Multiple DNA Extractions Coupled with Stable-Isotope Probing of Anthracene-Degrading Bacteria in Contaminated Soil[▽][†]

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In many of the DNA-based stable-isotope probing (SIP) studies published to date in which soil communities were investigated, a single DNA extraction was performed on the soil sample, usually using a commercial DNA extraction kit, prior to recovering the ^{13}C-labeled (heavy) DNA by density-gradient ultracentrifugation. Recent evidence suggests, however, that a single extraction of a soil sample may not lead to representative recovery of DNA from all of the organisms in the sample. To determine whether multiple DNA extractions would affect the DNA yield, the eubacterial 16S rRNA gene copy number, or the identification of anthracene-degrading bacteria, we performed seven successive DNA extractions on the same aliquot of contaminated soil either untreated or enriched with [U-^{13}C]anthracene. Multiple extractions were necessary to maximize the DNA yield and 16S rRNA gene copy number from both untreated and anthracene-enriched soil samples. Sequences within the order *Sphingomonadales*, but unrelated to any previously described genus, dominated the 16S rRNA gene clone libraries derived from ^{13}C-enriched DNA and were designated "anthracene group 1." Sequences clustering with *Variovorax* spp., which were also highly represented, and sequences related to the genus *Pigmentiphaga* were newly associated with anthracene degradation. The bacterial groups collectively identified across all seven extracts were all recovered in the first extract, although quantitative PCR analysis of SIP-identified groups revealed quantitative differences in extraction patterns. These results suggest that performing multiple DNA extractions on soil samples improves the extractable DNA yield and the number of quantifiable eubacterial 16S rRNA gene copies but have little qualitative effect on the identification of the bacterial groups associated with the degradation of a given carbon source by SIP.

Molecular methods are increasingly being used to explore the microbial diversity of environmental systems without needing to isolate microorganisms from their natural environment, especially because many relevant organisms have proven difficult to isolate from their environmental sources (3, 7, 18). The effectiveness of molecular methods to describe microbial diversity depends on our ability to efficiently extract and purify macromolecules from microbial cells native to an environmental sample (40). Commercially available kits are commonly used to extract nucleic acids from environmental samples by physical and/or chemical lysis of microbial cells, followed by purification of the nucleic acids from cell debris and other organic material. Feinstein et al. (10) recently demonstrated that extracting a soil aliquot only once with a commercial kit can lead to incomplete DNA extraction, thus biasing estimates of the genomic DNA mass yield, the small-subunit ribosomal gene copy number, and the bacterial groups identified; multiple extractions led to broader recovery of organisms in the soil community.

Bioremediation is the primary method of removing polycyclic aromatic hydrocarbons (PAHs) from PAH-contaminated environments (1), but our understanding of the roles of specific

organisms within PAH-degrading microbial communities and the metabolic mechanisms responsible for PAH degradation is still developing. Stable-isotope probing (SIP) is one cultivation-independent molecular technique that can link the identity of a microorganism with its metabolic function without isolating that organism from its natural environment (38). DNA-based SIP has been used to identify bacteria capable of degrading aromatic hydrocarbons in PAH-contaminated environments, and in some cases, it has revealed novel bacterial groups (15, 16, 33, 35, 43). Earlier SIP studies of pyrene-degrading bacteria conducted in our lab revealed members of previously uncultivated beta- and gammaproteobacterial groups, neither of which is related to any cultivated genus (16, 35). SIP investigations have also facilitated the isolation of ecologically relevant organisms (15, 19, 36) and have been used to reduce the complexity of community DNA slated for metagenomic analysis (4). To date, SIP of anthracene-degrading bacteria has not been reported.

In many of the DNA-based SIP studies published to date in which soil communities were investigated, a single DNA extraction was performed on the soil sample, usually using a commercial DNA extraction kit, prior to recovering the ^{13}C-labeled (heavy) DNA by density-gradient ultracentrifugation. In the present study, we performed successive DNA extractions on the same aliquot of PAH-contaminated soil either untreated or enriched with uniformly ^{13}C-labeled anthracene to determine whether multiple DNA extractions would affect the DNA yield, the eubacterial 16S rRNA gene recovery, or the identification of anthracene-degrading bacteria. In addition, we tested the effects of soil loading and multiple extrac-

tions on the efficiency of the FastDNA spin kit for soil (MP Biomedicals, Solon, OH).

MATERIALS AND METHODS

Soil processing. PAH-contaminated soil was collected from a former manufactured gas plant site in Salisbury, Rowan County, NC. The total PAH concentration was approximately 890 mg/kg, and the anthracene concentration was 32 mg/kg. Large objects were removed by hand. The soil was then sieved through a 10-mm wire screen, blended, and sieved again prior to storage in the dark at 4°C. The processed soil (64% sand, 30% silt, 6% clay, and 15% moisture; pH 7.6) was further prepared by manually removing any remaining small stones and other debris immediately before use in experiments.

Chemicals. Natural abundance isotopomer (unlabeled) anthracene (scintillation grade) was obtained from Eastman Kodak (Rochester, NY). [U-^{13}C]Anthracene was synthesized according to methods to be described elsewhere (Z. Zhang, L. M. Ball, and A. Gold, personal communication). Until the method is published, details of the synthesis can be obtained by contacting M.D.A. at mike_aitken@unc.edu. [1,2,3,4,4a,9a-^{14}C]Anthracene (17.3 mCi/mmol) was obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals used were at the highest purity available. All solvents were molecular biology or high-pressure liquid chromatography (HPLC) grade.

Enrichment with anthracene. Soil slurries were prepared in 125-ml flasks containing 1 g of soil (wet weight) and 30 ml of simulated groundwater amended with 0.37 mM NH_4NO_3 and 0.08 mM K_2HPO_4. The groundwater was prepared to reproduce the major ion concentrations in the groundwater of Rowan County, NC (0.7 mM $CaCl_2 \cdot H_2O$, 0.2 mM $MgSO_4 \cdot 7H_2O$, 1.0 mM $NaHCO_3$, 0.06 mM KCl, 1 N H_2SO_4; pH 7.5) (31) and was filter sterilized through a 0.1-μm-pore-size flowthrough, hollow-fiber membrane water filter (Sawyer Products, Safety Harbor, FL). Four sets of flasks containing the soil slurry were incubated in parallel. For each set, after 2 days of shaking (225 rpm) in the dark at room temperature to reduce the concentrations of native PAHs, the aqueous phase was replaced with fresh nitrogen- and phosphorus-amended groundwater. Duplicate clean flasks were spiked with 625 μg of either unlabeled anthracene (set 1) or [U-^{13}C]anthracene (set 2) (in acetone) to enrich the anthracene-degrading microorganisms. The acetone was allowed to evaporate before the slurry was introduced, and the flasks were returned to the shaker ($t = 0$). In addition, triplicate flasks containing unlabeled anthracene (set 3) were prepared to monitor the anthracene disappearance by HPLC and to archive community DNA over time. Another set of triplicate flasks containing a mixture of unlabeled and radiolabeled anthracene (20,000 dpm) (set 4) was prepared to monitor anthracene mineralization by liquid scintillation counting of $^{14}CO_2$ trapped in KOH-soaked filter paper (34). Inhibited controls were prepared by acidifying incubations to pH values of <2 using 200 μl of 85% phosphoric acid.

Monitoring anthracene disappearance. Soil slurry from each triplicate flask in set 3 (1 ml) was mixed with 1 ml of ethyl acetate in each of the triplicate 15-ml conical-bottom glass centrifuge tubes. The tubes were vortexed at maximum speed for 1 min and centrifuged for 5 min at 3,500 rpm. The organic layer of each resulting supernatant was filtered through a 0.45-μm-pore-size nylon filter and stored in a gas chromatography vial at −20°C prior to HPLC analysis. The extracts were diluted with acetonitrile as needed immediately before HPLC analysis. The HPLC system included a Waters 600E system controller (Milford, MA), a Waters 717 Plus autosampler, and a PerkinElmer LS40 fluorescence detector (Beaconsfield, United Kingdom). Analyte standards were prepared from an EPA 610 polynuclear aromatic hydrocarbons mixture stock (Sigma-Aldrich, St. Louis, MO) and used to create a four-point calibration curve for sample quantification. Samples were injected through a 3-μm-particle-size Supelcosil LC-PAH column (Sigma-Aldrich, St. Louis, MO) using a gradient mobile phase of acetonitrile and water and analyzed as previously described (34).

DNA extraction. After centrifugation, DNA was extracted from the soil in each flask from sets 1 and 2 in two 500-mg aliquots of the soil pellet by using the FastDNA spin kit for soil (MP Biomedicals, Solon, OH) according to the accompanying instructions with the following exceptions. Samples were secured horizontally to a bench-top vortexer set at maximum speed for homogenization. After each extraction, fresh sodium phosphate and MT buffers (MP Biomedicals) were added to the Lysing Matrix E tube containing the original soil aliquot, and the extraction procedure was repeated. DNA was eluted from each successive extraction with Tris-EDTA buffer (TE; pH 8.0) into a clean catch tube until seven extractions had been performed. The equivalent extracts of each 500-mg aliquot from a given incubation flask were pooled prior to further analysis; because there were duplicate incubation flasks, there were duplicate series of seven DNA extracts. For the flasks from set 3, the FastDNA spin kit for soil was used to perform a single DNA extraction on a soil pellet resulting from 1 ml of

soil slurry containing approximately 33 mg of soil. In a subsequent experiment, DNA was extracted from untreated soil in duplicate aliquots of 33, 100, 250, or 500 mg using the same multiple-extraction procedure described above, except that six successive extractions were performed.

DNA and 16S rRNA gene quantification. The DNA mass yield was quantified with a NanoDrop 3300 fluorospectrometer (NanoDrop Products, Wilmington, DE) using the Quant-iT PicoGreen double-stranded DNA (dsDNA) assay kit (Invitrogen, Eugene, OR). The 16S rRNA gene copy number of a targeted sequence was determined by quantitative PCR (qPCR) using 1 μl of DNA as the template, primers (final concentration, 600 nM) as identified in Table 1, and QuantiTect SYBR Green PCR master mix (Qiagen, Valencia, CA) with the SmartCycler platform (Cepheid, Sunnyvale, CA) in a 25-μl reaction. Primer sets for targeted quantification of several SIP-identified groups were designed and validated as described elsewhere (35), except that sequences were aligned within the myRDP personalized workspace (6). The qPCR temperature program used included 15 min at 95°C, followed by 45 cycles of 15 s at 95°C, 30 s at the annealing temperature (Table 1), and 30 s at 72°C. Data were collected during primer extension, and reaction products were analyzed by melt curve analysis between 65 and 95°C. The r^2 value for each qPCR standard curve (cycle threshold versus log gene copy number) was ≥0.995, and the amplification efficiencies of curves from eubacterial and group-specific primer sets were close to 2.0 (Table 1). To compare the abundance of the SIP-identified bacteria in the heavy DNA to their abundance in the light DNA, each group-specific primer set was used to quantify the corresponding sequences in each fraction from one ultracentrifuge tube. Two sets of primers for quantification of sequences for "anthracene group 1" (AG1) were developed. Primer set AG1F/AG1R was used to quantify AG1 sequences in the multiple extraction experiment, while primer set AG1.1F/AG1.1R, which possessed a lower detection limit during creation of standard curves, was used to quantify genes in collected SIP fractions and time course experiment samples.

DNA separation and recovery. DNA extracted from anthracene-enriched samples was mixed with 20 μl of SYBR Safe (Invitrogen, Carlsbad, CA), and the mixture was added to 6-ml polyallomer Ultracrimp tubes (Kendro Laboratory Products, Newtown, CT). SYBR Safe is an alternative to ethidium bromide in CsCl density gradients used in DNA-based SIP assays (25) that simplifies the cleanup of fractions collected from ultracentrifuge tubes because ethidium bromide does not have to be extracted. Inclusion of a fluorescent dye in the CsCl solution allowed us to visualize bands of DNA postseparation, thus locating the approximate positions of fractions containing DNA of interest. The tubes were filled with a cesium chloride solution (ρ = 1.72 g/ml), crimp sealed, and ultracentrifuged (RC70 ultracentrifuge; Sorvall, Newtown, CT) at 175,800 × g and 20°C for 40 h in a TV-1665 vertical rotor (Sorvall). A tube containing 1 μg each of *Escherichia coli* K-12 DNA from a culture grown in LB broth and a *Pseudomonas putida* G7 culture grown on uniformly labeled [^{13}C]glucose (Cambridge Isotope Laboratories, Inc.) was included as a control to verify separation of unlabeled DNA from ^{13}C-labeled DNA. DNA bands were visualized with the Safe Imager blue light transilluminator (Invitrogen, Carlsbad, CA) before collection of 24 fractions of 250 μl each from the bottom of each ultracentrifuge tube, as described by Singleton et al. (33). Separation of unlabeled and ^{13}C-labeled DNA achieved in the control tube is illustrated in Fig. S1 in the supplemental material. DNA in each fraction was recovered by ethanol precipitation (30) and resuspended in 100 μl of 0.2-μm-filter-sterilized TE (pH 8.0).

Identification of heavy and light DNA fractions. The DNA concentration and eubacterial 16S rRNA gene copy number in each DNA fraction recovered were quantified, and the eubacterial community profile for each fraction was visualized by denaturing gradient gel electrophoresis (DGGE). PCR for DGGE targeted the V1-V3 hypervariable region of the 16S rRNA gene and was performed with 5Prime Mastermix (Gaithersburg, MD) using 1 μl of DNA as the template and primers 63F-GC and 517R (final concentration, 200 nM each) in a 20-μl reaction as previously described (44). The temperature program was modified such that 10 cycles of touchdown PCR were followed by 15 cycles of conventional PCR. PCR products were loaded onto a 6.5% polyacrylamide gel without denaturant stacked on top of a 6.5% polyacrylamide gel with a urea-formamide denaturing gradient at between 30% and 60% and run for 16 h at 60 V on a DCode system (Bio-Rad Laboratories, Hercules, CA). DGGE gels were poststained with ethidium bromide, and bands were visualized under UV transillumination. Subsequent to identification of sequences recovered in putative heavy DNA from the SIP incubations, selected clonal insert sequences were amplified and analyzed by DGGE to associate the most prominent DGGE bands in the community DNA with specific sequences.

Consecutive fractions in the ultracentrifuge tube with similar DNA concentrations, 16S rRNA gene copy numbers, and community profiles were pooled and identified as the composite heavy or light DNA fraction, depending on the

TABLE 1. qPCR primers used in this study

Target group	Primer	Primer sequence (5′→3′)	T_m (°C)[a]	qPCR standard[b]	Amplicon length	Amplification efficiency (Bac; Group)[c]	No. of RDP hits[d]	Reference
Bacteria	341F 517R	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	60		177			26
Acidovorax	AcidF AcidR	TAACGGAGCGAAAGCTT GTCCGCGCAAGGCCTT	55	PHE7d8 (AY699582)	75	1.96; 1.90	493	32
Anthracene group 1[e]	AG1F AG1R	TTCGGAATAACTCCTC TCACCAACTAGCTAATCC	50	sbant93 (HM596265)	102	1.97; 2.03	100	This study
Anthracene group 1[f]	AG1.1F AG1.1R	TTGGCAAGTCAGGGGT CAAGCGAGGCAGTTTC	55.5	sbant93 (HM596265)	64	1.97; 1.98	7	This study
Herminiimonas	HERM.1F HERM.1R	TATCGGAACGTACCCTAG TATCGGCCGCTCCATG	52	sbant22 (HM596196)	116	1.97; 1.94	191	This study
Pigmentiphaga	PIGMF PIGMR	CAGGCGGTTCGGAAAG TGACATACTCTAGTTCGGGA	56	sbnap45 (GU266321)	63	1.97; 1.98	53	This study
Sphingobium	SGB.5F SBG.5R	ACAGTACCGGGAGAATAAGCTC CAAGCAATCCAGTCTCAAAGGCTA	56	sbant43 (HM596217)	158	1.98; 1.90	95	This study
Variovorax	VARIO.2F VARIO.2R	AGCTGTGCTAATACCGCATA TCCATTCGCGCAAGGTCTTG	55	sbant158 (HM596155)	67	2.11; 1.90	684	This study

[a] PCR annealing temperature.
[b] Name of the linear plasmid clone used as the DNA template to generate standard curves. GenBank accession numbers of the inserts are provided in parentheses.
[c] Amplification efficiency (28) with eubacterial (Bac) and group-specific (Group) primers.
[d] Number of sequences returned by the Ribosomal Database Project release 10.23 (5) (excluding sequences from this study) with no mismatches to both primers.
[e] Used to quantify AG1 sequences in the multiple-extraction experiment (Fig. 3).
[f] Used to quantify genes in collected SIP fractions and time course experiment samples (Fig. 4 and 5).

section of the tube from which the fractions were removed. Effort was made not to interrupt a peak when deciding which individual fractions to combine to create composite heavy and light fractions. DNA from the composite heavy fraction of each anthracene-enriched replicate was screened for archaeal rRNA gene sequences using primers 25F (8) and 1492R (22) and for fungal rRNA gene sequences using primers ITS1F (11) and ITS4 (42) before being used as the template to generate a eubacterial 16S rRNA gene clone library.

Clone library preparation and analysis. After identifying the fractions corresponding to heavy DNA in extracts from each [U-^{13}C]anthracene-enriched replicate, PCR was performed with 1 μl of heavy DNA as the template, primers 8F (9) and 1492R (22) (final concentration, 200 nM), and 5Prime Mastermix in a 50-μl reaction. The PCR temperature program used included 10 min at 94°C, followed by 25 cycles of 1 min at 94°C, 1 min at 50°C, and 3 min at 72°C, and ended with one 15-min cycle at 72°C. PCR products were cloned using a TOPO TA cloning kit for sequencing (Invitrogen, Carlsbad, CA), according to the accompanying instructions. Plasmid DNA from a random subset of clones was subjected to restriction analysis prior to sequencing to ensure successful ligation of the insert to the plasmid vector. Inserts were partially sequenced with primer 8F by Functional Biosciences, Inc. (Madison, WI).

Sequences were analyzed by VecScreen (http://www.ncbi.nlm.nih.gov /VecScreen) to remove vector contamination, Bellerophon (13) was used to screen for chimeric sequences, and the RDP Classifier (41) was used to determine the closest cultivated genus to each sequence. Each sequence was compared to those in public sequence databases using BLASTN (2) and RDP release 10.17 (5) to identify closely related sequences. Multiple sequence alignments and guide trees were produced using myRDP (6) and ClustalX (37), respectively, which facilitated the grouping of sequences most similar to one another and to those in GenBank. Rarefaction curves were generated at a 3% sequence distance to test whether clone libraries of adequate size had been generated. The UniFrac significance test (12) was used to determine whether the clone libraries were significantly different from one another. Clones were named to indicate the source soil (Salisbury [SB]) and growth substrate (anthracene [ANT]) and were numbered.

To confirm whether sequences identified in the clone libraries were ^{13}C enriched, we quantified each sequence in fractions spanning the putative heavy and light DNA in selected extracts from the SIP incubations with ^{13}C-labeled anthracene as well as in the extract from the incubation with unlabeled anthracene. To align equivalent fractions from separations of both unlabeled and ^{13}C-labeled anthracene, we also quantified sequences associated with the *Acidovorax* genus that were identified as degraders of other PAHs in this soil (17) but that were not

observed in the clone libraries generated from heavy DNA from the SIP incubations with ^{13}C-labeled anthracene. Thus, *Acidovorax* genes were expected to be associated with unlabeled DNA fractions, regardless of the incubation.

Nucleotide sequence accession numbers. Sequences recovered from this study were deposited in GenBank with accession numbers HM596084 to HM596270.

RESULTS

Anthracene removal and mineralization. Soil slurry was incubated in triplicate with unlabeled anthracene (to follow anthracene removal) or a mixture of unlabeled and radiolabeled anthracene (to follow mineralization) for 20 days. After 3 days, less than 10% of the added anthracene remained in the flasks containing unlabeled anthracene. However, the mineralization experiment continued until the rate appeared to decline at day 20 (Fig. 1), which was then selected as the time to terminate SIP incubations with [U-^{13}C]anthracene.

SIP with anthracene and identification of heavy DNA. After 20 days, seven successive DNA extractions were performed on duplicate aliquots of 500 mg (wet weight) of soil from each of the duplicate flasks containing unlabeled or [U-^{13}C]anthracene. For comparison, six successive extractions were performed on replicate 500-mg aliquots of the untreated original soil sample. Quantifiable amounts of DNA were obtained through the six extractions of the untreated soil (total over 6 extractions = 0.98 ± 0.01 μg/g dry soil), although the number of 16S rRNA genes in the sixth extract was negligible (total over 6 extractions = 2.24 × 10⁸ ± 1.99 × 10⁷ gene copies/g dry soil) (Fig. 2). The pairwise Wilcoxon signed rank test determined that the amount of DNA ($P = 0.85$) and the number of 16S rRNA genes ($P = 0.36$) recovered at each extraction step were similar in each of the duplicate extraction series. Both the total DNA (39.0 ± 2.65 μg/g dry soil) and 16S rRNA genes
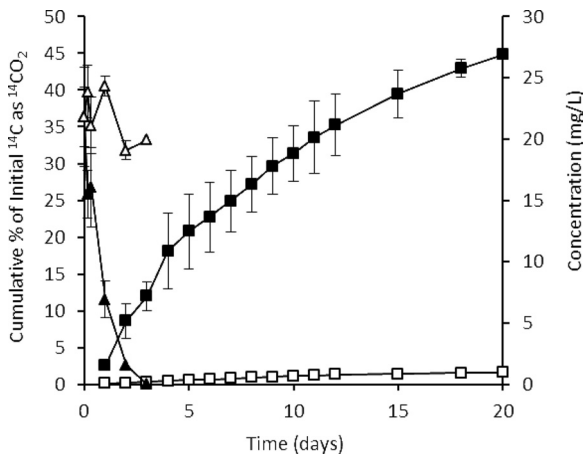
FIG. 1. Cumulative $^{14}CO_2$ recovered from incubations with [^{14}C]anthracene (squares) and anthracene removal from incubations with unlabeled anthracene (triangles) by the indigenous Salisbury soil microbial community. Filled and open symbols represent live and inhibited incubations, respectively. Values are the means and ranges of duplicate incubations for mineralization or the means and standard deviations ($n = 3$) for anthracene removal. Some error bars are smaller than the symbol.

TABLE 2. Bacterial groups recovered in clone libraries of putative heavy DNA from incubations with ^{13}C-labeled anthracene

Genus or group	No. of clones			
	Extract 1	Extracts 2-4	Extracts 5-7	Total
Variovorax	17	20	8	45
Anthracene group 1	14	25	42	81
Sphingobium	9	12	5	26
Pigmentiphaga	3	3	1	7
Herminiimonas	7	0	1	8
Other (no. of different groups)	11 (5)	3 (3)	6 (4)	20 (11)
Total	61	63	63	187

($2.37 \times 10^{11} \pm 2.60 \times 10^9$ gene copies/g dry soil) were quantifiable through the seventh extraction of anthracene-enriched soil (Fig. 2). The amount of DNA ($P = 0.30$) and the number of 16S rRNA genes ($P = 0.81$) recovered at each extraction step were similar in each of the duplicate extraction series.

DGGE analysis of anthracene-enriched samples revealed the same banding patterns for extracts 1 to 4, but additional bands were present in lanes containing amplicons from extracts 5 to 7 (not shown). Based on this analysis, for each incubation flask, the DNA from extracts 2 to 4 (19 μg) was pooled, and the DNA from extracts 5 to 7 (13 μg) was pooled prior to ultracentrifugation. The pooled extracts from each duplicate flask were loaded into separate ultracentrifuge tubes and centri-

fuged in parallel to duplicate tubes containing DNA from extract 1 (7 μg).

Fractions collected from each of the ultracentrifuge tubes were analyzed by measuring the DNA concentration and eubacterial 16S rRNA gene abundance and by DGGE. In the tubes containing DNA from incubations with unlabeled anthracene, there was a single peak over a range of fractions corresponding to where unlabeled ("light") DNA was observed in the control tube (see Fig. S2A in the supplemental material). No measurable DNA was observed in lower fractions, and 16S rRNA gene abundance was at a background level (2.0 to 2.5 log gene copies) in the range of fractions corresponding to the location of heavy DNA in the control tube. DGGE bands were visible in these lower fractions, but the banding patterns were identical to those of fractions containing unlabeled DNA (see Fig. S3A in the supplemental material). In the tubes containing DNA from incubations with ^{13}C-labeled anthracene, the fractions containing measurable DNA also had quantifiable eubacterial 16S rRNA genes of up to 3 orders of magnitude above the background level (see Fig. S2B to D). Differences in DGGE banding patterns (see Fig. S3B to D) between fractions were used to select the fractions corresponding to heavy DNA in each tube. No archaeal or fungal rRNA genes were detected in any heavy DNA fraction.

Analysis of sequences recovered from 16S rRNA gene clone libraries. A 16S rRNA gene clone library was generated from the heavy DNA recovered from each replicate of extract 1, pooled extracts 2 to 4, and pooled extracts 5 to 7. For each replicate, 32 clones were partially sequenced (192 total), of which 1 containing a vector sequence and 4 containing chimeras were excluded from further analyses. Phylogenetic analysis of the recovered sequences is illustrated in Fig. S4 in the supplemental material, and the major groups corresponding to these sequences are summarized in Table 2. Rarefaction analysis indicated that an adequate number of clones was sequenced (see Fig. S5 in the supplemental material). The pairwise UniFrac significance test (weighted and normalized to account for sequence abundance and branch lengths, respectively) determined that libraries from extract 1 and extracts 5 to 7 were significantly different from one another ($P \leq 0.002$); however, all groups identified in extracts 2 to 4 and 5 to 7 were also identified in extract 1. Other library pairs (extract 1 versus extracts 2 to 4 and extracts 2 to 4 versus extracts 5 to 7) were not significantly different from one another ($P > 0.1$). Se-
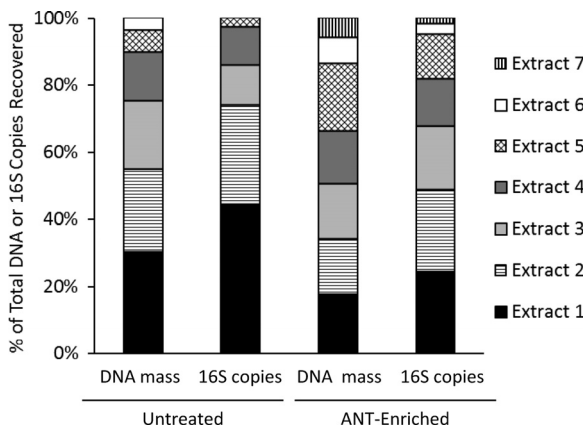

FIG. 2. Recovery of DNA mass and eubacterial 16S rRNA genes from successive DNA extracts of the original (untreated) or anthracene-enriched soil. DNA mass values are the means from triplicate measurements from each of the duplicate soil aliquots. Values for the 16S rRNA gene copy number are the means from a single analysis of each of two soil aliquots. ANT, anthracene.
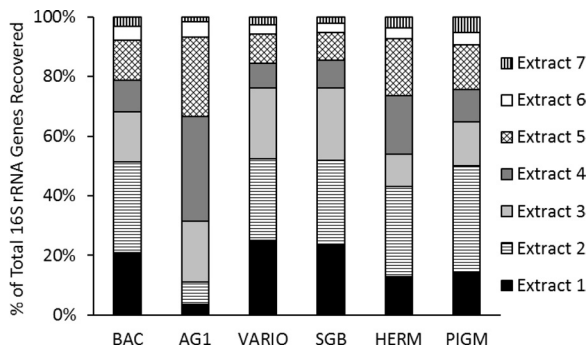
FIG. 3. Differential recovery of eubacterial (BAC) and group-specific 16S rRNA genes in unseparated DNA from each successive extract of soil enriched with [^{13}C]anthracene. The total number of gene copies recovered over the seven extractions was 2.94×10^7 for anthracene group 1 (AG1), 4.89×10^9 for *Variovorax* (VARIO), 8.34×10^9 for *Sphingobium* (SGB), 4.04×10^8 for *Herminiimonas* (HERM), and 3.36×10^9 for *Pigmentiphaga* (PIGM). Values are the means from triplicate qPCR analyses.

quences clustering with members of the order *Sphingomonadales*, but that are unrelated to any previously described genus, were abundant in each clone library (14 of 61 for extract 1, 25 of 63 for pooled extracts 2 to 4, and 42 of 63 for pooled extracts 5 to 7) and were designated "anthracene group 1" (AG1). Other well-represented sequences were similar to sequences representing the genera *Variovorax* (93% similar) and *Sphingobium* (93%); sequences related to *Herminiimonas* (99%) and *Pigmentiphaga* (99%) were found less frequently (Table 2).

Quantification of SIP-identified bacteria. Primers used for quantitative PCR that targeted the 16S rRNA gene were developed to measure the abundances of the major bacterial groups recovered in putative heavy DNA from the SIP incubations with ^{13}C-labeled anthracene (Table 1). Quantitative PCR analyses of unseparated DNA reserved from each of the ^{13}C-labeled anthracene enrichments revealed that there was differential recovery of each SIP-identified group compared to that of the eubacterial community with successive extractions (Fig. 3); the most disparate recovery pattern occurred with AG1 sequences. To test the hypothesis that the extraction patterns of 16S rRNA genes from the different groups are not correlated over the seven extractions, we calculated the corre-

lation between each pair of groups and assessed the significance of the hypothesis that each correlation is 0 using a permutation *P* value. Specifically, we permuted the data 2,000 times and calculated correlations using the permuted data. The permutation *P* value is the proportion of permutations in which we observed larger correlations than we observed using the unpermuted data (see Table S1 in the supplemental material). We could not reject the hypothesis of zero correlation for comparisons between AG1 and any other group ($P = 0.19$ to 0.57), which suggests that the pattern of 16S rRNA gene extraction for AG1 was different from the extraction pattern of each other group. The tests between *Sphingobium* and *Herminiimonas* ($P = 0.08$) and between *Variovorax* and *Herminiimonas* ($P = 0.10$) were marginally significant, suggesting a relatively weak correlation across the seven extracts. Tests between all other pairs were significant ($P < 0.02$), suggesting similar extraction patterns.

We also quantified growth over time of the SIP-identified bacterial groups during incubations in the presence of unlabeled anthracene. After 3 days of incubation in the presence of unlabeled anthracene, corresponding to when the majority of the added anthracene had been removed, the abundance of the 16S rRNA genes increased by at least an order of magnitude for all of the SIP-identified bacterial groups other than *Sphingobium* (Fig. 4). We also quantified sequences related to *Acidovorax* spp. known to be present in the soil but which were presumed not to be associated with anthracene degradation. *Acidovorax* sequences increased by 4-fold during the 3-day period over which anthracene was consumed, which was significantly lower than the increase in the SIP-identified groups, other than *Sphingobium* (Fig. 4). The abundances of AG1 and *Pigmentiphaga*-related sequences did not change significantly after day 3. *Variovorax*-, *Herminiimonas*-, and *Sphingobium*-related sequences continued to increase in abundance between day 3 and day 7, after which there was relatively little change in these sequences.

Each group-specific primer set was used to quantify the corresponding sequences in each DNA fraction recovered from extract 1 and pooled extracts 2 to 4 of the SIP incubation and in each fraction of separated DNA from the incubation with unlabeled anthracene (Fig. 5). Fractions from each of the separations (unlabeled anthracene; ^{13}C-labeled anthracene, extract 1; and ^{13}C-labeled anthracene, extracts 2 to 4) were
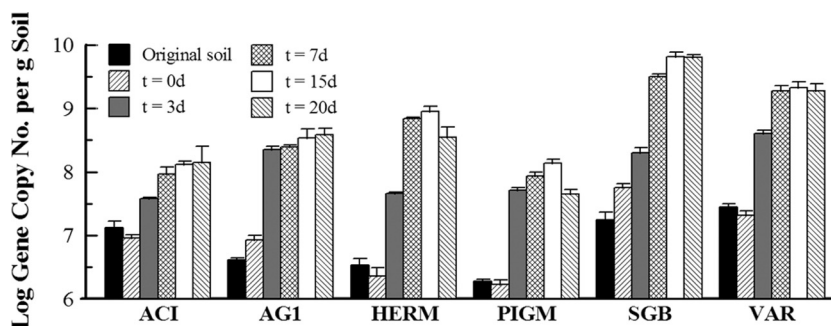

FIG. 4. Abundances of group-specific 16S rRNA genes over time (days) in response to enrichment with unlabeled anthracene; "$t = 0$" is when anthracene was first added to the incubation flask after 2 days of incubating the soil slurry without anthracene. Values are the means and standard deviations ($n = 3$). ACI, *Acidovorax*; VAR, *Variovorax*; other abbreviations are defined in the legend to Fig. 3.
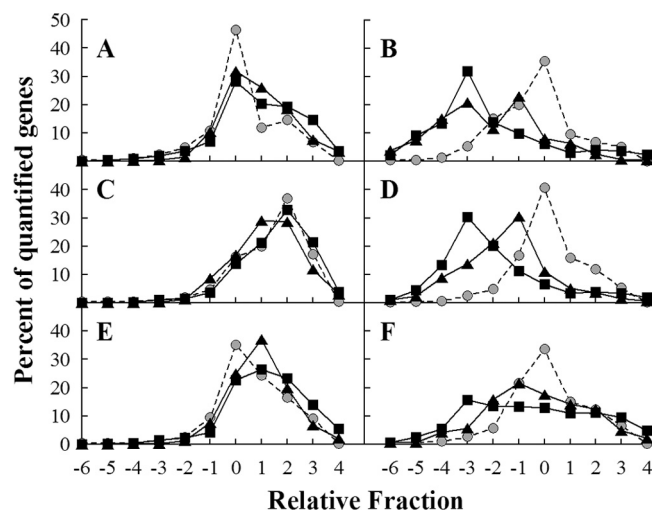
FIG. 5. Distribution of 16S rRNA genes in ultracentrifuge tube fractions for bacterial groups identified in clone libraries of putative heavy DNA. Values shown are the percentages of the total quantified genes per fraction from one replicate of extract 1 (■) and pooled extract 2 to 4 (▲) of SIP incubations with [^{13}C]anthracene as well as from DNA extracted from the incubation with unlabeled anthracene (○). Fractions were normalized relative to the peak of the *Acidovorax* sequence in each sample (*Acidovorax* peak = relative fraction 0). Each value was obtained from a single qPCR performed on each fraction using the group-specific primer sets listed in Table 1. (A) *Acidovorax*; (B) AG1; (C) *Herminiimonas*; (D) *Pigmentiphaga*; (E) *Sphingobium*; (F) *Variovorax*.

aligned based on the peak of the *Acidovorax* sequences in each sample. DNA shifting to lower fractions from incubations with ^{13}C-labeled anthracene compared to those from the unlabeled compound was evidence for incorporation of the label. Compared to the peak fraction in unlabeled DNA, there were shifts to lower fractions (heavier DNA) in the DNA from SIP incubations for AG1 sequences (Fig. 5B), *Pigmentiphaga*-related sequences (Fig. 5D), and *Variovorax*-related sequences (Fig. 5F). No such shifts were observed for the *Herminiimonas*- or *Sphingobium*-related sequences (Fig. 5C and E, respectively).

Effects of soil loading and multiple extractions on extraction efficiency. Using the FastDNA spin kit for soil, DNA was extracted six successive times from untreated soil in duplicate aliquots of 33, 100, 250, or 500 mg to determine the optimum soil load and number of extractions needed to maximize DNA mass yield and 16S rRNA gene recovery from the same aliquot of PAH-contaminated soil (see Fig. S6 in the supplemental material). With a single extraction, DNA mass yield decreased with an increasing soil load ($r^2 = -0.94$), but soil loads of up to 250 mg did not affect the yield of quantifiable 16S rRNA genes recovered in the first extraction ($P = 0.07$). Subsequent extractions resulted in further recovery of DNA and 16S rRNA genes, but there was relatively little recovery beyond the second extraction for soil loads of up to 250 mg.

DISCUSSION

In DNA-based SIP experiments with soil, it is important to recover as much of the ^{13}C-labeled DNA as possible. Feinstein et al. (10) recently demonstrated that multiple extractions of

an uncontaminated forest soil led to shifts in the relative abundance of various phyla and in operational taxonomic unit (OTU) composition in pyrosequence libraries between the first and sixth successive DNA extracts. The efficiency of DNA extraction kits used to recover DNA from SIP experiments has not been determined. We performed multiple, successive DNA extractions of 500-mg aliquots of PAH-contaminated soil that was enriched with anthracene to determine whether multiple extractions would affect DNA yield, eubacterial 16S rRNA gene recovery, or the identification of bacteria associated with anthracene degradation by SIP. Compared to a single DNA extraction, multiple DNA extractions maximized DNA yield and the recovery of 16S rRNA genes. Although an initial DGGE profile of each extract suggested that DNA from additional bacteria may have been extracted in later extractions, clone libraries with adequate sequence coverage (see Fig. S5 in the supplemental material) did not support this observation (Table 2). Successive DNA extractions did not result in the identification of additional bacterial groups beyond those identified in the first DNA extraction (Table 2), but there was a significant shift in the relative abundance of the identified genera across successive extracts, specifically from extract 1 to pooled extracts 5 to 7 ($P \leq 0.002$). Overall, a group of bacteria within the *Sphingomonadales*, but not similar to any known genus (designated "anthracene group 1"), and members of the *Pigmentiphaga* and *Variovorax* genera were newly associated with anthracene degradation as a result of this study.

Assessment of anthracene-degrading microbial community activity. The activity of the anthracene-degrading microbial community native to the PAH-contaminated soil sample was assessed by measuring parent anthracene removal and radiolabeled anthracene mineralization. Because [1,2,3,4,4a,9a-^{14}C]anthracene is labeled on only one of the two end rings of the symmetrical anthracene molecule, the actual amount of mineralization should be at least twice the accumulation of ^{14}CO$_2$ measured; thus, at least 25% of the anthracene was mineralized over the first 3 days, when the majority of anthracene was removed (Fig. 1). We infer that this would have been the period during which the majority of growth and assimilation of ^{13}C from the parent compound would have occurred during incubations with ^{13}C-labeled anthracene. However, mineralization continued through day 20 (Fig. 1), which might be attributed to the transformation of at least some of the anthracene to one or more extracellular metabolites over the first 3 days, followed by slower mineralization of such metabolites; continuing mineralization between day 3 and day 20 could also be due in part to turnover of previously assimilated carbon. Termination of the SIP experiment at a time point earlier than 20 days would have reduced the chances for dilution of ^{13}C in cellular macromolecules, but allowing enough incubation time for adequate initial labeling of the DNA is also important to consider. In our experience, termination of the SIP incubation once reduced microbial activity is observed (as indicated by a reduced mineralization rate) is a reasonable compromise.

Identification of anthracene-degrading bacteria. Substantial growth occurred over the first 3 days of incubation in the presence of unlabeled anthracene for all of the bacteria identified in putative heavy DNA from the SIP incubations with ^{13}C-labeled anthracene, other than those related to *Sphingo-*

bium (Fig. 4). *Acidovorax* organisms known to be well represented in the soil (17), but not found in the clone libraries representing DNA in heavy fractions from the SIP experiment, grew slightly over this period as well; however, the increase in abundance of *Acidovorax*-related sequences was significantly less than that of all the SIP-identified groups other than *Sphingobium*. The increases in abundance of sequences related to AG1 and *Pigmentiphaga* exclusively during the period of anthracene removal in the incubation with unlabeled anthracene (Fig. 4), combined with evidence for ^{13}C enrichment of their DNA in SIP incubations with [^{13}C]anthracene (Fig. 5), suggests that these organisms grew on anthracene itself as opposed to an extracellular metabolite derived from anthracene.

Increases in abundance during the incubation with unlabeled anthracene continued to occur after day 3 for sequences related to *Variovorax*, *Herminiimonas*, and *Sphingobium* (Fig. 4), which may have resulted from growth on any anthracene metabolite(s) that had accumulated over the first 3 days or from slow growth on substrates unrelated to anthracene. Of these three groups, only *Variovorax* showed any evidence of ^{13}C enrichment of its DNA during the SIP experiment with ^{13}C-labeled anthracene (Fig. 5). Although DNA from *Variovorax*-related sequences shifted to heavier fractions in SIP incubations, these sequences were spread over a wider range of fractions than that observed for sequences representing AG1 or *Pigmentiphaga* organisms. Combined with the continued increase in abundance of *Variovorax* sequences after the period over which anthracene had been removed (Fig. 4), these data suggest that assimilated ^{13}C in *Variovorax* DNA was diluted with unlabeled carbon over the 20-day incubation period of the SIP experiment.

Unlike *Variovorax* sequences, neither *Herminiimonas*- nor *Sphingobium*-related sequences showed evidence of ^{13}C enrichment of their DNA in the SIP experiment (Fig. 5). The presence of *Herminiimonas* sequences in the clone libraries representing heavy DNA from the SIP experiment (Table 2) and their significant increase in abundance during the period in which anthracene removal occurred (Fig. 4) are, therefore, puzzling; nevertheless, we cannot conclude that these organisms grew on anthracene. Sequences 99.4% similar to the *Herminiimonas*-related sequences identified in this study were associated with a phenanthrene-degrading isolate recovered from PAH-contaminated soil from a former coal gasification plant in Iowa City, IA (39), but members of the genus have not been associated with anthracene degradation.

Although *Sphingobium*-related sequences increased in abundance slightly after 3 days of incubation with unlabeled anthracene (Fig. 4), the greatest increase occurred after the added anthracene had been consumed. This observation, combined with the lack of evidence for assimilation of ^{13}C, suggests that *Sphingobium*-related bacteria grew on something other than anthracene. The presence of *Sphingobium*-related sequences in putative heavy DNA from the SIP experiment (Table 2) is likely due to their high initial abundance in the soil as well as their very high abundance at the 20-day time point, corresponding to the termination of the SIP experiment (Fig. 4). *Sphingobium* spp. have previously been associated with the degradation of PAHs composed of up to 5 rings (14, 21, 29), but only cooxidation of anthracene by a *Sphingobium* species has been reported (29).

Of the organisms associated with anthracene degradation by SIP, organisms related to AG1 have previously been associated with hydrocarbon-contaminated environments but not specifically with anthracene degradation. Sequences representing AG1 are 98.6% similar to sequences previously recovered from the Rancho La Brea Tar Pits in Los Angeles, CA (20). *Variovorax* spp. have previously been associated with the degradation of biphenyl (23) and naphthalene (15, 27) but not with anthracene degradation. In the only previous study to associate the genus *Pigmentiphaga* with PAH contamination, 16S rRNA gene sequences that were >97% identical to those of *Pigmentiphaga kullae* were recovered from creosote-contaminated soil, but an isolate was not recovered to be tested for specific PAH utilization (21, 27).

Effect of multiple DNA extractions. Similar to the findings of Feinstein et al. (10), multiple DNA extractions did not result in the identification of additional bacterial groups compared to those identified from a single extraction, but there were shifts in relative abundance across the range of extracts. However, a single extraction might have resulted in a much lower representation of sequences belonging to AG1 than that which we observed from the pooled extracts (23% versus 43%, respectively) (Table 2; Fig. 3). The DNA recovered from single extractions of multiple aliquots of soil from the same site is often pooled to reduce the potential effects of extraction bias with such heterogeneous material, but pooling of DNA will also increase the amount of total DNA recovered. Testing of the efficiency of the FastDNA spin kit for soil (see Fig. S6 in the supplemental material) revealed that a single extraction of the maximum 500-mg soil load recommended by the manufacturer was not optimum for the recovery of genomic DNA or 16S rRNA genes from a PAH-contaminated soil. It is likely that at this soil load, natural organic matter and/or contaminants in the soil that were coextracted with DNA resulted in competition for binding sites on the solid-phase sorbent and may also have interfered with PCR of the eluted material (24). We cannot extrapolate our observations to other soils, but we recommend that DNA extraction efficiency as a function of the soil load be determined before extensive experimentation with any given soil and DNA extraction kit.

The benefits of performing multiple DNA extractions on a sample will depend on the downstream applications of the DNA. In an SIP investigation, it is important to maximize DNA recovery, particularly if the heavy DNA in the metagenome is only partially labeled. Partial labeling leads to weaker separation from unlabeled DNA during ultracentrifugation than if the DNA were nearly 100% enriched in ^{13}C. Overall, this study demonstrated that optimizing the recovery of ^{13}C-enriched DNA from an SIP experiment may represent a compromise between the length of the incubations, the number of successive DNA extractions, and the number of soil aliquots for a given soil load.

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