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Phylogenetic analysis of aerobic freshwater and marine enrichment cultures efficient in hydrocarbon degradation: effect of profiling method

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Abstract

Aerobically grown enrichment cultures derived from hydrocarbon-contaminated seawater and freshwater sediments were generated by growth on crude oil as sole carbon source. Both cultures displayed a high rate of degradation for a wide range of hydrocarbon compounds. The bacterial species composition of these cultures was investigated by PCR of the 16S rDNA gene using multiple primer combinations. Near full-length 16S rDNA clone libraries were generated and screened by restriction analysis prior to sequence analysis. Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) was carried out using two other PCR primer sets targeting either the V3 or V6–V8 regions, and sequences derived from prominent DGGE bands were compared to sequences obtained via cloning. All data sets suggested that the seawater culture was dominated by α-subgroup proteobacteria, whereas the freshwater culture was dominated by members of the β- and γ-proteobacteria. However, the V6–V8 primer pair was deficient in the recovery of *Sphingomonas*-like 16S rDNA due to a 3′ terminal mismatch with the reverse primer. Most 16S rDNA sequences recovered from the marine enrichment were not closely related to genera containing known oil-degrading organisms, although some were detected. All methods suggested that the freshwater enrichment was dominated by genera containing known hydrocarbon-degrading species.

Keywords: Enrichment cultures; DGGE; Hydrocarbon degradation; Polymerase chain reaction; Phylogenetic analysis; 16S rDNA

1. Introduction

Hydrocarbon-degrading consortia of bacteria derived from contaminated environments are generated under laboratory conditions for a number of reasons. These include investigation of chemical degradation

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pathways, isolation and characterization of hydrocarbon-degrading organisms, and production of large-scale inocula for bioaugmentation to accelerate intrinsic remediation of contaminated environments. The species composition of cultures showing potential for use in bioaugmentation is of interest for scientific, regulatory and biotechnological reasons, and an understanding of the composition of hydrocarbon-degrading consortia may assist the development of bioremediation monitoring systems (Head, 1998).

Isolation of individual members of a consortium as pure cultures is not always practical due to its time-consuming nature and the lack of understanding of the growth requirements for most environmental bacteria (Skinner et al., 1952; White, 1983; Bakken, 1985; Tunlid and White, 1992; White et al., 1993). Therefore, molecular methods have been increasingly employed over recent years in the description of complex microbial communities in environmental samples, and environmentally derived enrichment cultures (Muyzer and Smalla, 1998; Torsvik et al., 1998, Rabus et al., 1999). Nucleic acid-based analyses of prokaryotic species in complex samples usually target the gene for 16S rRNA, due to the ubiquity of the gene for 16S rRNA and the presence of conserved regions suitable as amplification target sites. Moreover, the existence of an extensive database of sequences from cultured organisms (Lane et al., 1985, Marchesi et al., 1998, Maidak et al., 1999) enables confirmation of the in situ significance of isolates (Watanabe et al., 1998), and can provide clues as to proper growth conditions for the isolation of the dominant organisms (Juretschko et al., 1998).

In this study, we have applied 16S rDNA analytical techniques to two highly active hydrocarbon-degrading enrichment cultures derived from contaminated sediments. The kinetics of hydrocarbon-degradation by these enrichments has been described elsewhere (Holder et al., 1999). The choice of primers and PCR conditions are critical to the generation and interpretation of the community profile recovered. The 16S rDNA sequence database is growing rapidly, and as sequence information continues to accrue, it is becoming increasingly apparent that the available data represents only a small fraction of naturally occurring 16S rDNA sequence variation. Frequent revision of PCR primers is therefore necessary to achieve the most efficient and unbiased recovery of diverse bacterial templates (Marchesi et al., 1998). In order to avoid the limitations of one particular primer set and also to allow comparison of efficiency between primer sets, we used three previously described primer sets for the amplification of bacterial community 16S rDNA.

Subsequent analysis of the amplified products can be carried out in a number of ways, classed as either cloning dependent or cloning independent. The sequences of cloned 16S rDNA fragments allow comparison with the 16S rDNA sequences of cultured organisms, and because this type of information is additive, also with the sequences of cloned material from previous studies. At the time of writing, the GenBank database carries over 31 600 partial and complete 16S rDNA sequences (Schuler et al., 1996). A commonly employed cloning-independent method is based on denaturing gradient gel electrophoresis (DUGE; Muyzer et al., 1993), which can be used to separate mixed PCR products into the constituent sequence types based upon their differential melting behavior in a gradient of denaturant chemicals. Herein, one primer set was used to amplify near-full-length copies of the community 16S rRNA genes, for the generation of clone libraries to be subjected to sequence analysis (Lane et al., 1985). Two other primer sets were used for domain-level PCR-DGGE analysis of 16S rDNA of the major bacterial populations, with the excision of prominent bands for sequence analysis (Muyzer et al., 1993, Nübel et al., 1996).

The study described herein had two aims. (1) To determine the major bacterial groups comprising the oil-degrading consortia of two highly active enrichment cultures derived from polluted marine and freshwater sediments (previously described in Holder et al., 1999) and (2), to compare the community profiles obtained by various primer sets and post-amplification screening methods, thereby identifying their strengths and weaknesses as regards the diversity of sequences recovered in relation to the other methods employed.

2. Methods and materials

2.1. Origin of cultures

The microbial communities described herein com-

prised two hydrocarbon-degrading enrichment cultures capable of rapid degradation of both aliphatic and polyaromatic hydrocarbons (Holder et al., 1999). The cultures were derived from long-term accidentally contaminated marine and freshwater sediments. The marine enrichment culture (4-Org) was originally isolated from marine sediment contaminated in 1989 after the Exxon Valdez tanker ran aground. Sediment was collected from Disk Island (147°40′W, 60°29′30′N), and the enrichment was grown in GP2 medium supplemented with potassium nitrate (g/l; NaCl, 21.03; Na_2SO_4, 3.52; KCl, 0.61, KBr, 0.088; $Na_2B_4O_7 \cdot 10H_20$, 0.034; $MgCl_2 \cdot 6H_20$, 9.50; $CaCl_2 \cdot 2H_20$, 1.32; $SrCl_2 \cdot 6H_20$; $NaHCO_2$, 0.17; KNO_3, 2.89; $FeCl_3 \cdot 6H_20$, 0.05; $Na_5P_3O_{10}$, 0.297; pH 8.0). The freshwater culture was derived from an Ohio River site in Cincinnati, Ohio (Anderson Ferry), downstream of a petroleum storage area and constantly contaminated by hydrocarbon spillage for several decades. Sediment was collected from the north side of the Ohio River (84.62°W, 39.07°N) and the enrichment grown in Bushnell–Haas medium (g/l $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.02, KH_2PO_4, 1.0 K_2HPO, 1.0; NH_4NO_3 1.0; $FeCl_3$, 0.05; cholesterol 0.3; in distilled water pH 7.0–7.2). Both cultures were provided with Alaska North Slope crude oil [ANS521, 5 g l^{-1} weathered under vacuum at 250°C to remove the light hydrocarbons; (Anon, 1989)] and grown in 10-l batch culture with vigorous aeration at 20°C for 21 days per sub-culture. Each enrichment culture underwent a minimum of five subcultures (1% inoculum) prior to analysis.

2.2. Culture characterization

The marine culture (4-Org) degraded alkanes with a first-order rate constant of -0.425 ($r^2 = 0.939$) day^{-1} and aromatics at a rate of -0.186 ($r^2 = 0.908$) per day. The freshwater culture degraded alkanes at a rate of -0.43 ($r^2 = 0.97$) per day and aromatics at a rate of -0.036 ($r^2 = 0.76$) per day. Rates were calculated as described previously (Holder et al., 1999). Cultures were concentrated by centrifugation (Beckman J2-21 M centrifuge, JA10 rotor, 10 000 rpm, 4°C, 20 min) and resuspended in 2% NaCl (marine) or 0.85% NaCl (freshwater) containing 10% (v/v) glycerol prior to cell number assay and storage at −80°C. The marine culture (4-Org) was shown to contain 2.5×10^6–4.2×10^8 detectable alkane-degrading and 1.1×10^4–5.2×10^4 aromatic-degrading bacteria per ml as determined by most probable number (MPN) analysis (Wrenn and Venosa, 1996). The freshwater culture was similarly estimated to contain 2.7×10^7 alkane-degrading and 1.9×10^7 aromatic-degrading bacteria per ml.

2.3. Nucleic acid extraction

A portion of each enrichment culture (1 ml) was centrifuged at 13 000 rpm in a bench-top microcentrifuge for 10 min (Biofuge 13™, Hereus Sepatech, GmbH, Germany). The supernatant was discarded and the cell pellet was resuspended in sodium phosphate buffer (500 μl, 120 mM, pH 8.0). DNA was then extracted by mechanical lysis by bead-beating, phenol/chloroform extraction, and resin purification as described previously (Stephen et al., 1999) with the exceptions that an extra chloroform extraction was employed immediately after beating to remove contaminating hydrocarbons and a 'FastPrep™' beating system (Bio-101) was used.

2.4. Amplification of rDNA fragment

All primers and PCR conditions have been described previously. Primer names, PCR parameters and references are given in Table 1. All amplifications used Expand High Fidelity™ polymerase (1.2 U; Boehringer Mannheim, Indianapolis, IN, USA) executed on a Robocycler 96 Gradient™ (Stratagene) with 1 ng of template DNA in a 25-μl reaction volume.

2.5. Cloning and screening of 16S rDNA fragments

Amplification products from duplicate DNA extractions were cloned directly into the PCR-TOPO 2.1™ cloning vector (Invitrogen, Carsbad, CA, USA) according to manufacturer's instructions. For each enrichment, two independent libraries were constructed. Recombinant (white) clones were screened for the presence of insert-containing plasmids by PCR using vector-specific primers (M13 reverse and T7; Invitrogen) and a portion of a colony as template source. Amplification products were digested with restriction endonuclease *Msp*I and analyzed by agarose gel electrophoresis (2% agarose, 1 × TAE buf-

Table 1
PCR primers sets used in this study, amplification parameters and primary references

Primer set	Published pair name	PCR conditions[a]	Reference
1	27F and 1492r	93°C 2 min, followed by 35 cycles of 92°C, 30 s; 50°C, 30 s; 68°C, 90 s	Lane et al., 1985
2	341F-GC and 534r	93°C 2 min, followed by 35 cycles of 92°C, 30 s; 55°C, 30 s; 68°C, 30 s	Muyzer et al., 1993
3	968F-GC and 1401r	93°C 2 min, followed by 35 cycles of 92°C, 30 s; 55°C, 30 s; 68°C, 30 s	Nübel et al., 1996

[a] All amplification reactions finished with a 7-min incubation at 68°C.

fer) and ethidium bromide fluorescence. A total of 40 insert-containing clones from each enrichment were screened by *Msp*I digestion. Clones were selected to represent each restriction pattern (at least one clone for each pattern, two for common patterns) and subjected to sequence analysis.

2.6. Sequence analysis of cloned products

PCR products derived from clones after amplification with the vector-specific primers M13 reverse and T7 (Invitrogen) were purified by use of GeneClean Spin columns (BIO-101, Vista, CA, USA) and quantified by fluorimetry (Hoefer DyNA-Quant 200™ Fluorometer and Hoechst H33258 dye binding assay; Pharmacia Biotech, Piscataway, NJ, USA). Double-strand sequencing was carried out on an Applied Biosystems automated sequencer (model 373) with 'Prism'™ dye terminators. All clones were sequenced using the primers 27f, 519r, 907r, 1492r (Lane et al., 1985), and 1098r (AAGGGTTGCGCTCGTTGCG; *E. coli* numbering (Brosius et al., 1981; this study), except clones 4-Orgl-6 and 4-Orgl-38 for which the primer 1184r (ATGATTTGACGTCATCCCC; *E. coli* numbering (Brosius et al., 1981; this study) was substituted for 1098r due to primer site incompatibility. Sequences were assembled using 'Seqpup Version 0.6.' (Gilbert, 1996). Reference sequences were recovered from the RDP release 7.0 of July 1998 (Maidak et al., 1999). Supplemental sequences were retrieved from GenBank via the National Institute for Biotechnology Information (NCIB) internet node using the Entrez facility (Schuler et al., 1996). Crude alignments of recovered sequences were performed using the ALIGN facility of the RDP followed by manual alignment within Seqpup V. 0.6. Ambiguous bases and regions that could not be unambiguously aligned were deleted from phylogenetic analysis by use of the Genetic Data Environment 2.2 'mask' function operated within ARB (Strunk and Ludwig, 1998). Phylogenetic algorithms (DNA-DIST, NEIGHBOR and SEQBOOT) also operated within the ARB software environment. Compiled sequences were screened for possible chimeric origin by use of the RDP 'CHECK CHIMERA' program and by independent neighbor-joining analysis of the 5′ and 3′ halves of sequences within the dataset shown in Fig. 1. Similarity comparisons of partial clone sequences using the BLAST 2.0 algorithm (Altschul et al., 1997) were carried out via the NCBI internet node.

2.7. Nucleic acid accession numbers

The near-full length 16S rDNA sequences of the marine and freshwater hydrocarbon-degrading bacteria have been submitted to GenBank with the consecutive accession numbers AF143820–AF143844.

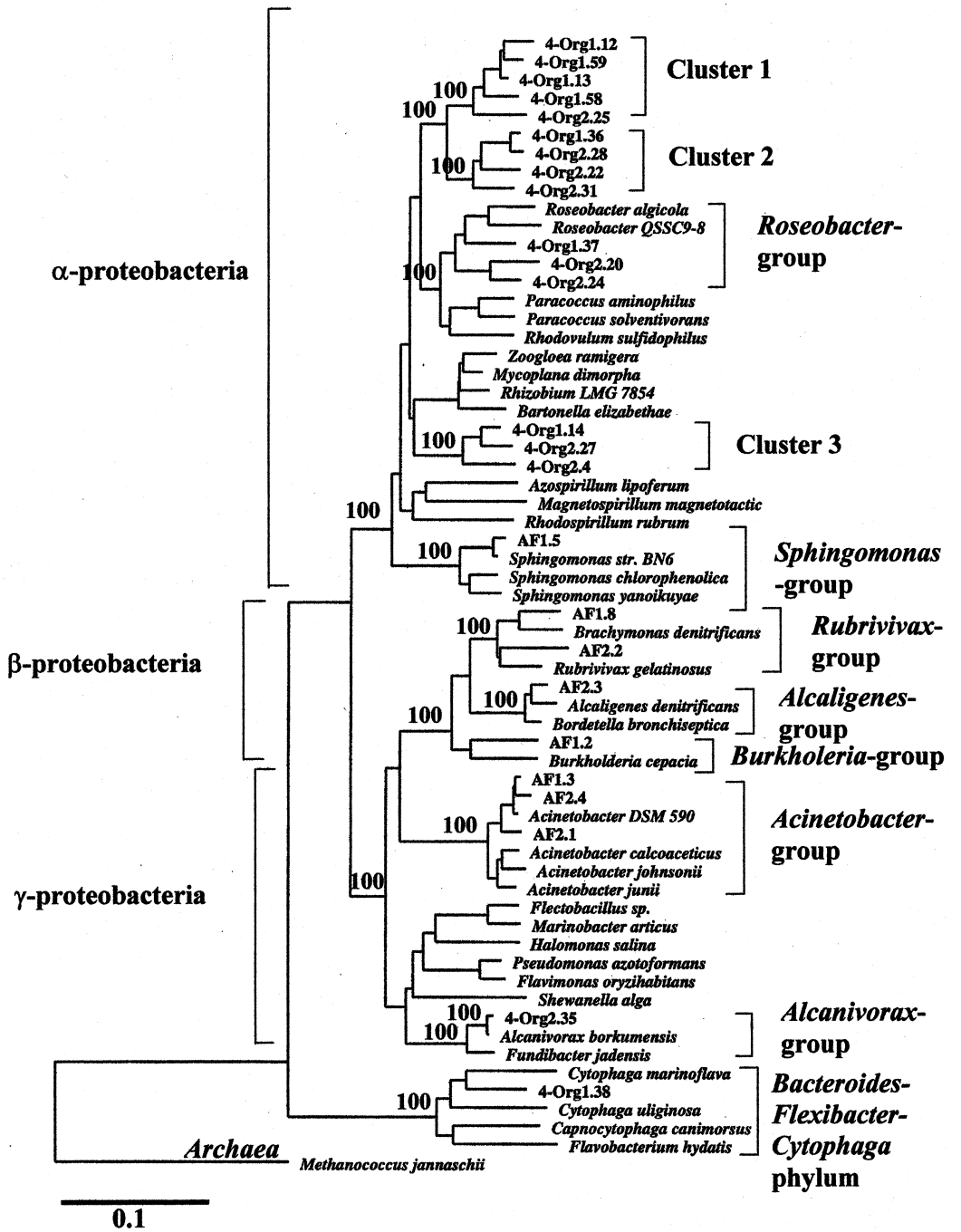

Fig. 1. Neighbor-joining dendrogram with Olsen correction of cloned near-full length sequences derived from marine (4-Org) and freshwater (AF) enrichment cultures with reference sequences derived from the Ribosomal Database Project (Maidak et al., 1999). Numbers represent Bootstrap support on 100 replicates for branches immediately to their right, and for simplicity are shown only for nodes of relevance to the novel sequences described. All analyses were carried out within the ARB sequence management system for the Linux operating system (Strunk and Ludwig, 1998). Scale-bar represents 10% estimated change.

2.8. DGGE analysis

All gradients, run times and percentages of acrylamide were as given in the appropriate reference for each primer set cited in Table 1. DGGE was performed using the D-Code™ system (16/16 cm gel; BioRad, Hercules, CA) maintained at a constant temperature of 60°C in 6 l of 0.5 TAE buffer (20 mM Tris–acetate, 0.5 mM EDTA, pH 8.0). The denaturant was defined as 100% = 7 M urea plus 40% v/v formamide. Gels were stained in purified water (Milli-Ro™, Millipore, Bedford, MA) containing ethidium bromide at 0.5 mg l^{-1}, and destained twice in 0.5 × TAE. Images were digitally captured using ALPHA-IMAGER™ software (Alpha-Innotech, San Leandro, CA).

The major bands were excised, re-amplified, and subjected to sequence analysis using one of the original PCR primers to generate up to 300 bases of sequence data. Sequencing and sequence analysis was as described in Section 2.6. Due to the presence of multiple PCR products migrating to the same point in the DGGE gel, not all DGGE bands generated high-quality sequence data. Illegible sequence data was discarded.

3. Results

3.1. Phylogenetic analysis of near-full-length 16S rDNA sequences

The number of clones selected for screening by restriction digestion was chosen to provide a similar level of sensitivity to detection of diversity as the PCR-DGGE methods, which can detect organisms that provide 1–2% of the total population in a mixture (Muyzer et al., 1993; Stephen et al., 1999). *Msp*I digestion patterns of clones randomly selected from the seawater (4-Org) and freshwater hydrocarbon-degrading enrichments (AF) generated 12 and 22 different restriction patterns respectively. The frequencies of recovery of each pattern are given in Table 2. At least one representative of each pattern-type was selected for complete sequencing of the circa 1.5-kb insert. A total of ten of these clones were determined to be of chimeric origin (four marine, six freshwater; data not shown). Chimera-check analysis of clone 4-Org 1–37 gave ambiguous results and we reserve judgement on the validity of this sequence as a genuine 16S rDNA. As clones were not selected randomly for sequence analysis, it

Table 2
Frequency of recovery of different *Mspl* restriction patterns from marine (4-Org) and freshwater (AF) hydrocarbon-degrading enrichment cultures. Single enzyme digests are useful for screening purposes, but should not be used to infer phylogenetic placement

	Restriction pattern class	Frequency %	Clone representatives sequenced
Culture 4-Org	1	3	4-Org24/4-Org114
	2	40	4-Org112/4-Org159
	3	3	4-Org113/
	4	7	4-Org136/4-Org227
	5	7	4-Org137/4-Org228
	6	3	4-Org138
	7	3	4-Org158
	8	10	4-Org220/4-0rg23 1
	9	3	4-Org222
	10	3	4-Org224
	11	7	4-Org225
	12	7	4-Org235
Culture AF	1	6	Af12
	2	48	AF-13/AF-24
	3	3	AF-15
	4	3	AF-18
	5	3	AF-21
	6	24	AF-22
	7	15	AF-23

is not possible to estimate the percentage of clones of chimeric origin. A neighbor-joining comparison of the remaining sequences from both enrichments is shown in Fig. 1. The frequency of recovery of each restriction-type are given in Table 2.

3.2. Marine enrichment

The majority of clones recovered from the seawater enrichment were related to the α-subgroup proteobacteria and fell within four sequence clusters (Fig. 1). Half (9/18) of the clones formed two strongly supported sister-groups within the α-proteobacteria, neither cluster being closely related to any known bacteria for which comparable-length sequences are available (clusters 1 and 2). As clones recovered from environmental samples are generally not subjected to full-length sequence analysis, the BLAST 2.0 algorithm was used to compare short fragments of cloned sequences to the GenBank database. This revealed that sequences belonging to clusters 1 and 2 showed a close relationship (at least 96 and 94% identity over the 443 bases of 5′ sequence, respectively) to a cloned sequence previously recovered from a marine environment (‘Tomales Bay 1g wh’; Accession number AF055829). A third group of three cloned sequences (cluster 3) also fell within the α-subdivision proteobacteria, showed a loose relationship to the genus *Roseobacter,* and fell within the *Rhodobacter* group (Maidak et al., 1999). The fourth cluster of three sequences (cluster 4) was not affiliated with any established 16S rDNA sequence cluster within the α-proteobacteria. These sequences showed no more than 90% similarity to any known sequences. The remaining two clones derived from this enrichment represented single sequences within the *Bacteroides–Flexibacter–Cytophaga* phylum and γ-subdivision of the class proteobacteria. The latter was found to be >99% identical to the 16S rDNA sequences of a cultured *Alcanivorax borkumensis* strain capable of degrading *n*-alkanes and producing biosurfactants (Yakimov et al., 1998).

3.3. Freshwater enrichment

Clones derived from the freshwater enrichment culture revealed a completely different community structure from that of the marine-derived clone library, with the majority of clones clearly related to known bacterial genera (Fig. 1). One clone was derived from the α-subgroup proteobacteria and fell within the *Sphingomonas* group, showing >98% sequence identity to the chlorophenol-degrading *Sphingomonas* sp. BN6 (Nohynek et al., 1996). Four clones fell within the β-subdivision proteobacteria. One of these fell within the *Burkholdaria* subgroup and one in the *Bordetella* subgroup. The remaining two clones could not be classified beyond the level of β-proteobacteria. The remaining three clones all fell within the *Acinetobacter* subgroup of the γ-subgroup proteobacteria.

3.4. Comparison of clone library structure with DGGE band analysis

Figs. 2 and 3 show the DGGE profiles obtained from the freshwater and marine enrichments with primer sets 2 and 3, respectively. These results demonstrated reasonable agreement between the clone libraries and the products amplified using both DGGE primer sets (Muyzer et al., 1993; Nübel et al., 1996; Heuer and Smalla, 1997). However, no *Sphingomonas*-like sequences were recovered from the freshwater culture using primer set 3. In both enrichments, both primer pairs 2 and 3 detected an *Actinomycete-like* sequence identical to several members of the genus *Rhodococcus* that was not recovered from the clone library. The groups detected by each approach are summarized in Table 3.

4. Discussion

4.1. Dominant community members

The two enrichment cultures studied, which were derived from different environments (freshwater and seawater) and grown in different media, represented two different consortia capable of rapid hydrocarbon degradation. The marine culture was apparently dominated by α-proteobacteria, the majority of which were inferred to have been recovered from, as yet uncultured, organisms. Based on 16S sequence similarity, which should not be taken as confirmation of species identity (Fox et al., 1992), two organisms

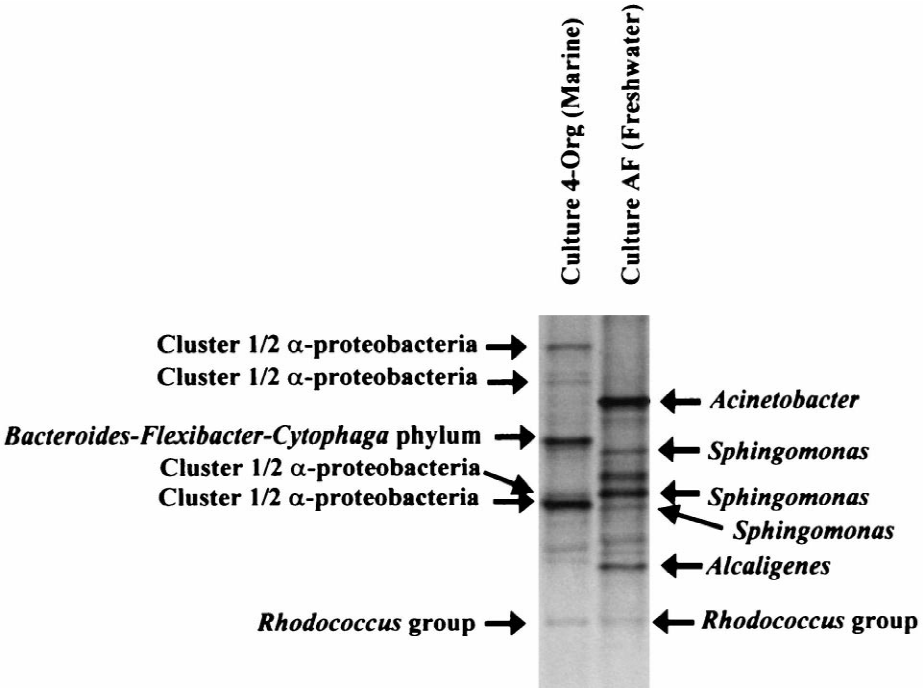

Fig. 2. DGGE analysis of marine (4-org) and freshwater (AF) enrichment cultures generated by the method of Muyzer et al., 1993 (primer set 2). Labeled bands generated legible sequences when excised and reamplified which were compared to clone sequences and databases. Unlabeled bands generated mixed sequences that could not be used for taxonomic ranking.

were detected with close relationship to known hydrocarbon-degrading and surfactant-producing genera, *Alcanivorax*, in the γ-subgroup proteobacteria (Yakimov et al., 1998) and *Rhodococcus* sp., an actinomycete lineage (Colquhoun et al., 1998a,b). The domination of this culture by α-proteobacteria may be interesting in the light of the recent discovery of α-proteobacteria as the dominant organisms colonizing an experimental beach oil-spill (Macnaughton et al., 1999). The freshwater enrichment culture appeared to be dominated by organisms closely related to cultured genera in the α- β- and γ-subgroups of the proteobacteria (including *Sphingomonas* (α-subgroup), *Alcaligenes* (β-subgroup), *Acinetobacter*, and *Pseudomonas* (γ-subgroup)) and an actinomycete identical over the regions sequenced to cultured members of the genus *Rhodococcus*, all of which contain efficient hydrocarbon-degrading species (Chaineau et al., 1999 and references cited therein).

4.2. Consistency of profile with different domain-level PCR primer sets

The congruence between the community profiles provided using the PCR-primer sets 1 through 3 was generally high, however none of the three profiles were identical (Table 3). Primer sets 1 and 2, used to create the clone library and to amplify the V3 region for DGGE analysis respectively, both appear to target highly conserved sites in the bacterial 16S rDNA gene. The primer set 2 forward primer is commonly used as a diagnostic probe site to differentiate *Bacteria* from *Archaea* (Amann et al., 1990, Snaidr et al., 1999), and the reverse partner is commonly considered a ‘universal’ conserved sequence within the small subunit rRNA genes of all known organisms (Stahl et al., 1988; Stapleton and Sayler, 1998). However, the region between these two sites is highly variable and less than 200 bp in length, and therefore not ideal for phylogenetic

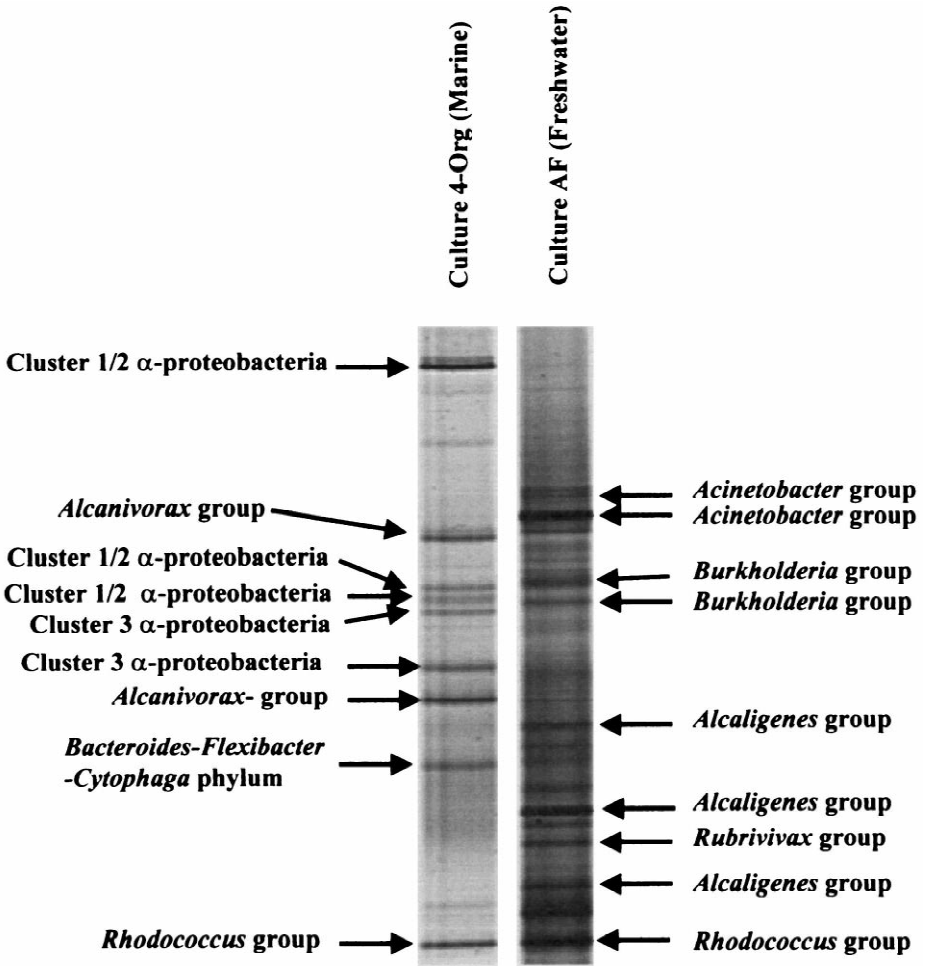

Fig. 3. DGGE analysis of marine (4-org) and freshwater (AF) enrichment cultures generated by the method of Nübel et al., 1996 (primer set 3). Labeled bands generated legible sequences when excised and reamplified which were compared to clone sequences and databases. Unlabeled bands generated mixed sequences that could not be used for taxonomic placement.

inference. Conversely, primer set 3 targeted somewhat less highly conserved regions of the bacterial 16S gene, but recovered a more informative and longer fragment. The near-full-length rDNA fragments recovered using cloning strategies carry the maximum amount of phylogenetic information, but are unsuitable for rapid community profiling (Fodde and Losekoot, 1994).

A comparison of the sequences of the primer set 3 oligonucleotides to the *Sphingomonas*-like clone sequence and *Sphingomonas* sequences from the RDP database provided a clear explanation for the inability of primer set 3 to amplify *Sphingomonas*-like sequences, a 3′ terminal mismatch with the reverse primer. The incompatibility of this primer set with the 16S rDNA of *Sphingomonas*-like organisms was confirmed by failure to generate an amplification product from genomic DNA extracted from *Sphingomonas aromaticivorans* B0695 (Balkwill et al., 1997; data not shown). The failure to recover several groups of sequences with primer set 2 is less easily explained, but it is possible that such products co-

Table 3
Summary of groups of organisms detected by the three community profiling methods applied to cultures 4-Org and AF [a]

Primer set	α-Proteobacteria Cl.1	α-Proteobacteria Cl.2	α-Proteobacteria Cl.3	*Roseobacter* group	*Alcanivorax* group	B–F–C [b] phylum	*Rhodococcus* group	*Pseudomonas* group
Culture 4-Org								
1	✓	✓	✓	✓	✓	✓	×	×
2	✓	✓	×	×	×	✓	✓	×
3	✓	✓	✓	×	✓	×	✓	×
Culture AF								
	Sphingomonas group	*Rubrivivax* group	*Alcaligenes* group	*Burkholderia* group	*Acinetobacter* group	*Rhodococcus* group		
1	✓	✓	✓	✓	✓	×		
2	✓	✓	✓	×	✓	✓		
3	×	✓	✓	✓	✓	✓		

[a] A tick (✓) indicates that the group was detected, a cross (×) indicates that no members of this group were detected by the profiling method used.
[b] *Bacteroides–Flexibacter–Cytophaga* phylum.

migrated with more dominant sequences and were not detected, or fell within mixed bands that did not generate legible sequences. Neither DGGE primer set recovered *Roseobacter*-like sequences from the marine culture, despite their apparent abundance in the clone library. However, based on both restriction analysis and clone sequencing (Table 2, Fig. 1), the *Roseobacter*-like sequences formed a rather diverse group, and it is possible that no individual sequence was sufficiently common to form a distinct band in DGGE analysis. The *Rhodococcus*-like actinomycete sequence detected in both cultures with both DGGE-PCR primer-pairs was not detected during screening of the clone-libraries. The high GC-content of this sequence made it particularly easy to recognize by DGGE, due to its formation of a distinct band lower in the gel than all other detectable 16S rDNA fragments. Detection of such a sequence in the clone library by screening of random clones is a function of the number of clones screened, whereas screening by DGGE biases detection towards sequences with unusual melting behavior. Thus, the structure of a bacterial community revealed by 16S-rDNA based PCR methods is to a considerable extent dependent on the method chosen to generate the profile, and it essential that this is appreciated when interpreting the results of such an analysis. The oligonucleotide primer pairs used in this study are some of the most widely used sets reported and target the most highly conserved regions of the 16S rRNA gene within the domain *Bacteria* (e.g., Marchesi et al., 1998), yet it is evident that intrinsic biases exist in each.

5. Conclusion

To our knowledge, this is the first report of the use of multiple primer sets to analyze unknown bacterial community structures at the kingdom level. Given that the true limitation and intrinsic bias of all primer sets is unknown, it may be advisable to take this precaution as a routine procedure in order to gain the most accurate insight possible into community compositions. Culture-independent methods such as those used here profile bacterial communities more accurately than traditional culture-based methods (Muyzer and Smalla, 1998; Torsvik et al., 1998). However, the fact that none of the three profiling methods used here generated identical results demonstrates that these profiles must still be considered method-dependent. Specifically, due to the likelihood of failure to detect *Sphingomonas* species, which are heavily implicated in degradation of a broad range of pollutants (White et al., 1996; Kastner et al., 1998; Barkay et al. 1999; Hamann et al., 1999; and references cited therein), this study has shown that the use of primer set 3 to study bioremediative communities in non-marine environments cannot be recommended.

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References

Anon, 1989. Crude petroleum oil-determination of distillation characteristics using 15 theoretical plates columns. International Organization for Standardization. Draft International Standard ISO/DIS 8708.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. et al., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402, http://www.ncbi.nlm.nih.gov/BLAST last accessed 8-25-1999.

Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., Stahl, D.A., 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56, 1919–1925.

Bakken, L.R., 1985. Separation and purification of bacteria from soil. Appl. Env. Microbiol. 49, 1188–1195.

Balkwill, D.L., Reeves, R.H., Drake, G.R., Reeves, J.Y., Crocker, F.H., King, M.B. et al., 1997. Phylogenetic characterization of bacteria in the subsurface microbial culture collection. FEMS Microbiol. Rev. 20, 201–216.

Barkay, T., Navon-Venezia, S., Ron, E.Z., Rosenberg, F., 1999. Enhancement of solubilization and biodegradation of polyaromatic hydrocarbons by the bioemulsifier alasan. Appl. Environ. Microbiol. 65, 2697–2702.

Brosius, J., Dull, T.L., Sleeter, D.D., Noller, H.F., 1981. Gene organization and primary structure of a ribosomal RNA operon from *Escherichia coli*. J. Mol. Biol. 148, 107–127.

Chaineau, C.H., Morel, J., Dupont, J., Bury, E., Oudot, J., 1999. Comparison of the fuel oil biodegradation potential of hydrocarbon-assimilating microorganisms isolated from a temperate agricultural soil. Sci. Total. Environ. 227, 237–247.

Colquhoun, J.A., Heald, S.C., Li, L., Tamaoka, J., Kato, C., Horikoshi, K. et al., 1998a. Taxonomy and biotransformation activities of some deep-sea actinomycetes. Extremophiles 2, 269–277.

Colquhoun, J.A., Mexson, J., Goodfellow, M., Ward, A.C., Horikoshi, K., Bull, A.T., 1998b. Novel *Rhodococci* and other mycolate actinomycetes from the deep sea. Ant. Van Leeuwen. 74, 27–40.

Fodde, R., Losekoot, M., 1994. Mutation detection by denaturing gradient gel electrophoresis (DGGE). Human Mutation 3, 83–94.

Fox, G.E., Wisotzkey, J.D., Jurtshuk, Jr. P., 1992. How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. Int. J. Syst. Bacteriol. 42, 166–170.

Hamann, C., Hegemann, J., Hildebrandt, A., 1999. Detection of polycyclic aromatic hydrocarbon degradation genes in different soil bacteria by polymerase chain reaction and DNA hybridization. FEMS Microbiol. Lett. 1731, 255–263.

Head, I.M., 1998. Bioremediation: towards a credible technology. Microbiol. 144, 599–608.

Holder, E.L., Miller, K.M., Haines, J.R., 1999. Crude oil component biodegradation kinetics by marine and freshwater consortia. In: Alleman, B.C., Leeson, A. (Eds.), In Situ Bioremediation of Petroleum Hydrocarbon and Other Organic Compounds, Vol. 5(3), Battelle Press, Columbus, Richland, USA.

Heuer, H., Smalla, K., 1997. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) for studying soil microbial communities. In: van Elsas, J.D., Wellington, E.M.H., Trevors, J.T. (Eds.), Modern Soil Microbiology, Marcel Dekker, New York, pp. 353–373.

Gilbert, D.G., 1996. SeqPup sequence alignment editor. Bloomington, Indiana. Available from author by ftp (ftp.bio.indiana.edu).

Juretschko, S., Timmermann, G., Schmid, M., Schleifer, K.H., Pommerening-Roser, A., Koops, H.P. et al., 1998. Combined molecular and conventional analyses of nitrifying bacterium diversity in activated sludge: *Nitrosococcus mobilis* and *Nitrospira*-like bacteria as dominant populations. Appl. Environ. Microbiol. 64, 3042–3051.

Kastner, M., Breuer-Jammali, M., Mahro, B., 1998. Impact of inoculation protocols, salinity, and pH on the degradation of polycyclic aromatic hydrocarbons (PAHs) and survival of PAH-degrading bacteria introduced into soil. Appl. Environ. Microbiol. 64, 359–362.

Lane, D.J., Pace, B., Olsen, G.J., Stahl, D.A., Sogin, M.L., Pace, N.R., 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc. Natl. Acad. Sci. USA 82, 6955–6959.

Macnaughton, S.J., Stephen, J.R., Venosa, A.D., Davis, G.A., Chang, Y.-J., White, D.C., 1999. Microbial population changes during bioremediation of an experimental oil spill. Appl. Environ. Microbiol 65, 3566–3574.

Maidak, B.L., Cole, J.R., Parker, Jr. C.T., Garrity, G.M., Larsen, N., Li, B., Lilburn, T.G. et al., 1999. A new version of the RDP. Nucleic Acids Res. 27, 171–173.

Marchesi, J.R., Sato, T., Weightman, A.J., Martin, T.A., Fry, J.C., Hiom, S.J. et al., 1998. Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16s rRNA. Appl. Environ. Microbiol. 64, 795–799, Published erratum appears in Appl. Environ. Microbiol. 64 (1998) 2333.

Muyzer, G., de Waal, E.C., Uitterlinden, A.G., 1993. Profiling of microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction amplified genes coding for 16S rRNA. Appi. Environ. Microbiol. 59, 695–700.

Muyzer, G., Smalla, K., 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Ant.Van Leeuwen. 73, 127–141.

Nohynek, L.J., Nurmiaho-Lassila, F.L., Suhonen, E.L., Busse, H.J., Mohammadi, M., Hantula, J., 1996. Description of chlorophenol-degrading *Pseudomonas* sp. strains KE1T, KF3, and NKF1 as a new species of the genus *Sphingomonas Sphingomonas subarctica* sp. nov. Int. J. Syst. Bacteriol. 46, 1042–1055.

Nübel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R.I. et al., 1996. Sequence heterogeneities of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature-gradient gel electrophoresis. J. Bacteriol. 178, 5636–5643.

Rabus, R., Wilkes, H., Shramm, A., Harms, G., Behrends, A., Amann, R., Widdel, F., 1999. Anaerobic utilization of alkylbenzenes and *n*-alkanes from crude oil in an enrichment culture of dentitrifying bacteria affiliated with the 13-subclass of Proteobacteria. Environ. Microbiol. 1, 145–158.

Schuler, G.D., Epstein, J.A., Ohkawa, H., Kans, J.A., 1996. Entrez: molecular biology database and retrieval system. Meth. Enzymol. 266, 141–162, (http://www.ncbi.nlm.nih.gov/Entrez last accessed 8-25-1999).

Skinner, F.A., Jones, P.C.T., Mollison, J.E., 1952. A comparison of direct and plate counting techniques for the quantitative estimation of soil microorganisms. J. Gen. Microbiol. 6, 261–271.

Snaidr, J., Fuchs, B., Wallner, G., Wagner, M., Schleifer, K.-H., Amann, R., 1999. Phylogeny and in situ identification of a morphologically conspicuous bacterium, *Candidatus Magnospira bakii*, present at very low frequency in activated sludge. Environ. Microbiol. 1, 125–135.

Stahl, D.A., Flesher, B., Mansfield, H.R., Montgomery, L., 1988. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. Appl. Environ. Microbiol. 54, 1079–1084.

Stapleton, R.D., Sayler, G.S., 1998. Assessment of the microbiological potential for the natural attenuation of petroleum hydrocarbons in a shallow aquifer system. Microb. Ecol. 36, 349–361.

Stephen, J.R., Chang, Y.-J., Macnaughton, S.J., Whitaker, S.L.,

Hicks, C.L., Leung, K.T. et al., 1999. Fate of a mixture of five metal resistant bacteria introduced into pristine and heavγ-metal-contaminated soil microcosms assessed by DGGE. Environ. Toxicol. Chem. 18, 1118–1123.

Strunk, O., Ludwig, W., 1998. ARB: a software environment for sequence data. http://www.mikro.biologie.tu-muenchen.de. Department of Microbiology, Technische Universiffit Mtinchen, Germany.

Torsvik, V., Daae, F.L., Sandaa, R.A., Ovreas, L., 1998. Novel techniques for analysing microbial diversity in natural and perturbed environments. J. Biotechnol. 64, 53–62.

Tunlid, A., White, D.C., 1992. Biochemical analysis of biomass, community structure, nutritional status and metabolic activity of the microbial community in soil. In: Bollag, J.-M., Stotzky, G. (Eds.), Soil Biochemistry, Vol. 7, Marcel Dekker Inc, New York, pp. 229–262.

Watanabe, K., Teramoto, M., Futamata, H., Harayama, S., 1998. Molecular detection, isolation, and physiological characterization of functionally dominant phenol-degrading bacteria in activated sludge. Appl. Environ. Microbiol. 64, 4396–4402.

White, D.C., 1983. Analysis of microorganisms in terms of quantity and activity in natural environments. In: Slater, J.H., Whittenbury, R., Wimpenny, J.W.T. (Eds.), Microbes in Their Natural Environments, Society For General Microbiology, Vol. 34, Cambridge University Press, London, pp. 37–66.

White, D.C., Ringelberg, D.B., Hedrick, D.B., Nivens, D.E., 1993. Rapid identification of microbes from clinical and environmental matrices by mass spectrometry. In: Fenselau, C. (Ed.), Identification of Microorganisms By Mass Spectrometry, American Chemical Society Symposium Series, Vol. 541, American Chemical Society, Washington, DC, pp. 8–17.

White, D.C., Sutton, S.D., Ringelberg, D.B., 1996. The genus *Sphingomonas*: physiology and ecology. Curr. Opin. Biotechnol. 7, 301–306.

Wrenn, B.A., Venosa, A.D., 1996. Selective enumeration of aromatic and aliphatic hrdrocarbon degrading bacteria by a most-probable-number procedure. Can. J. Microbiol. 42, 252–258.

Yakimov, M.M., Golyshin, P.N., Lang, S., Moore, E.R., Abraham, W.R., Lunsdorf, H. et al., 1998. *Alcanivorax borkumensis* gen. nov., sp. nov., a new, hydrocarbon-degrading and surfactant-producing marine bacterium. Int. J. Syst. Bacteriol. 48, 339–348.