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Stable-Isotope Probing of Bacteria Capable of Degrading Salicylate, Naphthalene, or Phenanthrene in a Bioreactor Treating Contaminated Soil

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[$^{13}C_6$]salicylate, [U-^{13}C]naphthalene, and [U-^{13}C]phenanthrene were synthesized and separately added to slurry from a bench-scale, aerobic bioreactor used to treat soil contaminated with polycyclic aromatic hydrocarbons. Incubations were performed for either 2 days (salicylate, naphthalene) or 7 days (naphthalene, phenanthrene). Total DNA was extracted from the incubations, the "heavy" and "light" DNA were separated, and the bacterial populations associated with the heavy fractions were examined by denaturing gradient gel electrophoresis (DGGE) and 16S rRNA gene clone libraries. Unlabeled DNA from *Escherichia coli* K-12 was added to each sample as an internal indicator of separation efficiency. While *E. coli* was not detected in most analyses of heavy DNA, a low number of *E. coli* sequences was recovered in the clone libraries associated with the heavy DNA fraction of [^{13}C]phenanthrene incubations. The number of *E. coli* clones recovered proved useful in determining the relative amount of light DNA contamination of the heavy fraction in that sample. Salicylate- and naphthalene-degrading communities displayed similar DGGE profiles and their clone libraries were composed primarily of sequences belonging to the *Pseudomonas* and *Ralstonia* genera. In contrast, heavy DNA from the phenanthrene incubations displayed a markedly different DGGE profile and was composed primarily of sequences related to the *Acidovorax* genus. There was little difference in the DGGE profiles and types of sequences recovered from 2- and 7-day incubations with naphthalene, so secondary utilization of the ^{13}C during the incubation did not appear to be an issue in this experiment.

Polycyclic aromatic hydrocarbons (PAHs) are always present as complex mixtures in PAH-contaminated soil and sediment, and many organisms isolated from contaminated systems have been found to grow on a number of different PAHs as sole carbon sources (2). Our knowledge of the diversity of PAH metabolism is almost exclusively limited, however, to studies with organisms isolated from these systems. While various molecular techniques can be used to identify organisms present in such complex systems, linking identity to function remains one of the more difficult problems in microbial ecology. This problem can be partially addressed by the application of stable-isotope probing (SIP) (32).

In this study we applied SIP to identify the microbial communities capable of growing on salicylate, naphthalene, or phenanthrene in a bench-scale, aerobic bioreactor used to treat contaminated soil from a former manufactured-gas plant site. Both naphthalene and phenanthrene are growth substrates for a diverse range of bacteria, and they are generally present in PAH-contaminated systems at concentrations capable of supporting bacterial growth. Jeon et al. and Padmanabhan et al. previously used SIP to identify naphthalene-degrading organisms in contaminated and uncontaminated soils in situ (19, 30). An additional study used SIP to evaluate phenanthrene degradation in contaminated soil, but the organisms that incorporated [^{13}C]phenanthrene were not identified (20). Whether the same organisms are primarily responsible for degrading both naphthalene and phenanthrene in any PAH-contaminated system has not yet been evaluated by SIP.

Salicylate is an intermediate in the degradation of naphthalene as well as an inducer of naphthalene catabolism (11, 42). It is also an intermediate in one of the aerobic bacterial pathways for phenanthrene metabolism (15), and it has been found to induce phenanthrene degradation in some organisms (7). The addition of salicylate to soil has been shown to increase the numbers and activity of naphthalene-degrading bacteria (8, 28, 29). Its application to PAH-contaminated systems has therefore been proposed as a potential means of stimulating the biodegradation of a range of PAHs (7, 28).

The goals of this study were to identify the organisms primarily responsible for degrading naphthalene or phenanthrene in a bioreactor actively degrading PAHs and to determine whether the addition of salicylate would select for these PAH-degrading organisms. Additionally, incubations of different lengths were performed with naphthalene to test the potential for secondary incorporation of the labeled compound (i.e., "cross-feeding") in the longer incubation.

MATERIALS AND METHODS

Bioreactor conditions and operation. PAH-contaminated soil was obtained from a manufactured-gas plant site in Charlotte, North Carolina. The reactor is constructed of stainless steel with a working volume of approximately 2.5 liters and is gas tight except for inlet and outlet ports for humidified air and off-gas, respectively. The reactor contains a slurry of the soil at a solids concentration of 20% (wt/wt), is continuously mixed and aerated, and is operated in a semicon-

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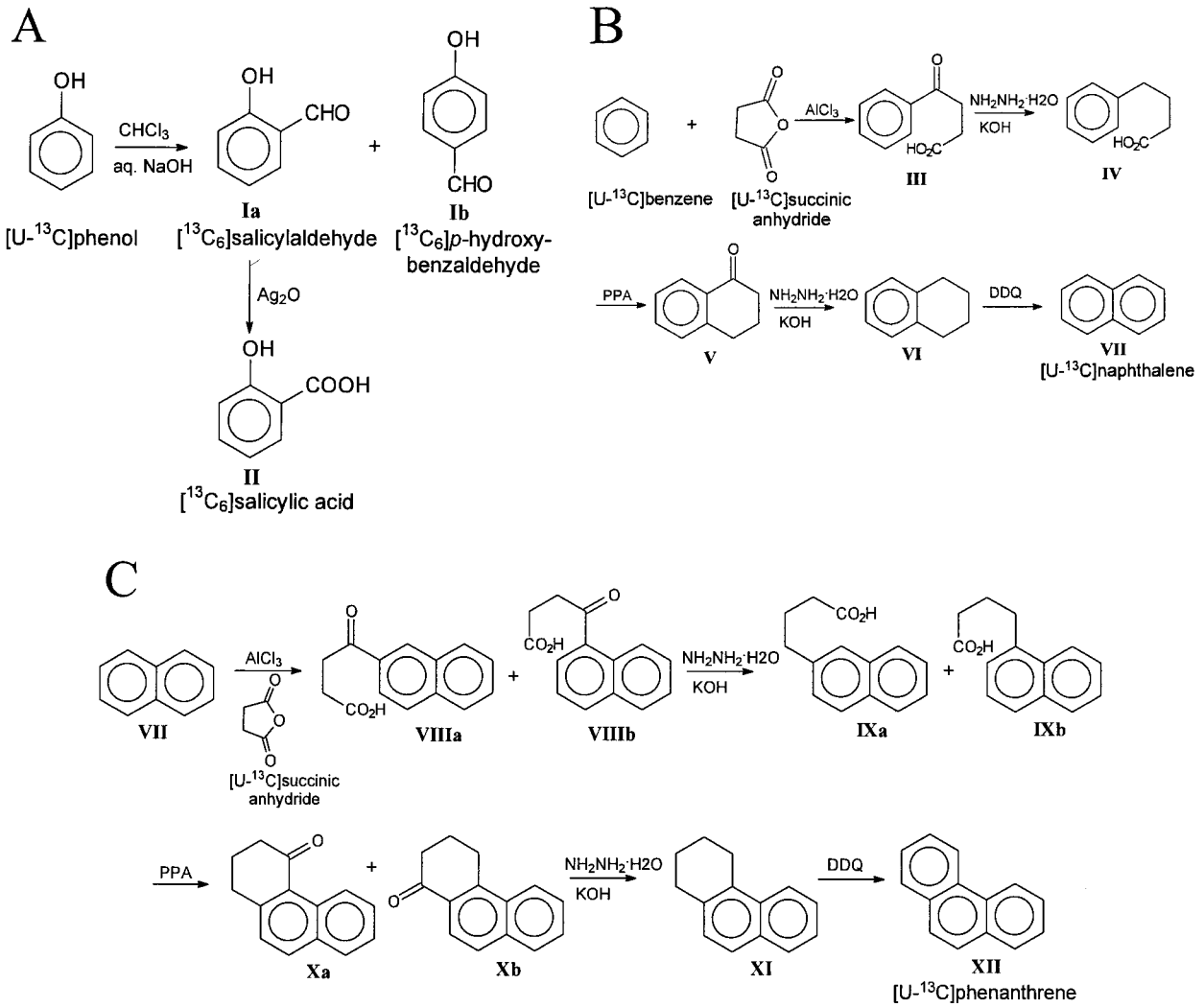

FIG. 1. Schemes for the synthesis of (A) [$^{13}C_6$]salicylic acid, (B) [U-^{13}C]naphthalene, and (C) [U-^{13}C]phenanthrene. DDQ, 2,3-dichloro-5,6-dicyanobenzoquinone; PPA, polyphosphoric acid.

tinuous (draw-and-fill) manner. At selected intervals, one-fifth of the reactor volume is removed and then replaced with an equal amount of untreated soil slurried in phosphate buffer (10 mM, pH 7.0) containing ammonium nitrate (2.3 mM) as a nitrogen source ("reactor buffer"). The interval between draw-and-fill cycles was approximately every 2 weeks, corresponding to a solids retention time of approximately 70 days.

Concentrations of naphthalene and phenanthrene in the untreated soil as determined by high-pressure liquid chromatography (HPLC) (3) were 22 ± 10 and 35 ± 8 mg/kg, respectively (based on triplicate subsamples of soil from each of four storage containers). Over a period in which the treated slurry from the bioreactor was monitored regularly for PAH concentrations, the naphthalene concentration was 12 ± 4 mg/kg and the phenanthrene concentration was 14 ± 4 mg/kg (based on triplicate subsamples from each of three different sampling events).

Chemicals. The natural abundance isotopomers of naphthalene (>99%) and phenanthrene (>96%) were purchased from Sigma-Aldrich. Natural abundance isotopomer salicylic acid (Sigma-Aldrich) was American Chemical Society grade. [U-^{13}C]benzene, [U-^{13}C]succinic acid, and [U-^{13}C]phenol were purchased from Cambridge Isotope Laboratories, Inc. (Andover, Mass.). [U-^{13}C]succinic acid was converted to the anhydride by treating with acetic anhydride. The purity of synthetic products was confirmed by analytical HPLC with UV detection (254 nm). Purity of labeling with ^{13}C was confirmed by gas chromatography-electron impact mass spectrometry (GC/EIMS) on an S973N mass spectrometer coupled to an Agilent 6890N GC equipped with a J&W Scientific DB5 column (60 m by 0.25 μm). All solvents were HPLC grade.

Synthesis of ^{13}C-labeled salicylic acid, naphthalene, and phenanthrene. (i) [$^{13}C_6$]salicylic acid. Synthesis of [$^{13}C_6$]salicylic acid (II) was accomplished via Reimer-Tiemann formylation (39) of commercially available [U-^{13}C]phenol (Fig. 1A). Formylation yielded a 1:1 mixture of [$^{13}C_6$]salicylaldehyde and [$^{13}C_6$]*p*-hydroxybenzaldehyde. Following separation of the mixture by preparative thin-layer chromatography, the [$^{13}C_6$]salicylaldehyde was oxidized with silver oxide to afford [$^{13}C_6$]salicylic acid. The synthesized salicylic acid was dissolved in 2 ml of acetonitrile and purified by semipreparative HPLC on a Supelcosil PLC-8 column (Supelco, Bellefonte, Pa.). Mobile phase (6.5 ml/min) was 60% acetonitrile: 40% water containing 0.1% trifluoroacetic acid. Fractions corresponding to elution of the salicylic acid peak were collected from each of multiple 190-μl injections, combined, and concentrated by evaporation using a vacuum pump with dry ice-acetone cooling of the condenser (caution: evaporation of the solvents by overnight lyophilization resulted in loss of the salicylic acid). GC/EIMS of disilylated [$^{13}C_6$]salicylic acid: m/z 273 $[M - CH_3]^+$.

(ii) [U-^{13}C]naphthalene. [U-^{13}C]naphthalene (VII) was synthesized from commercially available [U-^{13}C]benzene and [U-^{13}C]succinic anhydride as starting materials by the Haworth reaction for annelation of arenes (1) (Fig. 1B). Condensation of [U-^{13}C] benzene in tetrachloroethane (34) with [U-^{13}C]succinic anhydride catalyzed by anhydrous aluminum chloride gave 4-oxo-4-phenylbutanoic acid (III) in 87% yield. Wolff-Kishner reduction of 4-oxo-4-phenylbu-

tanoic acid followed by cyclization furnished [^{13}C]tetralone (V) with an overall 80% yield. Wolff-Kishner reduction of [^{13}C]tetralone and aromatization with 2,3-dichloro-5,6-dicyanobenzoquinone in refluxing benzene gave [U-^{13}C]naphthalene in 73% yield. The structure was confirmed by comparison of the UV-visible spectrum (Hitachi U-3300) and the analytical thin-layer chromatography of the product with those of the natural abundance isotopomer. GC/EIMS: *m/z* 138 M^+.

(iii) [U-^{13}C]phenanthrene. [U-^{13}C]naphthalene was the starting point for the synthesis of phenanthrene (XII) by the Haworth strategy (Fig. 1C). Acylation with [U-^{13}C]succinic anhydride yielded isomeric keto acids (VIIIa and VIIIb) through attack at C-1 and C-2 in a 1:2 ratio in 78% total yield. Since the cyclodehydration step was anticipated to yield the phenanthrene framework from both keto acids, the mixture was not separated. Treatment of the mixture of keto acids in the same manner as described above for the synthesis of naphthalene gave phenanthrene in 70% yield. GC/EIMS: *m/z* 192 M^+.

Setup of incubations. All incubations were performed in 125-ml sterilized glass screw-top Erlenmeyer flasks with 5 ml of freshly sampled bioreactor slurry as inoculum. The caps of flasks containing naphthalene were lined with aluminum foil to prevent sorption of volatilized naphthalene. The target compound was added to each flask with 25 ml of reactor buffer (nominal concentrations of 25 mg/liter for naphthalene or phenanthrene and 43 mg/liter for salicylate), either in unlabeled or ^{13}C-labeled form. Naphthalene (as a saturated solution in reactor buffer) and salicylate (dissolved to the target concentration in reactor buffer) were both added directly to the incubation flasks. For phenanthrene, a concentrated stock was first prepared in dichloromethane. A volume of dichloromethane containing the desired mass of phenanthrene was added to the incubation flask and the solvent was allowed to evaporate. Reactor buffer (25 ml) was then added to the phenanthrene crystals in the flask. The concentrations of each carbon source were selected to provide approximately equal concentrations of electron equivalents, which are known to correlate with free energy yields on complete oxidation (6). In addition, the masses of naphthalene and phenanthrene added to the flasks were 40 to 50 times greater than the masses carried over in the soil slurry, based on the concentrations in the treated soil indicated above.

Flasks containing each carbon source in unlabeled form were set up to measure disappearance of the compound over time. Additional abiotic incubations with unlabeled carbon sources were created by adding sodium azide (6 mM final concentration) to inoculated flasks to confirm that the disappearance of the carbon source was due to biological activity. Incubations containing inoculum and reactor buffer but no additional carbon source were also performed. All incubations were performed in duplicate.

Monitoring disappearance of carbon sources. Samples for salicylate analysis were obtained by centrifuging the slurry, filtering the supernatant with a 0.2-μm-pore-size syringe filter, and reducing the pH to <2 by the addition of 20% phosphoric acid. The solutions were stored in amber, crimp-top gas chromatography vials at −20°C before HPLC determination of salicylic acid concentrations. A mild extraction method was used to extract primarily the added naphthalene or phenanthrene from the slurry. An aliquot of slurry (1 ml) was added to a conical-bottom glass centrifuge tube containing 850 μl of ethyl acetate. After vortexing and centrifugation to pellet the solids, the supernatant was filtered with a 0.4-μm-pore-size filter unit (Whatman, Clifton, N.J.) and stored as described above.

Concentrations of each carbon source were determined with an HPLC system consisting of a Waters (Milford, Mass.) 600E system controller, a Waters 717 Plus autosampler, and a Kratos (Chestnut Ridge, N.Y.) Spectroflow 757 UV absorbance detector. Salicylate was analyzed isocratically with a Supelcosil LC-8 column (Supelco) and mobile phase of 25% acetonitrile:75% water containing 0.1% trifluoroacetic acid. Naphthalene and phenanthrene were analyzed with a Supelco LC-PAH column. The mobile phase was run with a gradient elution program of 60% acetonitrile:40% water for 5 min, ramping with a linear gradient to 100% acetonitrile over 20 min, and holding with 100% acetonitrile for 5 min.

DNA extraction. With the exception of the 7-day [^{13}C]naphthalene incubations, all incubations were sacrificed shortly after the bulk of the added compound had been consumed, as determined by HPLC analyses. The entire volume of slurry was transferred to a 50-ml tube and centrifuged to pellet the solids. The supernatant was discarded and the solids resuspended in 3 ml of sterile water. The slurry was then redistributed to two sterile, 2-ml screw-cap microcentrifuge tubes. DNA was extracted from each of these tubes with a MoBio (Carlsbad, Calif.) UltraClean soil DNA kit per the manufacturer's instructions, including a 10-min incubation of the extraction mixture at 70°C prior to physical disruption of the cells. The recovered DNA from each aliquot of the original sample was recombined into a single sample before the quality and quantity of DNA was estimated by agarose gel electrophoresis.

Separation and isolation of DNA. Separation of the unlabeled and ^{13}C-labeled DNA was accomplished by density gradient ultracentrifugation in cesium chloride according to the method of Radajewski et al. (33), except that a Sorvall OTD60B ultracentrifuge with a TV-1665 vertical rotor and appropriate ultracentrifuge tubes were used. All separations were performed in parallel with a tube containing 1 μg each of *Escherichia coli* K-12 DNA from a culture grown on nutrient broth and a *Pseudomonas putida* G7 culture grown on uniformly labeled [^{13}C]glucose (Cambridge Isotope Laboratories, Inc.). Additionally, 100 ng of unlabeled *E. coli* DNA was added to each experimental tube as an indicator of incomplete DNA separation.

Separated DNA was recovered by collecting fractions from the bottom of the ultracentrifuge tube. The tube was first vented at the top with a needle, which was then closed with an attached Luer-lock valve. The bottom of the tube was pierced with a second needle. A syringe pump was used to add distilled water via a third needle to the top of the tube at a flow rate of 800 μl min^{-1}, displacing the denser cesium chloride gradient. Eight to nine 400-μl fractions (each equivalent to approximately 4 mm of liquid in the ultracentrifuge tube) were collected for each sample. Under these conditions there was approximately 13 to 15 mm of separation between the peaks of "light" and "heavy" DNA. DNA in each fraction was recovered using butanol extractions to remove ethidium bromide and isopropanol precipitation (36) before resuspension in a total of 75 μl of Tris-EDTA buffer (pH 8.0).

Molecular analyses. A PCR for denaturing gradient gel electrophoresis (DGGE) was performed using 1 to 3 μl of DNA from each fraction as template, primers (final concentrations of 0.65 mM) 907r (23) and 341GC (27), and a Ready-to-Go PCR bead (Amersham Biosciences Co., Piscataway, N.J.) in a final volume of 25 μl. The PCR program consisted of 35 cycles of 45 s at 94°C, 45 s at the annealing temperature (a touchdown of 65 to 55°C was used over the first 10 cycles, 55°C for the remaining 25 cycles), and 1.5 min at 72°C. Products were loaded on a 6% polyacrylamide gel with formamide-urea denaturing conditions between 35 and 55% and run for 16 h at 60 V on a Dcode system (Bio-Rad Laboratories, Hercules, Calif.). DGGE gels were poststained with ethidium bromide.

PCR for the construction of clone libraries used primers 8f (13) and 1492r (22). The PCR program consisted of 25 cycles of 1 min at 94°C, 1 min at 50°C, and 3 min at 72°C. Reaction products were cloned into a TA cloning kit (Invitrogen, Carlsbad, Calif.) per the manufacturer's instructions. Clones were screened for unique restriction patterns by reamplification of the insert followed by digestion with either HaeIII or a double digest with RsaI and EcoRI (New England Biolabs, Beverly, Mass.). Digestion reactions were visualized on either a 2% agarose gel or a 5% polyacrylamide gel. Representative inserts with unique restriction patterns were nearly completely sequenced at the University of North Carolina—Chapel Hill Genome Analysis Facility using primers M13F1, M13R3, 8f, 907r, and 1492r. Some infrequently appearing inserts were partially sequenced using primer 8f. Phylogenetic trees were constructed using the pileup program of the Genetics Computer Group (Wisconsin Package version 10.3; Accelrys Inc., San Diego, Calif.) and the ClustalX program (38). Trees were bootstrapped within ClustalX 1,000 times and gaps were not considered during tree construction. Close relatives of recovered sequences were obtained by testing the sequences against public databases by using the BLASTN (5) or FASTA (31) programs. Chimeras were determined by analyses using the CHIMERA_CHECK program on the Ribosomal Database Project II website (9) as well as additional BLAST or FASTA analyses.

To screen for *E. coli* DNA in various fractions, PCR was performed using the primers and conditions in Sabat et al. (35) with either 25 or 40 cycles. Heavy DNA fractions were screened for archaeal sequences using the primers 25f (12) and 1492r as well as for fungal sequences using the primers ITS1F (17) and ITS4 (40).

Nucleotide sequence accession numbers. Nearly complete sequences obtained in this study have been deposited in GenBank with the accession numbers AY699582 to AY699604.

RESULTS

Incubations and compound disappearance. Soil slurry removed from an active bioreactor was incubated with salicylate or naphthalene for 2 days, while an additional set of slurry samples was incubated with naphthalene or phenanthrene for 7 days before DNA was extracted. The concentrations of salicylate, naphthalene, and phenanthrene all decreased over the course of the experiment as determined in incubations con-

taining unlabeled carbon sources that were run in parallel to the incubations with the ^{13}C-labeled carbon sources. Salicylate was undetectable after 24 h, and naphthalene concentrations dropped to less than 10% of the initial concentration during the same time period. Most of the phenanthrene added to the flasks was detected in the slurry at 24 h, but the concentration decreased steadily to less than 20% of the added amount after 5 days. Loss of any of the compounds in the abiotic controls was not significant during the time in which the compounds were being degraded. Naphthalene was gradually lost from the abiotic 7-day incubations, likely due to volatilization when the vessel was opened to collect samples. Because the incubations with [^{13}C]naphthalene were not opened to collect samples and the majority of naphthalene was degraded in the first 24 h, loss of the labeled compound due to volatilization in these samples is believed to have been minimal.

Approximately 3 μg of DNA was obtained from each of the 2-day incubations with salicylate and naphthalene. Less DNA (~1 μg) was recovered from the 7-day incubations with naphthalene and phenanthrene. No significant differences in the quantity or quality of DNA were observed between replicates or between incubations with unlabeled and ^{13}C-labeled forms of the same compound. Nearly the entire amount of recovered DNA was used in the separations.

***E. coli* internal indicator DNA.** Unlabeled genomic DNA from *E. coli* K-12 was added to each of the extracted DNA samples prior to separation by ultracentrifugation to serve as a measure of the separation of light (unlabeled) and heavy (^{13}C-enriched) DNA. We estimate that in incubations containing a ^{13}C-labeled compound the added *E. coli* DNA was comparable in abundance to a fairly well-represented indigenous organism (~6 to 18% of the total light DNA). Detection of *E. coli* DNA in fractions representing heavy DNA after ultracentrifugation was therefore indicative of incomplete separation, and the extent of this contamination was further estimated by the frequency of appearance of *E. coli* sequences in 16S rRNA gene clone libraries. PCR screens with *E. coli*-specific PCR primers (35) of unamended slurry from the bioreactor before and after the incubations did not produce a product.

When a 25-cycle PCR program with *E. coli*-specific primers was used, *E. coli* DNA was always detected in light DNA fractions but was only infrequently detected in fractions containing heavy DNA. In one instance where there appeared to be slight contamination of the heavy DNA from a [^{13}C]phenanthrene incubation, a faint but visible *E. coli* product was present in heavy DNA fractions (Fig. 2A; fractions 2 to 4). Encouragingly, these products were much lighter than those obtained from the light DNA fractions. The separation of light and heavy DNA was more successful with other incubations in this experiment. For example, in a 2-day incubation with [^{13}C]naphthalene, no *E. coli* PCR product was observed in the fractions corresponding to heavy DNA (Fig. 2B).

A more-sensitive, 40-cycle PCR program using the *E. coli*-specific primers produced an amplicon in most of the fractions tested regardless of the compound, incubation time, or position in the ultracentrifuge tube (data not shown). Therefore, in an attempt to minimize the amplification of this ubiquitous, unseparated DNA during the construction of 16S rRNA gene clone libraries derived from heavy fractions, the less-sensitive 25-cycle PCR program was employed. Despite this precaution,

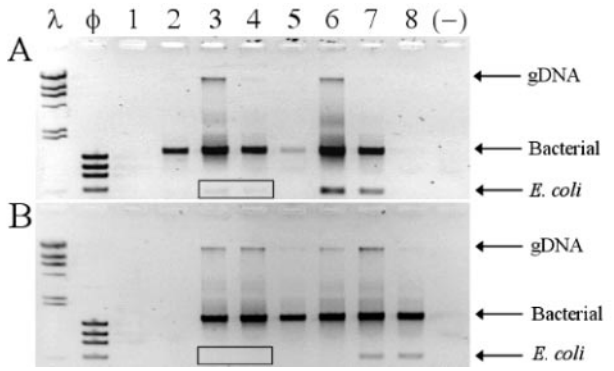

FIG. 2. Negative images of gel electrophoresis (1% agarose) screens of all fractions collected from ultracentrifuge tubes initially containing DNA extracted from (A) a 7-day incubation with [^{13}C]phenanthrene or (B) a 2-day incubation with [^{13}C]naphthalene. Numbers corresponding to each fraction are shown above each lane, with fraction 1 representing the bottom of the tube (containing the heaviest DNA). The numbered lanes were loaded with 2 μl each of a bacterial-specific 16S rRNA gene PCR product (Bacterial), *E. coli*-specific PCR product (*E. coli*), and the genomic DNA (gDNA; out of 75 μl) recovered from a given fraction. The box outlines weak *E. coli* PCR products in the heavy fractions from a [^{13}C]phenanthrene incubation in panel A, and the absence of those products in the equivalent fractions of panel B. λ and ϕ, 250 ng of a λHindIII or ϕX174HaeIII DNA ladder, respectively; (−), negative DNA PCR control.

two *E. coli* sequences were recovered from the clone libraries of heavy DNA from the [^{13}C]phenanthrene incubations (out of 51 total clones). No *E. coli* sequences were recovered from any of the libraries associated with the heavy DNA of [^{13}C]naphthalene or [^{13}C]salicylate incubations.

DGGE analysis of fractions. For all incubations, one fraction from the ultracentrifuge tubes was selected as representative of the light DNA and another fraction was selected as representative of the heavy DNA. These fractions generally corresponded to the highest and lowest positions in the tubes, respectively, from which a strong PCR product could be obtained. Incubations performed with unlabeled forms of the compounds did not possess heavy DNA, so a fraction equivalent to the heavy fraction in the corresponding ^{13}C incubation was selected.

The total community DNA for each selected fraction from each of the incubations was examined by DGGE (Fig. 3). The heavy DNA fractions from incubations with [^{13}C]salicylate and [^{13}C]naphthalene (2- and 7-day incubations) were very similar, with a single band dominating the profile (lanes 6, 10, and 14). Subsequent analyses on PCR-amplified DNA from clone libraries indicated that multiple sequences from different genera contributed to this dominant band (data not shown). The heavy DNA from incubations with [^{13}C]phenanthrene had a different profile (lane 18) than those from incubations with either [^{13}C]salicylate or [^{13}C]naphthalene.

There were no significant differences between the DGGE profiles of any of the replicate incubations (data not shown). Incubations in which no additional carbon source was added (Fig. 3; lanes 1 and 19) appeared similar to the fractions containing light DNA from incubations with ^{13}C-labeled carbon sources (lanes 5, 9, 13, and 17). The PCR program and primers used for DGGE did not appear to amplify *E. coli* DNA or

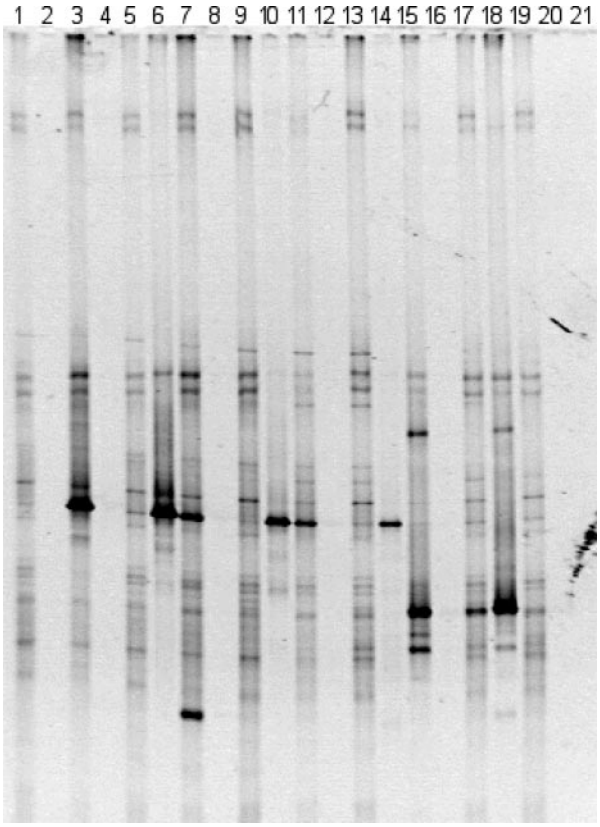

FIG. 3. Negative image of the DGGE gel of recovered DNA fractions from incubations with various carbon sources. Lanes 1 to 10 represent 2-day incubations and lanes 11 to 21 represent 7-day incubations. Lanes correspond to incubation conditions identified by the carbon source (SAL, salicylate; NAP, naphthalene; PHE, phenanthrene) and the fraction from the ultracentrifuge tube as follows: (1) no added carbon source, light fraction; (2) no added carbon source, heavy fraction; (3) unlabeled SAL, light fraction; (4) unlabeled SAL, heavy fraction; (5) [^{13}C]SAL, light fraction; (6) [^{13}C]SAL, heavy fraction; (7) unlabeled NAP, light fraction; (8) unlabeled NAP, heavy fraction; (9) [^{13}C]NAP, light fraction; (10) [^{13}C]NAP, heavy fraction; (11) unlabeled NAP, light fraction; (12) unlabeled NAP, heavy fraction; (13) [^{13}C]NAP, light fraction; (14) [^{13}C]NAP, heavy fraction; (15) unlabeled PHE, light fraction; (16) unlabeled PHE, heavy fraction; (17) [^{13}C]PHE, light fraction; (18) [^{13}C]PHE, heavy fraction; (19) no added carbon source, light fraction; (20) no added carbon source, heavy fraction; (21) negative DNA PCR control.

other light DNA sequences from fractions representing heavy DNA, and no bands appeared in DGGE lanes corresponding to the heavy DNA fraction of incubations with unlabeled carbon sources (lanes 2, 4, 8, 12, 16, and 20).

16S rRNA gene clone libraries. 16S rRNA gene clone libraries constructed from the fractions containing heavy DNA from replicate incubations with ^{13}C-labeled carbon sources were screened by restriction analysis prior to sequencing. For the [^{13}C]salicylate, [^{13}C]naphthalene (2-day), [^{13}C]naphthalene (7-day), and [^{13}C]phenanthrene incubations, a total of 34, 57, 52, and 51 clones were screened, respectively. Representatives of restriction patterns that appeared more than once were fully sequenced. This screening process led to the recovery of few identical sequences between treatments, even though some unique restriction patterns displayed high sequence similarity and a close phylogenetic relationship. In addition, some apparently identical restriction patterns represented different (albeit very similar) sequences (e.g., clones NAP2d2 and Sal2d22; Fig. 4). Approximately 8% of all sequenced clones appeared to be chimeric; by sequencing only clones with unique restriction patterns, more chimeras were likely recovered than if equivalent numbers of random clones were sequenced.

There was overlap between sequences recovered from clone libraries associated with heavy DNA from incubations with [^{13}C]salicylate and [^{13}C]naphthalene. Sequences from both sets of clone libraries shared high similarity with sequences in the *Pseudomonas* genus of the γ-proteobacteria and the *Ralstonia* genus of the β-proteobacteria (Fig. 4). However, the representatives of the majority of clones from the 2-day incubations with [^{13}C]naphthalene and some of the representative sequences from the 7-day incubations with [^{13}C]naphthalene grouped in clades within the pseudomonads (with similarity to *Pseudomonas jessenii* and *Pseudomonas mandelii*) that did not include a representative sequence from the [^{13}C]salicylate incubations. Another group of clones had high similarity to other pseudomonads (particularly *P. putida* KT2440) and contained representatives from the 2- and 7-day incubations with [^{13}C]naphthalene as well as the incubations with [^{13}C]salicylate. Interestingly, the lack of a cultured *Pseudomonas* species with >98% similarity to this group suggests that these organisms may be underrepresented in culture collections of PAH degraders.

In general, there was good overlap between sequences that were recovered from 2- and 7-day incubations with [^{13}C]naphthalene (Fig. 4). Along with the DGGE profiles, these data suggest that continued incubation of the soil slurry after the [^{13}C]naphthalene had been consumed did not substantially affect the sequences recovered. One exception was a sequence recovered from the 7-day incubation with [^{13}C]naphthalene (NAP7d18) that represented 6 of 52 screened clones but shared little similarity to other sequences obtained in the study (Fig. 4).

The majority of clones from incubations with [^{13}C]phenanthrene (34 of 51) were represented by two closely related sequences with high similarity to *Acidovorax* sequences (Fig. 4). No other clone restriction pattern appeared more frequently than recovered *E. coli* sequences (two clones) in the libraries derived from incubations with [^{13}C]phenanthrene, and therefore they were not considered to be significant representatives of the phenanthrene-degrading community in the bioreactor. No sequence that appeared more than once was shared between the libraries derived from incubations with [^{13}C]phenanthrene and [^{13}C]naphthalene, consistent with the differences in the DGGE profiles of these incubations.

Many unique restriction patterns from cloned 16S rRNA genes were recovered from the fractions containing heavy DNA but were not fully sequenced or examined in detail because they appeared infrequently. In general, the partial sequences derived from the [^{13}C]naphthalene and [^{13}C]salicylate clone libraries shared high similarity with other sequences observed in this study. However, a variety of β-proteobacterial sequences other than *Acidovorax* were obtained from the incubations with [^{13}C]phenanthrene that were not frequent in the other libraries. Despite their similarity to abundant sequences in this experiment or to known PAH-degrading bac-

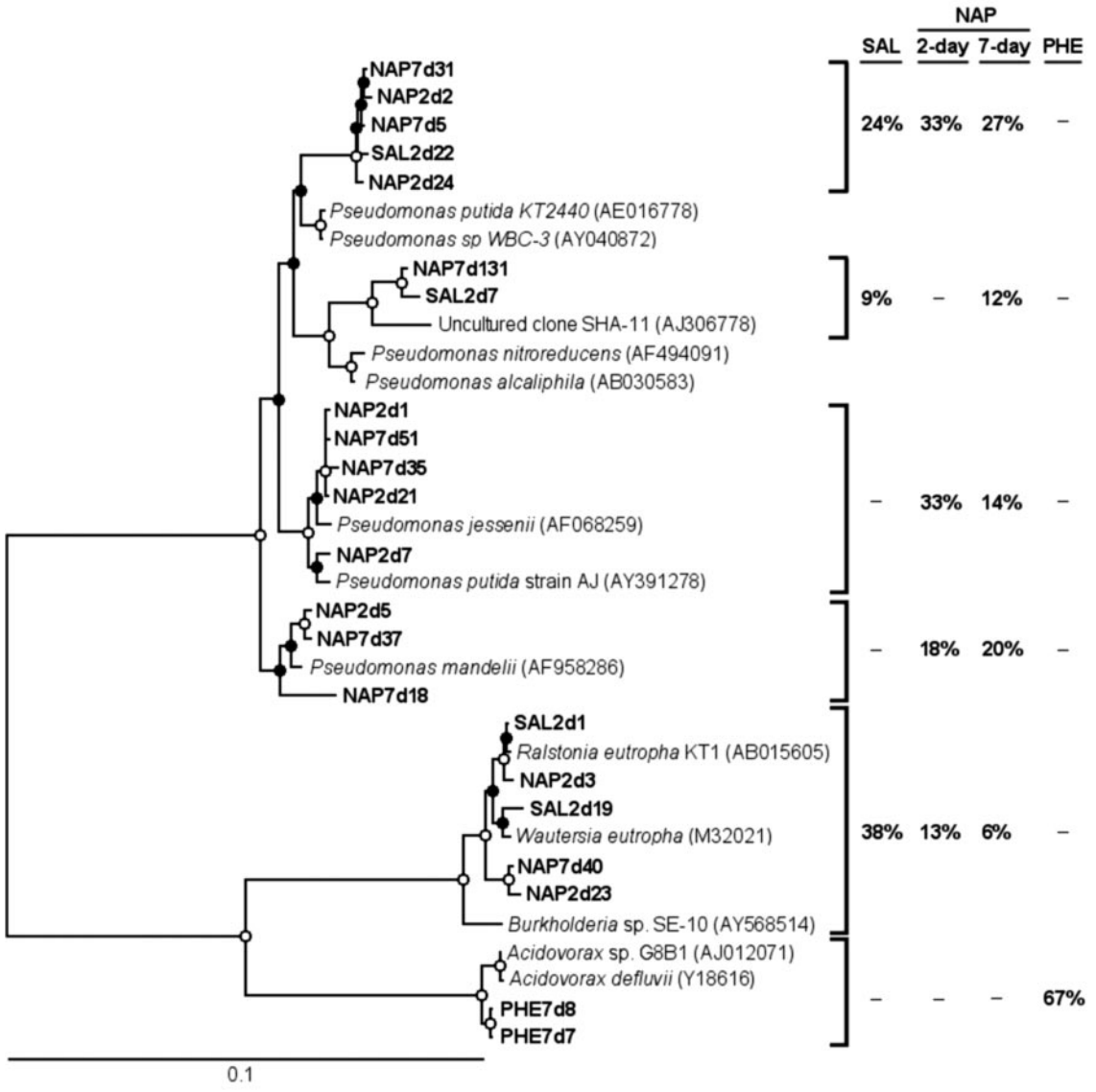

FIG. 4. Phylogenetic tree showing sequences recovered from heavy (^{13}C-enriched) fractions in this study and their closest relatives. Clones from this study are shown in bold and follow the naming scheme of growth substrate, number of days incubated (2d, 2 days; 7d, 7 days), and a number assigned to each clone for identification purposes. The percentage of screened clones represented by each group of sequences in each of the clone libraries is shown to the right of the tree. Reference sequences are shown in normal or italic font with the GenBank accession number in parentheses. The tree was based on the complete sequence of the amplified portions of the 16S rRNA gene (~1,500 bp). Some nodes are marked with either a closed or open circle indicating greater than 50 or 95% bootstrap support, respectively. SAL, salicylate; NAP, naphthalene; PHE, phenanthrene.

teria, it was uncertain as to whether sequences recovered at low frequency from any of the incubations actually represented organisms capable of growth on the ^{13}C-labeled carbon source. In any case, no singleton sequence from any fraction containing heavy DNA was considered as representative of an organism capable of incorporating the labeled carbon source, regardless of the presence or absence of the internal indicator sequence in the clone library.

Archaeal and fungal screens. Fractions containing heavy DNA from all incubations with ^{13}C-labeled carbon sources were screened for the presence of archaeal or fungal sequences by using domain-specific PCR primers. No archaeal or fungal products were produced.

DISCUSSION

The 16S rRNA gene sequences recovered from incubations with ^{13}C-labeled forms of naphthalene and phenanthrene indicated that these PAHs were degraded by a number of frequently encountered PAH-degrading organisms with limited phylogenetic diversity. *Pseudomonas* and *Ralstonia* sequences are common in PAH-contaminated soils (10, 14, 26, 41) and

were associated with growth on salicylate and naphthalene in this study. *Acidovorax* species represented the majority of sequences associated with growth on phenanthrene, and they have also been found at PAH-contaminated sites (14, 26, 37).

It is possible that the sequences detected in this study may only represent a portion of the total community capable of growth on the added compounds. The relatively high concentrations of the compounds in readily available form (i.e., not associated with the solid phase) could have influenced the growth of some organisms. For example, while *Sphingomonas* spp. are commonly encountered in PAH-contaminated environments (10, 24, 26), high levels of readily available phenanthrene have been shown to influence *Sphingomonas* diversity (24). We also recognize that the method of incubation may have preferentially enriched faster-growing organisms, potentially influencing the recovered 16S rRNA gene sequences. Such bias is less likely for phenanthrene, since the majority of the phenanthrene mass was not dissolved at the start of the experiment and a significant fraction of the added phenanthrene remained after 5 days of incubation.

Salicylate has been proposed as a supplemental carbon source that could selectively enrich for PAH-degrading bacteria in contaminated systems that contain limited amounts of bioavailable growth substrates (7). In this study, the addition of salicylate to the microbial community in the bioreactor led to the enrichment of many bacteria that were similar to those capable of degrading naphthalene, although it is possible that some recovered sequences may represent organisms capable of growing on salicylate that do not possess any PAH degradation capability. In contrast, sequences associated with enrichment with salicylate were not recovered at a significant frequency in incubations with phenanthrene. The apparent selection of naphthalene-degrading bacteria by the addition of salicylate may have implications for the use of salicylate to enhance the degradation of higher-molecular-weight PAHs, since bacteria that have been isolated by growth on naphthalene generally metabolize a narrower range of PAHs than organisms isolated by growth on other compounds (10, 16, 21).

It is also interesting that there was no significant overlap between organisms capable of growing on naphthalene and those growing on phenanthrene in this study. Although a number of phenanthrene-degrading isolates can also grow on naphthalene (4), many others cannot (4, 16, 18). Additionally, the presence of naphthalene has been shown to inhibit the growth of some phenanthrene-degrading bacteria, including an *Acidovorax* isolate (37).

We conducted two incubations with [^{13}C]naphthalene, one for a period consistent with the time required for substantial naphthalene depletion (2 days) and one for a period consistent with the time required for substantial depletion of phenanthrene (7 days). There was little difference in the types of 16S rRNA gene sequences associated with heavy DNA of [^{13}C]naphthalene incubations over these two periods, suggesting that cross-feeding of the labeled carbon was not a problem in this study. Less total DNA was recovered, however, from the longer incubations, which may be attributable to cell turnover in the 6 days during which low amounts of exogenous carbon were available. Even though cross-feeding did not appear to be a major concern in this study, in future SIP studies it may be advisable to sacrifice microcosms soon after depletion of the labeled compound. This will not only reduce the possibility of cross-feeding but may also maximize the concentrations of the labeled macromolecules with which to work.

One challenge when using SIP is verifying that the sequences recovered from a heavy DNA fraction represent only organisms with ^{13}C-enriched DNA. For example, the G+C content of an organism's DNA will influence its buoyant density in a cesium chloride gradient; the magnitude of the density difference between extremes in G+C content can approach that of differences based on isotopic labeling (25). However, we did not observe any heavy DNA in incubations with unlabeled substrates or in the initial slurry sample, indicating that the heavy DNA we recovered from incubations with the ^{13}C-labeled substrates was associated with the isotopic labeling and was not an artifact of differences in G+C content. As an additional complication in SIP, a small percentage of the total DNA added to a cesium chloride gradient for separation can appear in any fraction of the ultracentrifuge tube (25). While we were able to detect this "background" DNA with a 40-cycle PCR program, the absence of the internal indicator in most other reactions and lack of reaction products entirely from heavy fractions of unlabeled substrates indicated that background DNA was likely not amplified.

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