1) was yellow pigmented, opaque, and formed distinct colonies. Metal-resistant isolate 2 (MRI2) was not pigmented, but was also opaque and formed distinct colonies. Despite the inclusion of cycloheximide in the culture media, after 1 week, fungal biomass dominated the plates. As such, no more bacteria were isolated after 5 days.

Membrane hybridizations

Hybridizations of DNA transferred from DGGE gels were performed using a cloned amplification product derived from NB1 and a product derived from whole MRI1 cells as probes. The probe generated from NB1 hybridized to the bands designated NB1 and NB2 for weeks 1 through 8, while the probe derived from whole cells of MRI1 hybridized with both band NB3 (with which it comigrated during DGGE) and NB4, again through weeks 1 through 8. Neither probe hybridized with bands visible at week 0 (data not shown).

Analysis of sequence data

The 16S rDNA sequences obtained for bands NB1 through NB4 (from positions 341–534 according to *Escherichia coli* enumeration) enabled identification as follows: NB1, *Acinetobacter* sp. (99% similarity to *Acinetobacter* sp. strain ATCC 10095, accession number Z93450); NB2, *Acinetobacter* sp. (98% similarity to *Acinetobacter haemolyticus* ATCC 17922, accession number Z9346); NB3, (100% similarity to an unidentified β proteobacterium strain G21019, accession number ABO11739, 98% similarity to *Burkholderia* sp. strain CRE57, accession number U37340); and NB4, *Burkholderia* sp. (98% similarity to *Burkholderia* sp. strain CRE57, accession number U37340). The corresponding 16S rDNA positions of two members of each morphological type of metal-resistant bacterial isolate were also sequenced. Again, based on analysis of 16S rDNA from positions 341–534 (*E. coli* enumeration), both colony types (MRI1 and MRI2) proved to belong to the genus *Burkholderia*, with each having 100% sequence homology with either NB3 (MRI1) or NB4 (MRI2). A DGGE analysis of PCR-amplified DNA from the week 8 metal-treated soil and the amplified 16S rDNA fragments from duplicate isolates of MRI1 and MRI2 are shown in Fig. 2. The indigenous soil organism responsible for generation of the single strong band, which persisted through weeks 0 to 8 under both soil treatments (CB1), was identified as having 100% sequence homology with an unidentified α proteobacterium closely related to *Caulobacter subvibroides*, *Rhizomonas suberifacians*, and *Sphingomonas* sp. (Mitsui et al. 1997).

Phospholipid and hydroxy fatty acid analysis

The PLFA profiles for isolates MRI1 and MRI2 were dominated by 16:1ω7c, 16:0, 18:1ω7c, and 18:0, while the 3OH-FA profiles were dominated by 3OH 14:0 and 3OH 16:0. Full PLFA and 3OH-FA characterizations of the strains are shown in Tables 1 and 2, respectively. The principal difference in the lipid profiles was that MRI2 contained substantially more 18:0, but less 18:1ω7c than did MRI1.

The amounts of total PLFA and monoenoic PLFA present in the pristine and metal-treated soils, as well as the amounts of the specific fatty acids that were also detected in the isolates, are shown in Table 3. The total biomass, represented by the PLFA content, and the Gram-negative biomass, represented by the monoenoic PLFA (Wilkinson 1988), decreased between weeks 0 and 8. However, at each single time point there was no significant difference between the total PLFA or monoenoic PLFA content of the two soil treatments. Of

Table 1. Phospholipid fatty acid profiles of novel isolates MRI1 and MRI2.

PLFA	MRI1	MRI2
14:1	0.04 (0.0)	0.02 (0.02)
14:1ω5c	0.14 (0.02)	0.14 (0.00)
14:0	0.34 (0.02)	0.26 (0.02)
4me14-x	0.21 (0.07)	0.24 (0.08)
unk 15mono	0	0.07 (0.00)
15:0	0	0.02 (0.00)
16:1ω7c	31.61 (0.30)	29.36 (0.14)
16:1ω7t	1.12 (0.02)	0.41 (0.05)
16:1ω5c	0.12 (0.00)	0.17 (0.03)
16:0	18.43 (0.08)	21.61 (0.02)
x:2 (a)	0.16 (0.02)	0
x:2 (b)	0.43 (0.11)	0
cy17:0	1.32 (0.00)	2.04 (0.02)
2OH 16:0	0.23 (0.06)	0.51 (0.00)
3OH 16:0	0.05 (0.03)	0.04 (0.01)
18:2ω6	0.06 (0.00)	0.08 (0.00)
18:1ω9c	0.13 (0.01)	0.12 (0.00)
18:1ω7c	35.5 (0.04)	41.66 (0.06)
18:1ω7t	0.19 (0.02)	0.16 (0.09)
18:0	9.3 (0.08)	1.38 (0.05)
cy19:0	0.27 (0.02)	0.58 (0.02)
20:1ω9c	0.17 (0.03)	0.06 (0.00)

Notes: Values given as mole percent (mean); $N = 3$; standard deviations are shown in brackets.

Table 2. Hydroxy fatty acid profiles of novel isolates MRI1 and MRI2.

OH-FA	MRI1	MRI2
3OH 14:0	63.72 (1.35)	63.20 (0.19)
3OH 16:1	0.97 (0.12)	1.87 (0.31)
3OH 16:0	35.32 (1.40)	34.92 (0.50)

Notes: Values given as mole percent (mean); $N = 3$; standard deviations are shown in brackets.

those specific PLFA also detected in the isolates, there were increases in the amount of 16:0 and 18:0 and decreases in the amount of 16:1ω7c and 18:1ω7c over the 8 weeks of the study.

Discussion

The PCR-based approach used here employed primers that recognise the rDNA targets of all eubacteria. Therefore, the scope of this study has been constrained in so much as it detected changes only in the eubacterial population, and only highly pronounced changes within this group. Nonetheless, domain-level PCR–DGGE analysis of rDNA target molecules enabled visualization of some positive changes among the indigenous soil eubacterial population induced by the addition of heavy metals.

Sequence analysis of the excised bands and the metal-resistant isolates placed the bacteria within the genera *Acinetobacter* (NB1 and NB2) and *Burkholderia* (NB3, NB4, MRI1, and MRI2), both of which form coherent monophyletic groups based on 16S rDNA analysis (Yabuuchi et al. 1992; Ibrahim, et al. 1997). This strongly supported the inference that the impact of toxic metal ions resulted in an increase in the relative abundance of at least two members of each genus in the soil microcosms described.

DGGE analysis of the pristine nonmetal-treated soils throughout the 8-week study revealed patterns typical of the complex microbial population present in healthy soils. Microbial communities containing a large number of approximately equally abundant bacterial species produce such a complex banding pattern upon DGGE that few, if any bands can be resolved (Heuer and Smaller 1997). The DGGE analysis enabled detection of the microbial response to the presence of metals within a week of metal treatment. Of the four dominant novel bands, NB1 and NB2 showed significant increases only between weeks 0 and 1, whilst NB3 and NB4 increased in intensity over the 8 weeks of the study (Fig. 1). A band was visible at the NB4 position at week 0 of the study, however, this band did not hybridize with the MRI1 probe, indicating that this band was representative of rDNA from another organism that comigrated with NB4. Only the *Burkholderia* spp. (representing NB3 and NB4) were isolated on the metal-amended tryptic soy agar. The presence of detectable organisms that are not necessarily culturable has been extensively documented (see Amann et al. 1995 and references therein), although positive correlations between culture-based and molecular retrieval-based techniques for microbial population characterizations have also been reported (Groβkopf et al. 1998). Generally, DNA-based techniques have shown the diversity of natural environments to far exceed that which had been determined using culture-based approaches (Heuer and Smalla 1997; Borneman et al. 1997). The genus *Acinetobacter* is well represented in culture collections; *Acinetobacter* spp. are abundant in a wide range of environments and metal resistance is a common phenotype. The culture conditions used here are commonly used for the propagation of *Acinetobacter* sp. (ATCC 1992), with the exception of the addition of metals. It is not clear why no *Acinetobacter* colonies corresponding to NB1 and NB2 were recovered, but it may be suggested that the metal resistance demonstrated by these strains in situ is dependent on some component of the soil matrix (chemical or physical) not available on the agar culture medium.

According to rDNA sequence analysis, the isolates MRI1 and MRI2 were closely related members of the genus *Burkholderia*, differing in 16S rDNA sequence at only one position in the V3 region (Neefs et al. 1993). MRI1 carried a T residue at position 469 (*E. coli* numbering, Brosius et al. 1981), whereas MRI2 carried a C, which accounted for the higher denaturant resistance of the amplified fragment from MRI2 and correspondingly lower gel position on DGGE analysis. However, their colonies were morphologically distinct, and lipid analysis also indicated small but discernible differences between them. The PLFA and 3OH-FA profiles of both organisms were typical of the genus *Burkholderia* (specifically *Burkholderia cepacia*, previously known as *Pseudomonas cepacia*) (Wilkinson 1988), however, MRI1 contained less monoenoic but more normal saturate PLFA than did MRI2 (Table 1). Use of PLFA to help infer phylogenetic relationships can be complicated by the fact that

Table 3. Shifts in isolate-specific PLFA concentrations of pristine and metal-treated soils from weeks 0 and 8.

	Pristine soil		Metal-treated soil	
PLFA	Week 0	Week 8	Week 0	Week 8
16:0	4341 (397)	3597 (231)	4196 (219)	4086 (35)
18:0	907 (66)	756 (56)	998 (131)	870 (37)
16:1ω7c	1880 (224)	1223 (88)	1614 (73)	966 (45)
16:1ω7t	85 (5)	53 (8)	74 (8)	53 (1.4)
Cy17:0	1014 (78)	879 (120)	1001 (33)	879 (83)
18:1ω7c	3071 (261)	2158 (137)	2561 (47)	1928 (60)
Total PLFA	31 075 (2 517)	25 052 (3 597)	31 109 (1 524)	24 893 (382)
Total monoenoic PLFA	12 284 (981)	9553 (750)	11 756 (510)	9092 (126)

Notes: Values for PLFA concentrations are in pmoles/g dry weight soil; $N = 3$; standard deviations are shown in brackets.

lipid profiles change, depending upon both the nutrient media used and the stage in the cell growth cycle at which the culture was sampled (Kohring et al. 1994). In this case, however, these cultures were grown using the same media to the same stage in the growth cycle and the PLFA profiles were therefore comparable. The significant phenotypic differences between these closely related isolates may be explained by the genomic plasticity associated with the genus *Burkholderia* (Lessie et al. 1996). However, significant physiological differences between similarly closely related *Cyanobacteria* spp. have been inferred from DGGE-defined distribution in hot spring mats (Ferris et al. 1996).

The impact of toxic metals on microbial communities has been primarily explored using techniques such as viable cell counts (Barkay et al. 1985), ATP assays (Babich and Stotzky 1985), select enzyme activity assays (Barnhart and Vestal 1983), [^{14}C]acetate incorporation into lipids (Barnhart and Vestal 1983), community respiration (Flemming and Trevors 1988), and target toxicant biodegradation rates (Said and Lewis 1991), the majority of which require some soil manipulation and (or) culture techniques. More recently, PLFA analysis has been used for the characterization of the soil microbial response to toxic metals (Bååth et al. 1998; Frostegård et al. 1996). Such PLFA analysis provides a measure of the environmentally mediated changes in the physiological and nutritional status of Gram-negative bacteria (see White and Macnaughton 1997 and references therein). Additionally, using PLFA, a broad community structure analysis of the total microbial population can be obtained, i.e., differentiation can be made between Gram-positive, Gram-negative, fungal, or protozoal biomass (White and Macnaughton 1997 and references therein) with species level analysis possible when specific fatty acids of known origin are present (Bååth et al. 1998 and Frostegård et al. 1996). However, PLFA does have limitations for the analysis of the Gram-negative bacterial community structure. The PLFA profiles of Gram-negative bacteria are generally dominated by monoenoic (e.g., 16:1ω7c and 18:1ω7c), saturated (16:0 and 18:0) and cyclopropane fatty acids (Wilkinson et al. 1988; Zelles 1997), the vast majority of which are broadly distributed and, as such, uninformative in species-level community structure analyses. The study described herein provides a case-in-point in that the shift in the eubacterial community structure detected using PCR–DGGE analysis towards one which was dominated by *Acinetobacter* sp. and *Burkholderia* sp. was not reflected in shifts in the PLFA profiles (Table 3). The increase in the 16:0 and 18:0 normal saturates has been linked to a decrease in microbial diversity (D. Ringelberg 1998), however, as a consequence of the broad distribution of Gram-negative-type PLFA, no other shifts within the Gram-negative populations of the soils that correlated with the PCR–DGGE analysis could be detected.

Within the past decade, immobilization of metals by microorganisms has been demonstrated (Volosky and Holan 1995) and it has potential as a procedure to assist in treatment of toxic metal and radionuclide contamination (Gadd 1997). Microorganisms have been shown to immobilize metals by the formation of insoluble precipitates (Beveridge 1989), e.g., at neutral or alkaline pH, *Cyanobacteria* sp. formed Sr calcite from groundwater discharge (Ferris et al. 1995) and *Citrobacter* sp. produced Cd phosphate in significant quantities (Macaskie et al. 1987). It is reasonable, therefore, to suggest that the indigenous microbial populations could significantly affect metal mobility and availability in soil.

To conclude, using a kingdom-level PCR–DGGE based analysis, a rapid and pronounced response of the indigenous eubacterial population in soil microcosms to the addition of high levels of toxic metals was demonstrated and the major positively selected components were identified to the level of genus. The response to metal impact was rapid (ca. 1 week). A gradual increase in the relative abundance of the two *Burkholdaria* species was detected over the following 7 weeks. During the study, the metal bioavailability decreased slightly but consistently. We are unable to predict the contribution, if any, of the species identified here to this phenomenon. Sequence analysis of bands excised from the DGGE gel enabled the identification of the major eubacteria positively selected by the presence of the metal impact as members of the genera *Acinetobacter* and *Burkholderia*. Of these, only the two *Burkholderia* strains were culturable on metal-amended tryptic soy agar growth media. Future work will focus on developing and utilizing a combination of DGGE and lipid analysis techniques for the comprehensive assessment of the indigenous microbial response at metal-contaminated sites.

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