Applied and Environmental Microbiology, Feb. 2008, p. 1176–1182 Vol. 74, No. 4
0099-2240/08/$08.00+0 doi:10.1128/AEM.01518-07

Enrichment, Isolation, and Phylogenetic Identification of Polycyclic Aromatic Hydrocarbon-Degrading Bacteria from Elizabeth River Sediments▽†§

Edward J. Hilyard,[1]‡ Joanne M. Jones-Meehan,[2] Barry J. Spargo,[1] and Russell T. Hill[3]*

Naval Research Laboratory, Washington, DC 20375[1]; Department of Homeland Security, Washington, DC 20528[2]; and Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Baltimore, Maryland 21202[3]

Received 5 July 2007/Accepted 12 December 2007

The diversity of indigenous bacteria in sediments from several sites in the Elizabeth River (Virginia) able to degrade multiple polycyclic aromatic hydrocarbons (PAHs) was investigated by the use of classical selective enrichment and molecular analyses. Enrichment cultures containing naphthalene, phenanthrene, fluoranthene, or pyrene as a sole carbon and energy source were monitored by denaturing gradient gel electrophoresis (DGGE) to detect changes in the bacterial-community profile during enrichment and to determine whether the representative strains present were successfully cultured. The DGGE profiles of the final enrichments grown solely on naphthalene and pyrene showed no clear relationship with the site from which the inoculum was obtained. The enrichments grown solely on pyrene for two sample sites had >80% similarity, which suggests that common pyrene-degrading strains may be present in these sediments. The final enrichments grown on fluoranthene and phenanthrene remained diverse by site, suggesting that these strains may be influenced by environmental conditions. One hundred and one isolates were obtained, comprising representatives of the actinomycetes and alpha-, beta-, and gammaproteobacteria, including seven novel isolates with 16S rRNA gene sequences less than 98% similar to known strains. The ability to degrade multiple PAHs was demonstrated by mineralization of ^{14}C-labeled substrate and growth in pure culture. This supports our hypothesis that a high diversity of bacterial strains with the ability to degrade multiple PAHs can be confirmed by the combined use of classical selective enrichment and molecular analyses. This large collection of diverse PAH-degrading strains provides a valuable resource for studies on mechanisms of PAH degradation and bioremediation.

Polycyclic aromatic hydrocarbons (PAHs) are common ubiquitous compounds found worldwide in soils and sediments as a result of both natural and anthropogenic production (7). High-molecular-weight PAHs from anthropogenic sources can reach toxic concentrations that are detrimental to the environment and human health (1, 23, 25, 33). These compounds persist in the environment and, due to their hydrophobicity, become associated with particulate matter, such as clays and humics, that are deposited in soils and sediment. PAHs are lipophilic and have the potential to biomagnify through the food chain (18). Recently, the U.S. Department of Health and Human Services listed 15 PAHs as being carcinogenic (32).

The use of conventional remediation approaches, such as dredging and incineration, can be costly and may cause further damage to the environment by dispersing PAHs and making them more bioavailable. PAHs are naturally degraded by microorganisms (18, 19, 26, 27), so an understanding of the microbial processes occurring at contaminated sites may suggest bioremediation strategies that could be effective in reducing PAH concentrations below toxic levels. Previous studies have resulted in the successful isolation of PAH-degrading bacteria from contaminated environmental samples (4, 5, 10, 35), including aquatic sediments (8, 9, 12, 13, 14, 15). In general, these studies have relied on selective enrichment in minimal media supplemented with naphthalene, pyrene, and/or phenanthrene, but the diversity of bacterial assemblages in enrichments has not been monitored by molecular approaches and it has not been clear whether many of the strains enriched in the liquid media have subsequently been successfully isolated on plates and obtained in pure culture. The diversity of microbes involved in PAH degradation in the environment has also been studied by molecular approaches using primers or probes that can be used to measure PAH degradation potential (3, 22, 30).

There are advantages to successfully isolating and growing PAH-degrading isolates compared to using molecular techniques to demonstrate their presence in environmental samples. First, isolates can be examined in detail to elucidate links between phylogeny and catabolic capability and to examine processes such as horizontal gene transfer. For example, Hedlund and Staley (15) found that *Pseudoalteromonas* strains with identical 16S rRNA gene sequences varied in the suite of PAHs that they could degrade and had likely acquired a gene coding for a naphthalene-degrading enzyme by horizontal

* Corresponding author. Mailing address: Center of Marine Biotechnology, Columbus Center Suite 236, 701 E. Pratt Street, Baltimore, MD 21202. Phone: (410) 234-8883. Fax: (410) 234-8896. E-mail: hillr@umbi.umd.edu.

† Supplemental material for this article may be found at http://aem.asm.org/.

§ Contribution no. 07-164 from the Center of Marine Biotechnology.

‡ Present address: Naval Medical Research Center, Silver Spring, MD 20910.

▽ Published ahead of print on 21 December 2007.

transfer. Second, isolates can be identified that degrade PAHs but do not contain genes with high homology to known genes implicated in PAH degradation (15, 36). These isolates can then be studied as sources of highly divergent or novel PAH-degrading enzymes. Third, strains may be useful in bioaugmentation of PAH-contaminated sites for bioremediation. Microcosm and pilot-scale studies testing bioaugmentation have often given poor results (17), but it is conceivable that outcomes could be better with novel isolates that have not yet been tested for environmental persistence and PAH degradation metabolic capability.

Our hypothesis was that a high diversity of bacterial strains with the ability to degrade multiple PAHs can be confirmed by combining the use of selective enrichment and molecular analyses to follow the enrichment process and to characterize those isolates that could be maintained in pure culture. We used naturally occurring aerobic bacterial communities in estuarine sediments contaminated with PAHs as inocula to establish a series of enrichment cultures. Our goal was twofold: (i) to monitor the population shifts during enrichment on different substrates by using molecular approaches, including denaturing gradient gel electrophoresis (DGGE), PCR, and 16S rRNA gene sequencing, and (ii) to obtain novel isolates with the ability to degrade multiple PAHs.

MATERIALS AND METHODS

Sediment samples. Sediment samples were collected from three sites designated L, RC, and P16 in the Elizabeth River, Norfolk, VA (Fig. 1). Each site was located just offshore from oil storage tanks and next to fueling piers. The site L sediment sample was collected at a water depth of 5 m at 15°C, pH 7.2, 1.7% (wt/wt) salinity, and dissolved oxygen (DO) at 76% saturation. The sample from site RC was collected at a water depth of 1.4 m at 19°C, pH 7.3, 1.6% (wt/wt) salinity, and DO at 85% saturation. The sample from site P16 was collected at a water depth of 1 m at 20°C, pH 7.2, 1.5% (wt/wt) salinity, and DO at 89% saturation. The samples were transported to the laboratory on ice and stored at 4°C until they were analyzed. The presence of PAH contamination in the sediment samples was confirmed by solvent extraction (accelerated solvent extractor; Dionex, Sunnyvale, CA) and gas chromatography-mass spectrometry analysis using a Hewlett-Packard model 6890 gas chromatograph with a 5973 mass selective detector (Agilent Technologies, Palo Alto, CA), as described previously (34).

Media. Bushnell-Haas (BH) minimal medium (Sigma-Aldrich Co., St. Louis, MO) with 1.5% NaCl was used for enrichment cultures and isolation. Solid medium contained 1.5% (wt/vol) Noble agar (Difco Laboratories, Detroit, MI). PAHs were purchased from Sigma-Aldrich Co. Stock concentrations (50 mg/ml) of phenanthrene, fluoranthene, pyrene, chrysene, and benzo[*a*]pyrene were prepared in acetone, while naphthalene was dissolved in methanol at a final concentration of 100 mg/ml. Phenanthrene, fluoranthene, and pyrene were oversprayed onto a solid agar medium after streak plating of bacteria (20). Naphthalene dissolved in methanol was applied to a petri dish lid, the methanol was allowed to evaporate, and volatile naphthalene was the sole carbon and energy source for microbes on BH agar plates.

Enrichment cultures and isolation. Four ~1-g sediment samples were inoculated into separate Erlenmeyer flasks containing 100 ml BH minimal medium and 1.5% (wt/vol) NaCl. One of the following PAHs (5 mg) served as the sole carbon and energy source for growth: naphthalene, phenanthrene, fluoranthene, or pyrene. The PAHs were added to empty flasks, the solvent was allowed to evaporate, and liquid medium was added, followed by an inoculum. An additional culture flask was set up for each sample site with no added carbon source to serve as a negative control. Two successive enrichments were prepared, with 1% (vol/vol) culture fluid used as the inoculum for each subculture at 3-week intervals. The cultures were incubated aerobically at room temperature with shaking at 175 rpm. The initial enrichment culture was designated A, and the second and third subcultures were designated B and C. To obtain PAH-degrading isolates, the B and C enrichments were diluted in BH, plated on BH agar plates, and sprayed with the same PAH used in the enrichment. Colonies surrounded by zones of clearing were subcultured to BH agar plates and sprayed with the same PAH. Replicate plating was done using LB agar plates, BH agar plates sprayed with PAH, and BH agar plates without PAH (control). The criterion for selection of naphthalene-degrading strains was enhanced growth on plates with added substrate compared to the control plates without added substrate. Selected pyrene-degrading isolates from pyrene spray plates were subcultured to BH agar plates and oversprayed with either fluoranthene, chrysene, benzo[*a*]pyrene, dibenzo[*a*,*i*]pyrene, or a mixture of phenanthrene/pyrene for cometabolism studies.

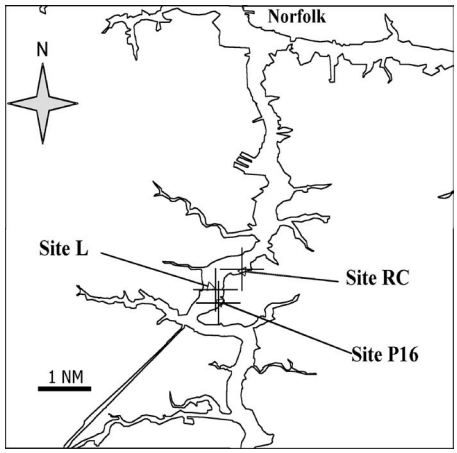

FIG. 1. Locations of the three sample sites along the southern branch of the Elizabeth River in Virginia.

Liquid culture and mineralization. Twelve isolates representing each genus were grown in liquid culture tubes containing 4 ml (BH) minimal medium and 0.5 mg of one of the following PAHs: naphthalene, phenanthrene, fluoranthene, pyrene, chrysene, or benzo[*a*]pyrene. Percent transmittance was measured at 540 nm on a Spectronic 20 (Milton Roy Company) to monitor growth. Overnight cultures grown in LB broth (Difco Laboratories) were diluted 1:20 in BH minimal medium for use as inocula in the mineralization experiment. PAH mineralization was determined with ^{14}C-labeled substrates by a method modified from previous studies (6, 11, 31). One milliliter of the diluted culture fluid was injected into a 40-ml prestoppered serum vial charged with one of the following ^{14}C-labeled substrates: 1.76 μg [^{14}C]naphthalene, 18.6 mCi/mmol; 1.33 μg [^{14}C]phenanthrene, 9 mCi/mmol; 0.91 μg [^{14}C]fluoranthene, 45 mCi/mmol; or 0.34 μg [^{14}C]pyrene, 55 mCi/mmol (Sigma-Aldrich Co.). Vials were prepared in quadruplicate, with one vial serving as an acidified control. Each stopper also contained NaOH-soaked filter paper suspended in the headspace. After incubation for 46 h, samples were acidified with 1 ml 1 M H_2SO_4 and incubated overnight. The filter papers were removed and placed into vials containing 5 ml scintillation cocktail. Disintegrations per minute were measured on a Beckman LS6500 scintillation counter, and the values were used to calculate mineralization. Mineralization rates were calculated from the $^{14}CO_2$ recovered and the specific activity of the initial ^{14}C-labeled substrate.

DNA preparation. Total-community DNA was extracted from each sediment sample using a PowerSoil DNA extraction kit (Mo Bio Laboratories, Inc., Carlsbad, CA) in accordance with the manufacturer's instructions. Total DNA from enrichment cultures was similarly extracted at 3-week intervals at the time of subculturing. Genomic DNA was obtained from isolates by using an UltraClean microbial DNA extraction kit (Mo Bio Laboratories, Inc.).

DGGE-PCR. Whole-community DNA from each enrichment was subjected to PCR amplification using primers p3-GC (29) and p907R (28) targeting the V3-to-V5 region of the 16S rRNA gene. The final PCR mixture (50 μl) consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.0 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, 1.0 μM (each) of forward and reverse primers, 1.0 U of AmpliTaq DNA Polymerase LD (Applied Biosystems, Foster City, CA), and 2.5 μl of template DNA. Amplification was performed in a Perkin-Elmer GeneAmp PCR System 9700 thermocycler (Applied Biosystems) with cycling as follows: an initial denaturation step at 95°C for 5 min, followed by 30 cycles of 92°C for 1 min, 56°C for 1 min, and 72°C for 3 min. A final extension step at 72°C for 30 min was included to reduce the possibility of double band formation (16). DGGE was performed on the PCR products as follows. Samples were loaded onto a 6% polyacrylamide gel with a denaturing gradient of 40% to 60%. Electrophoresis was accomplished

TABLE 1. Substrate utilization by 12 representative isolates: mineralization rates and sole source degradation[a]

Isolate	Source	Mineralization (μg C cell^{-1} day^{-1}) × 10E−11				Degradation					
		N	Ph	F	Py	N	Ph	F	Py	C	B[*a*]P
13-24	L-C-N	95.7 ± 16.1	15.5 ± 2.5	0	0	+	−	−	−	−	−
15-27	P16-C-N	1,005.2 ± 473.9	0	0	0	+	−	+	−	−	−
16-17	R-C-Ph	0	414.9 ± 306.4	0	6.2 ± 2.4	+	+	−	+	−	−
21-13	L-C-Py	0	539.3 ± 129.7	323.4 ± 108.8	170.0 ± 89.2	+	+	+	+	−	+
22-27	P16-C-Py	0	54.5 ± 9.0	11.8 ± 6.7	2.5 ± 1.5	+	+	+	+	+	+
22-29	P16-C-Py	0	581.7 ± 438.6	149.6 ± 44.4	168.6 ± 29.5	+	+	−	−	−	−
22-34	P16-C-Py	0	175.2 ± 160.3	174.3 ± 7.2	72.2 ± 43.2	+	+	+	+	−	−
22-39	P16-C-Py	0	323.8 ± 82.1	225.2 ± 49.5	194.6 ± 13.5	+	+	+	−	−	−
23-01	RC-C-Py	0	0	0	30.1 ± 6.0	+	+	+	+	+	+
23-04	RC-C-Py	0	0	0	0	+	−	+	+	+	−
23-11	RC-C-Py	0	454.3 ± 120.7	7.7 ± 4.0	126.6 ± 37.9	−	+	+	+	−	−
23-44	RC-C-Py	0	149.3 ± 0.7	20.9 ± 4.2	34.4 ± 7.8	+	+	+	+	+	+

[a] L, RC, and P16 are the sediment sampling sites; C indicates the enrichment culture; N, naphthalene; Ph, phenanthrene; F, fluoranthene; Py, pyrene; C, chrysene; B[*a*]P-benzo[*a*]pyrene. Microbial growth was measured as the increase in turbidity (percent transmittance at 540 nm). + indicates growth in medium with the specified PAH as the sole carbon and energy source, and − indicates no growth.

using a DCode Universal Mutation Detection System (Bio-Rad, Hercules, CA) run at 75 V and 60°C for 18 h. Bands were visualized by staining them with SYBR Green I (Molecular Probes, Eugene, OR). The gel was digitized with the UVP/EC^3 BioImaging System (UVP, Inc., Upland, CA) and analyzed with BioNumerics software (Applied Maths, Inc., Austin, TX). Dendrograms were constructed using percent similarities from unweighted-pair group method using average linkages and Dice coefficients. Band position tolerance and optimization were set at 1.3 and 0.17%, respectively.

16S rRNA gene sequencing. 16S rRNA genes from PAH-degrading isolates were PCR amplified from genomic DNA with primers 27F and 1492R (21). The final PCR mixture (25 μl) consisted of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, 1.0 μM each of forward and reverse primer, 1.0 U of AmpliTaq DNA Polymerase LD (Applied Biosystems), and 0.5 μl of template DNA. Amplification was performed in the thermocycler with cycling as follows: an initial denaturation step at 95°C for 5 min, followed by 40 cycles of 92°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and finally a 3-min extension step at 72°C. The amplified PCR products were excised from a 1.0% agarose gel and purified using the UltraClean GelSpin DNA purification kit (Mo Bio Laboratories, Inc.). The purified product was directly sequenced using published universal primers. Bands chosen for sequencing were excised with a sterile razor blade. The gel slices were soaked in 200 μl double-distilled H_2O at room temperature for 1 h and centrifuged, and the supernatant was discarded. One hundred microliters double-distilled H_2O was added to the tube, which was incubated at 4°C overnight. One microliter was used for reamplification with the same primers used for DGGE-PCR (the GC clamp was removed from primer p3-GC). The products were purified as described above and sequenced with the same primers used for reamplification. Partial 16S rRNA gene sequences were obtained using BigDye cycle sequencing on an ABI 310 genetic analyzer (Applied Biosystems). All sequences obtained were compared to available databases using the Basic Local Alignment Search Tool (BLAST) (2).

Nucleotide sequence accession numbers. The 16S rRNA gene sequences have been deposited in GenBank under accession numbers EU167937 to EU168037 and EU168038 to EU168066 for isolates and DGGE bands, respectively.

RESULTS

Enrichment and isolation. Enrichment cultures were harvested 3 weeks after inoculation. Positive growth was determined by an increase in the turbidity of the flasks containing PAH as a sole carbon and energy source compared to the negative control flasks. Serial dilutions of enrichment cultures were plated on solid media and oversprayed with PAHs, enabling selection of colonies surrounded by zones of clearing. Representative colonies of different morphotypes from each final enrichment were selected for further characterization (see Table S1 in the supplemental material).

Substrate utilization. Selected isolates were tested for utilization of additional PAHs (fluoranthene, chrysene, benzo[*a*]pyrene, dibenzo[*a*,*i*]pyrene, and a mixture of phenanthrene/pyrene) (see Table S2 in the supplemental material). Degradation was indicated by zones of clearing surrounding individual colonies. The isolates designated as showing growth with no clearing demonstrated no obvious visible degradation of PAH. The rate of degradation for these isolates may have been too low to produce detectable clearing of the substrate; however, their growth was enhanced compared with the no-PAH control plate, strongly suggesting use of the substrate for growth. The main finding here was that pyrene-degrading isolates were generally able to degrade several additional PAHs. Twelve isolates comprising representatives from each bacterial genus isolated from the final enrichments were selected for mineralization studies. Seven of the nine isolates from enrichments grown on pyrene mineralized phenathrene and fluoranthene. These isolates were related to *Pusillimonas* sp., *Alcaligenes* sp., *Mycobacterium* sp., *Pseudomonas* sp., *Parvibaculum* sp., *Stappia* sp., and *Microbacterium* sp. In general, isolates degraded two or more PAHs on the basis of mineralization (Table 1). In some cases, strains showed growth in minimal medium with PAHs as the sole carbon and energy source, but mineralization could not be detected by assay for release of labeled CO_2, possibly because this release was below the detection limit for the assay.

DGGE profile analysis. Fragments of 16S rRNA genes were amplified from total-community genomic DNA by using primers p3-GC and p907r. The resulting fragments were approximately 566 bp in size and encompassed the variable region V3 to V5 of the 16S rRNA gene. Community DGGE profiles were obtained from the three sampling site enrichment cultures (Fig. 2). The C enrichment cultures showed a shift in diversity and the emergence of a small number of dominant bands compared to the corresponding A enrichments (Fig. 2). Dendrograms of banding patterns from A and C enrichment cultures were generated to examine the differences between sites and substrates (Fig. 3). For the initial enrichment A, sites clustered separately, indicating likely geographical differences in the microbial communities in the sediment samples used as

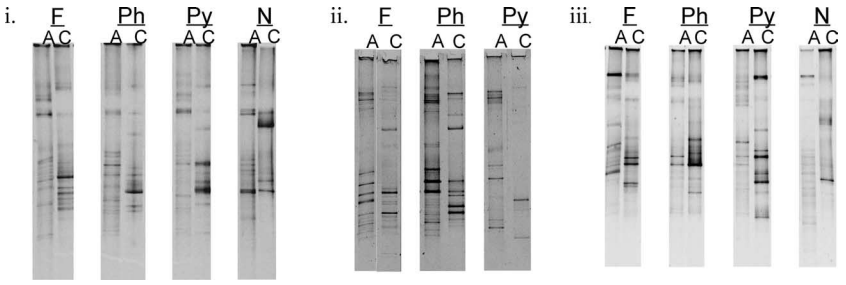

FIG. 2. Comparison of site enrichments by substrate, demonstrating the shift of predominant bands from the first enrichment (A) to the third enrichment (C). For site P16, the A enrichment for naphthalene did not amplify (no DGGE gel). (i) Site L enrichments. (ii) Site P16 enrichments. (iii) Site RC enrichments. PAHs are indicated as follows: F, fluoranthene; Ph, phenanthrene; Py, pyrene; N, naphthalene.

inocula. After two further enrichments, banding patterns for fluoranthene and phenanthrene in C enrichment cultures (Fig. 3b) remained clustered according to the initial sampling sites from which the enrichments were inoculated. However, the C enrichment cultures grown on naphthalene and pyrene were not clustered according to the site from which the inoculum was obtained.

16S rRNA gene sequencing. A total of 101 PAH-degrading isolates were obtained in culture and characterized by 16S rRNA sequence analysis. Seven isolates were novel, according to the criterion that they were 97% or less similar to known isolates based on the 16S rRNA gene sequence (see Table S1 in the supplemental material).

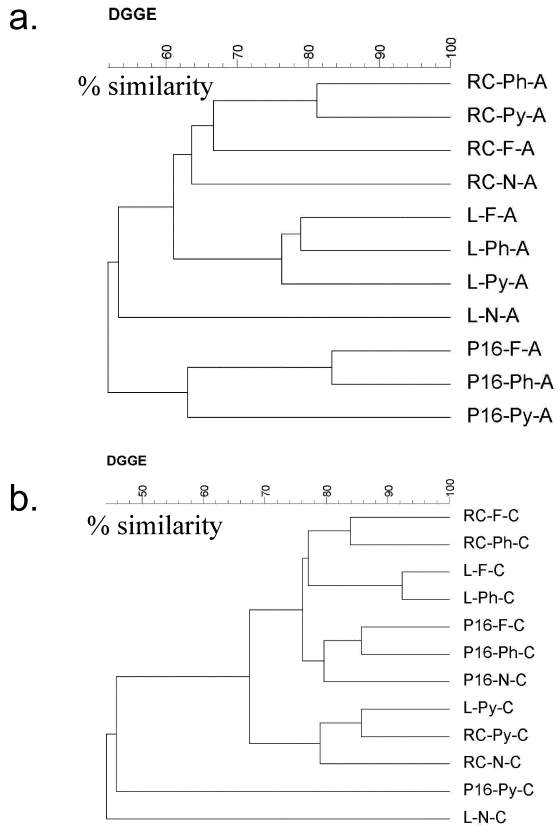

FIG. 3. Dendrograms of DGGE lanes from the initial enrichment culture, A (a), and the final enrichment culture, C (b), of sediments from the three sampling sites. Samples are labeled with the site identifier, the PAH used in the enrichment (F, fluoranthene; Ph, phenanthrene; Py, pyrene; N, naphthalene), and an A or C, designating the enrichment.

Selected bands from DGGE gels were characterized by 16S rRNA gene sequence analysis (Fig. 4). A common band in enrichments grown on the substrates fluoranthene and phenanthrene for all three sites is represented by RC-F-C3, L-F-C2, P16-F-C2, RC-Ph-C3, L-Ph-C2, and P16-Ph-C4 (Fig. 4). The respective sequences from these six bands had 100% identity and clustered together with a sequence from *Novosphingobium pentaromativorans* (AF502400) (Fig. 5). A band in the corresponding position was also present in the enrichments grown solely on pyrene, but only from site L.

Isolates obtained from C enrichments were compared with DGGE analyses of those enrichments. A dendrogram illustrating phylogenetic relationships between the isolates and bands excised from DGGE gels is shown in Fig. 5. The DGGE sequences formed six phylogenetic groups on the basis of 16S rRNA gene sequence analysis, with representatives from the alphaproteobacteria, betaproteobacteria, gammaproteobacteria, *Actinomycetes*, *Planctomycetes*, and the *Cytophaga-Flexibacter-Bacteroides* (CFB) group. Isolates were related to the *Actinobacteria* (48.5%), alphaproteobacteria (31.7%), gammaproteobacteria (14.8%), and betaproteobacteria (5%). None belonged to the *Planctomycetes* or the CFB group, although these groups were represented in the DGGE analysis. Most isolates grouping with the *Actinomycetes* were related to the genus *Mycobacterium*. These isolates showed strain differences based on growth substrates, with isolates obtained from the enrichments grown solely on fluoranthene and pyrene grouping with *Mycobacterium sacrum* and *Mycobacterium gilvum*, respectively.

Sequences from five DGGE bands showed high homology to sequences of isolates from the same enrichment from which the DGGE bands were excised. The sequence for band L-Ph-C2 from the site L enrichment grown solely on phenanthrene is most similar to those for isolates 17-30 to 17-34 (see Table S1 in the supplemental material) and was matched by BLAST to the genus *Novosphingobium*. Matching the genus *Mycobacterium* were the sequences recovered from site P16 enrichment grown solely on pyrene from DGGE band P16-Py-C4 and the isolates 22-28, 22-29, and 22-35 to 22-37. Band P16-N-C7 from the P16 site enrichment grown solely on naphthalene was found to be most similar to isolates 15-27, 15-31, 15-39, and 15-41 and matched an uncultured bacterium. From site RC, the sequence obtained for band RC-Ph-C3 was most

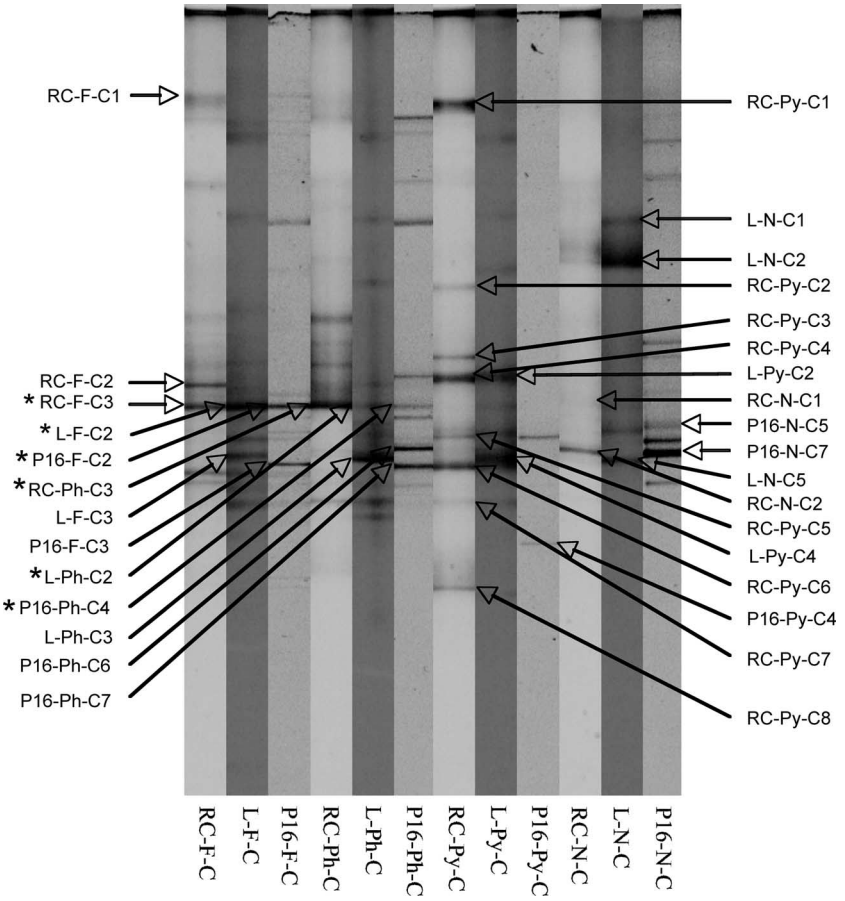

FIG. 4. DGGE comparison of microbial communities in C enrichments on various PAH substrates. The gel lane labels correspond to the enrichment cultures in Fig. 3b. The arrows indicate the bands excised and sequenced. Sequences common to all three sites are indicated by asterisks.

similar to those for isolates 16-13, 16-15 to 16-17, and 16-21 cultured from the enrichment grown solely on phenanthrene. These sequences were matched by BLAST to the genus *Novosphingobium*. Also from site RC, band RC-Py-C8 was most similar to isolates 23-03, 23-05 to 23-10, 23-12, 23-14 to 23-18, 23-22 to 23-27, 23-29 to 23-34, 23-37, 23-42, 23-45, and 23-46 cultured from the enrichment grown solely on pyrene and matched the genus *Mycobacterium* by BLAST analysis. The length of the DGGE fragment analyzed was only ca. 560 bp. Phylogenetic assignment based on this fragment should therefore be made cautiously, and we have limited assignments to the genus rather than the species level.

DISCUSSION

Selective cultures for enrichment of PAH-degrading strains were monitored by DGGE to detect changes in the bacterial-community profile during enrichment and to determine whether representative strains present in the enrichments were successfully cultured. Culturable PAH-degrading strains were obtained from these enrichments. Comparison of 16S rRNA gene sequences from bands excised from DGGE gels and from these isolates confirmed that key groups of PAH-degrading strains present in contaminated aquatic sediments from the Elizabeth River were successfully isolated and obtained in pure culture. Of the collection of 101 PAH-degrading isolates obtained in culture, 7 isolates were novel, using the criterion that they were 97% or less similar to known isolates based on the 16S rRNA gene sequence. This finding supports the hypothesis that a high diversity of bacterial strains with the ability to degrade multiple PAHs can be confirmed by the combined use of classical selective enrichment and molecular analyses. The collection of PAH-degrading bacteria from Elizabeth River sediments is a valuable resource for studies on mechanisms of PAH degradation and bioremediation.

Many of the bacteria present in the enrichments were successfully brought into culture. Four of the major groups (actinomycetes and alpha-, beta, and gammaproteobacteria) shown to be present by the DGGE analysis were represented in the set of isolates, although representatives of the *Planctomycetes* and the CFB group were not cultured. PAH-degrading strains in the *Mycobacterium* and *Novosphingobium* groups were particularly well represented in both the DGGE analysis and the collection of isolates. The cultivation methods and approach of selective enrichments carefully monitored by molecular analyses used in this study may be of general utility in successfully obtaining novel PAH-degrading strains from contaminated environments other than the Elizabeth River.

The dendrogram for initial enrichments (Fig. 3a) shows the

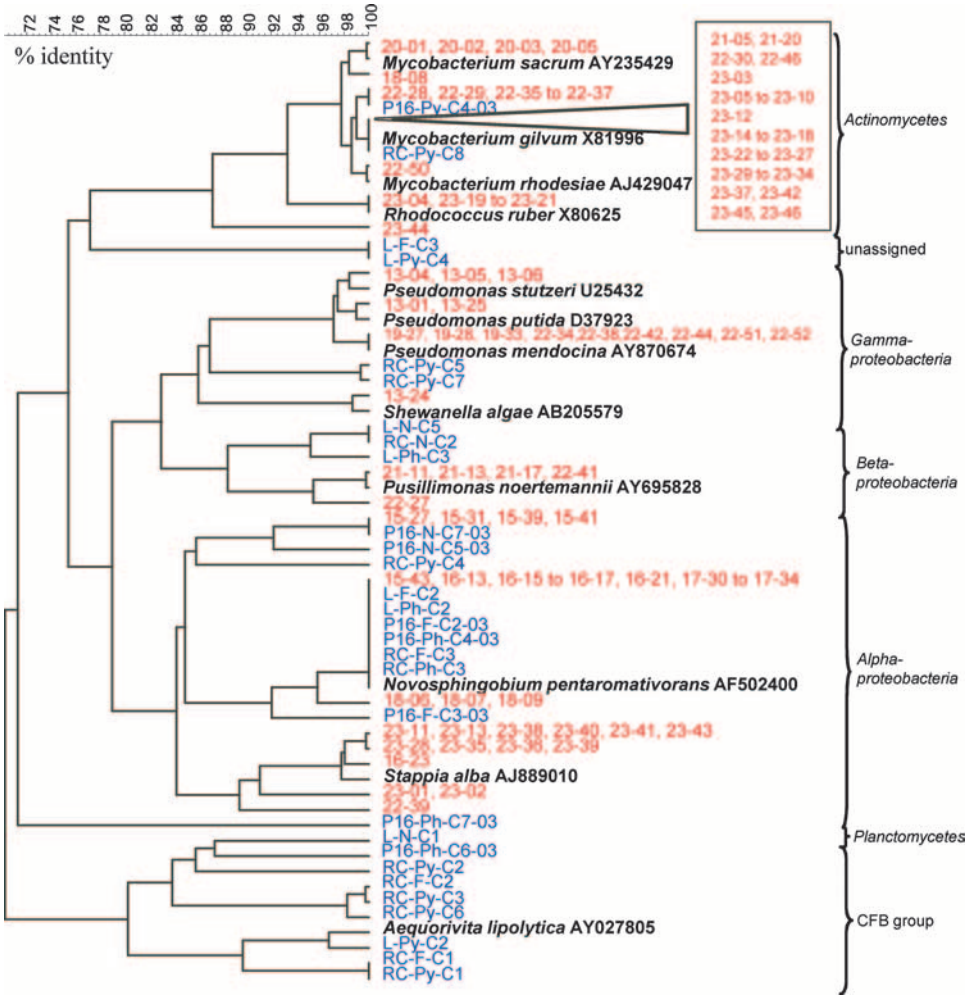

FIG. 5. Dendrogram of 16S rRNA gene sequences from isolates obtained from C enrichments and from bands excised from DGGE gels obtained from C enrichments. Isolate and DGGE bands are distinguished by red and blue labels, respectively.

three sampling sites clustering separately. Since the initial enrichments most closely resemble the original sediment composition, this clustering indicates a difference in the indigenous microbial populations over a short geographic distance (the three sites are within 1 mile of each other). DGGE profile differences in bacterial communities enriched on different PAHs (Fig. 4) were clearly evident in final enrichments for all substrates tested. The DGGE profiles of the final enrichments grown solely on naphthalene and pyrene showed no clear relationship between the site from which the initial inocula were obtained and the resultant microbial-community profiles after enrichment on these substrates. In fact, the enrichments grown solely on pyrene for sample sites L and RC had a greater than 80% similarity in banding patterns, which suggests that common pyrene-degrading strains may be present in these sediments. On the other hand, the final enrichments grown solely on fluoranthene and phenanthrene remained clustered by site (Fig. 3b), suggesting that fluoranthene- and phenanthrene-degrading strains may be more specialized for conditions found at each site rather than being ubiquitous in Elizabeth River sediments.

Very few bands present in the DGGE of the initial enrichments comigrated with dominant bands present in the DGGE from each of the respective substrate-specific final enrichments. In a previous study (24), an experimental inoculum of natural hydrocarbon-degrading strains was isolated from beach sand near the experimental site and enriched on light crude oil before being sprayed onto experimental plots. Comparison of the DGGE gel obtained from the enriched inoculum with the DGGE gels obtained from the contaminated sites showed markedly different microbial-community profiles (24). This observation and our finding of major shifts in bacterial-community structure during enrichment on specific substrates demonstrate the power of the selection process in the isolation of strains that are present only as minor components in the original environmental communities. Further study is necessary to determine the contributions of these minor components to the degradation of PAHs in the environment. The large collection of PAH-degrading strains obtained during our study provides a valuable resource for detailed examination of the PAH-catabolic potential of environmental isolates and identification of highly divergent or novel functional genes associated with the degradation of PAHs and may include isolates of utility for the bioaugmentation of contaminated sites.

ACKNOWLEDGMENTS

We are grateful to M. T. Montgomery and L. Hamden for their help with the mineralization experiments. We thank Deanna Colton and Marybeth Sciarretta for technical assistance.

This research was supported by the Naval Research Laboratory CORE Work Unit MA013-01-44 (6.1 Base Program).

The opinions and assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the U.S. Navy.

REFERENCES

1. **Agency for Toxic Substances and Disease Registry.** 1995. Toxicological profile for polycyclic aromatic hydrocarbons. U.S. Department of Health and Human Services, Atlanta, GA.
2. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
3. **Baldwin, B. R., C. H. Nakatsu, and L. Nies.** 2003. Detection and enumeration of aromatic oxygenase genes by multiplex and real-time PCR. Appl. Environ. Microbiol. **69:**3350–3358.
4. **Bastiaens, L., D. Springael, P. Wattiau, H. Harms, R. deWachter, H. Verachtert, and L. Diels.** 2000. Isolation of adherent polycyclic aromatic hydrocarbon (PAH)-degrading bacteria using PAH-sorbing carriers. Appl. Environ. Microbiol. **66:**1834–1843.
5. **Boldrin, B., A. Tiehm, and C. Fritzsche.** 1993. Degradation of phenanthrene, fluorene, fluoranthene, and pyrene by a *Mycobacterium* sp. Appl. Environ. Microbiol. **59:**1927–1930.
6. **Boyd, T. J., M. T. Montgomery, J. K. Steele, J. W. Pohlman, S. R. Reatherford, B. J. Spargo, and D. C. Smith.** 2005. Dissolved oxygen saturation controls PAH biodegradation in freshwater estuary sediments. Microb. Ecol. **49:**226–235.
7. **Chung, W. K., and G. M. King.** 2001. Isolation, characterization, and polyaromatic hydrocarbon degradation potential of aerobic bacteria from marine macrofaunal burrow sediments and description of *Lutibacterium anuloederans* gen. nov., sp. nov., and *Cycloclasticus spirillensus* sp. nov. Appl. Environ. Microbiol. **67:**5585–5592.
8. **Churchill, S. A., J. P. Harper, and P. F. Churchill.** 1999. Isolation and characterization of a *Mycobacterium* species capable of degrading three- and four-ring aromatic and aliphatic hydrocarbons. Appl. Environ. Microbiol. **65:**549–552.
9. **Daane, L. L., I. Harjono, G. J. Zylstra, and M. M. Häggblom.** 2001. Isolation and characterization of polycyclic aromatic hydrocarbon-degrading bacteria associated with the rhizosphere of salt marsh plants. Appl. Environ. Microbiol. **67:**2683–2691.
10. **Dagher, F., E. Deziel, P. Lirette, G. Paquette, J. Bisaillon, and R. Villemur.** 1996. Comparative study of five polycyclic aromatic hydrocarbon degrading bacterial strains isolated from contaminated soil. Can. J. Microbiol. **43:**368–377.
11. **Deming, J. W.** 1993. ^{14}C tracer method for measuring microbial activity in deep-sea sediments, p. 405–414. *In* P. F. Kemp, B. F. Sherr, E. B. Sherr, and J. J. Cole (ed.), Handbook of methods in aquatic microbial ecology. Lewis Publishers, Ann Arbor, MI.
12. **Geiselbrecht, A. D., B. P. Hedlund, M. A. Tichi, and J. T. Staley.** 1998. Isolation of marine polycyclic aromatic hydrocarbon (PAH)-degrading *Cycloclasticus* strains from the Gulf of Mexico and comparison of their PAH degradation ability with that of Puget Sound *Cycloclasticus* strains. Appl. Environ. Microbiol. **64:**4703–4710.
13. **Geiselbrecht, A. D., R. P. Herwig, J. W. Deming,and J. T. Staley.** 1996. Enumeration and phylogenetic analysis of polycyclic aromatic hydrocarbon-degrading marine bacteria from Puget Sound sediments. Appl. Environ. Microbiol. **62:**3344–3349.
14. **Hedlund, B. P., A. D. Geiselbrecht, T. J. Bair, and J. T. Staley.** 1999. Polycyclic aromatic hydrocarbon degradation by a new marine bacterium, *Neptunomonas naphthovorans* gen. nov., sp. nov. Appl. Environ. Microbiol. **65:**251–259.
15. **Hedlund, B. P., and J. T. Staley.** 2006. Isolation and characterization of *Pseudoalteromonas* strains with divergent polycyclic aromatic hydrocarbon catabolic properties. Environ. Microbiol. **8:**178–182.
16. **Janse, I., J. Bok, and G. Zwart.** 2004. A simple remedy against artificial double bands in denaturing gradient gel electrophoresis. J. Microbiol. Methods **57:**279–281.
17. **Johnsen, A. R., S. Schmidt, T. K. Hybholy, S. Hendriksen, C. S. Jacobsen, and O. Andersen.** 2007. Strong impact on the polycyclic aromatic hydrocarbon (PAH)-degrading community of a PAH-polluted soil but marginal effect on PAH degradation when priming with bioremediated soil dominated by mycobacteria. Appl. Environ. Microbiol. **73:**1474–1480.
18. **Kanaly, R. A., and S. Harayama.** 2000. Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. J. Bacteriol. **182:**2059–2067.
19. **Kastner, M., M. Breuer-Jammali, and B. Mahro.** 1994. Enumeration and characterization of the soil microflora from hydrocarbon-contaminated soil sites able to mineralize polycyclic aromatic hydrocarbons (PAH). Appl. Microbiol. Biotechnol. **41:**267–273.
20. **Kiyohara, H., K. Nagao, and K. Yana.** 1982. Rapid screen for bacteria degrading water insoluble, solid hydrocarbons on agar plates. Appl. Environ. Microbiol. **43:**454–457.
21. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115–175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, Hoboken, NJ.
22. **Leys, N. M. E. J., A. Ryngaert, L. Bastiaens, W. Verstraete, E. M. Top, and D. Springael.** 2004. Occurrence and phylogenetic diversity of *Sphingomonas* strains in soils contaminated with polycyclic aromatic hydrocarbons. Appl. Environ. Microbiol. **70:**1944–1955.
23. **Lipiatou, E., and A. Saliot.** 1991. Fluxes and transport of anthropogenic and natural polycyclic aromatic hydrocarbons in the western Mediterranean Sea. Mar. Chem. **32:**51–71.
24. **MacNaughton, S. J., J. R. Stephen, A. D. Venosa, G. A. Davis, Y. J. Chang, and D. C. White.** 1999. Microbial population changes during bioremediation of an experimental oil spill. Appl. Environ. Microbiol. **65:**3566–3574.
25. **Menzie, C. A., B. B. Potocki, and J. Santodonato.** 1992. Exposure to carcinogenic PAHs in the environment. Environ. Sci. Technol. **26:**1278–1284.
26. **Meyer, S., R. Moser, A. Neef, U. Stahl, and P. Kampfer.** 1999. Differential detection of key enzymes of polyaromatic-hydrocarbon-degrading bacteria using PCR and gene probes. Microbiology **145:**1731–1741.
27. **Mueller, J., S. Lantz, R. Devereux, J. Berg, and P. Pritchard.** 1994. Studies on the microbial ecology of polycyclic aromatic hydrocarbon degradation, p. 218–230. *In* R. Hinchee, A. Leeson, L. Semprini, and S. Ong (ed.), Bioremediation of chlorinated and polycyclic aromatic hydrocarbons. Lewis Publishers, Boca Raton, FL.
28. **Muyzer, G., T. Brinkhoff, U. Nübel, C. Santegoeds, H. Schäfer, and C. Wawer.** 1998. Denaturing gradient gel electrophoresis (DGGE) in microbial ecology, p. 1–27. *In* A. D. L. Akkermans, J. D. van Elsas, and F. J. deBruijn (ed.), Molecular microbial ecology manual. Kluwer Academic Publishers, Dordrecht, The Netherlands.
29. **Muyzer, G., E. C. De Waal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59:**695–700.
30. **Ní Chadhain, S. M., R. S. Norman, K. V. Pesce, J. J. Kukor, and G. J. Zylstra.** 2006. Microbial dioxygenase gene population shifts during polycyclic aromatic hydrocarbon degradation. Appl. Environ. Microbiol. **72:**4078–4087.
31. **Pfaender, F. K., and G. W. Bartholomew.** 1982. Measurement of aquatic biodegradation rates by determining heterotrophic uptake of radiolabelled pollutants. Appl. Environ. Microbiol. **44:**159–164.
32. **Public Health Service.** 2005. Report on carcinogens, 11th ed. U.S. Department of Health and Human Services, Atlanta, GA.
33. **Samanta, S. K., O. V. Singh, and R. K. Jain.** 2002. Polycyclic hydrocarbons: environmental pollution and bioremediation. Trends Biotechnol. **20:**243–248.
34. **Straube, W. L., C. C. Nestler, L. D. Hansen, D. Ringleberg, P. H. Pritchard, and J. Jones-Meehan.** 2003. Remediation of polyaromatic hydrocarbons (PAHs) through landfarming with biostimulation and bioaugmentation. Acta Biotechnol. **23:**179–196.
35. **Uyttebroek, M., S. Vermeir, P. Wattiau, A. Ryngaert, and D. Springael.** 2007. Characterization of cultures enriched from acidic polycyclic aromatic hydrocarbon-contaminated soil for growth on pyrene at low pH. Appl. Environ. Microbiol. **73:**3159–3164.
36. **Widada, J., H. Nojiri, K. Kasuga, T. Yoshida, H. Habe, and T. Omori.** 2002. Molecular detection and diversity of polycyclic aromatic hydrocarbon-degrading bacteria isolated from geographically diverse sites. Appl. Environ. Microbiol. **58:**202–209.