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Effect of Model Sorptive Phases on Phenanthrene Biodegradation: Molecular Analysis of Enrichments and Isolates Suggests Selection Based on Bioavailability

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Reduced bioavailability of nonpolar contaminants due to sorption to natural organic matter is an important factor controlling biodegradation of pollutants in the environment. We established enrichment cultures in which solid organic phases were used to reduce phenanthrene bioavailability to different degrees (R. J. Grosser, M. Friedrich, D. M. Ward, and W. P. Inskeep, Appl. Environ. Microbiol. 66:2695–2702, 2000). Bacteria enriched and isolated from contaminated soils under these conditions were analyzed by denaturing gradient gel electrophoresis (DGGE) and sequencing of PCR-amplified 16S ribosomal DNA segments. Compared to DGGE patterns obtained with enrichment cultures containing sand or no sorptive solid phase, different DGGE patterns were obtained with enrichment cultures containing phenanthrene sorbed to beads of Amberlite IRC-50 (AMB), a weak cation-exchange resin, and especially Biobead SM7 (SM7), a polyacrylic resin that sorbed phenanthrene more strongly. SM7 enrichments selected for mycobacterial phenanthrene mineralizers, whereas AMB enrichments selected for a *Burkholderia* sp. that degrades phenanthrene. Identical mycobacterial and *Burkholderia* 16S rRNA sequence segments were found in SM7 and AMB enrichment cultures inoculated with contaminated soil from two geographically distant sites. Other closely related *Burkholderia* sp. populations, some of which utilized phenanthrene, were detected in sand and control enrichment cultures. Our results are consistent with the hypothesis that different phenanthrene-utilizing bacteria inhabiting the same soils may be adapted to different phenanthrene bioavailabilities.

We hypothesize that some contaminant-degrading microorganisms have evolved specialization to low-bioavailability microenvironments that occur due to the propensity of nonpolar contaminants to adsorb strongly to natural organic matter (NOM). Most previously cultivated contaminant-degrading bacteria have been isolated under selection conditions that do not mimic such microenvironments. As a result, they may not exhibit the properties associated with such specialization that may be important for in situ bioremediation. In the accompanying paper (9a), we describe new enrichment strategies that simulated such microenvironments by selecting for microorganisms capable of metabolizing a model nonpolar contaminant. Phenanthrene was presorbed to model organic solids, such as the carboxylic acid cation-exchange resin Amberlite IRC-50 (AMB) (sorption coefficient [log K_D] = 2.99 liters kg^{-1}) and the polyacrylate-based resin Biobead SM7 (SM7) (log K_D = 3.47 liters kg^{-1}), that reduced its bioavailability to different degrees in the range of bioavailabilities observed with soil NOM (log K_D = 2.5 to 3.5 liters kg^{-1}). We used this strategy to enrich phenanthrene-degrading microorganisms from two contaminated soils in order to evaluate whether similar microbial populations were recovered from geographically distant sites when the same selection pressure was used. Phenanthrene degradation was slower in enrichment cultures containing organic solids than in controls containing sand or no sorptive phase. AMB reduced bioavailability to a lesser extent than did SM7. It was found that an isolate from SM7

enrichment cultures exhibited higher relative rates of metabolism of sorbed phenanthrene than did isolates from enrichment cultures without sorptive phases, suggesting that different microbial populations were selected under different phenanthrene bioavailability conditions.

In this study, we determined the compositions of the microbial assemblages that developed in the enrichment cultures by using cultivation-independent molecular tools (i.e., analysis of the 16S ribosomal DNA [rDNA] gene, a universal genetic marker). The application of molecular biology methods to microbial ecology has proven that the naturally occurring rRNA sequences differ from the rRNA sequences of species cultivated from the same habitat (7, 8, 11, 24, 28–30), in part due to a mismatch between adaptations of the native species and the selective nature of the culture methods (23). A cultivation-independent molecular approach for community structure analysis also facilitates detection of microorganisms that are difficult to cultivate or that cannot be cultivated with our current understanding of microbial growth requirements. Consequently, we monitored changes in the compositions of our phenanthrene enrichment cultures by denaturing gradient gel electrophoresis (DGGE) analysis of PCR-amplified 16S rDNA gene segments. DGGE separates double-stranded DNA segments of equal length based on sequence differences (17, 18). Although the segments were only a few hundred nucleotides long and this may have limited resolution of closely related molecules, the resulting DGGE band patterns facilitated detection of differences among the microbial communities in our different enrichment cultures. In addition, individual bands from DGGE profiles can be directly sequenced to identify populations by their 16S rDNA sequences (4, 16). As 16S rRNA gene sequence data do not permit inferences concerning a microbial population's ability to metabolize phenan-

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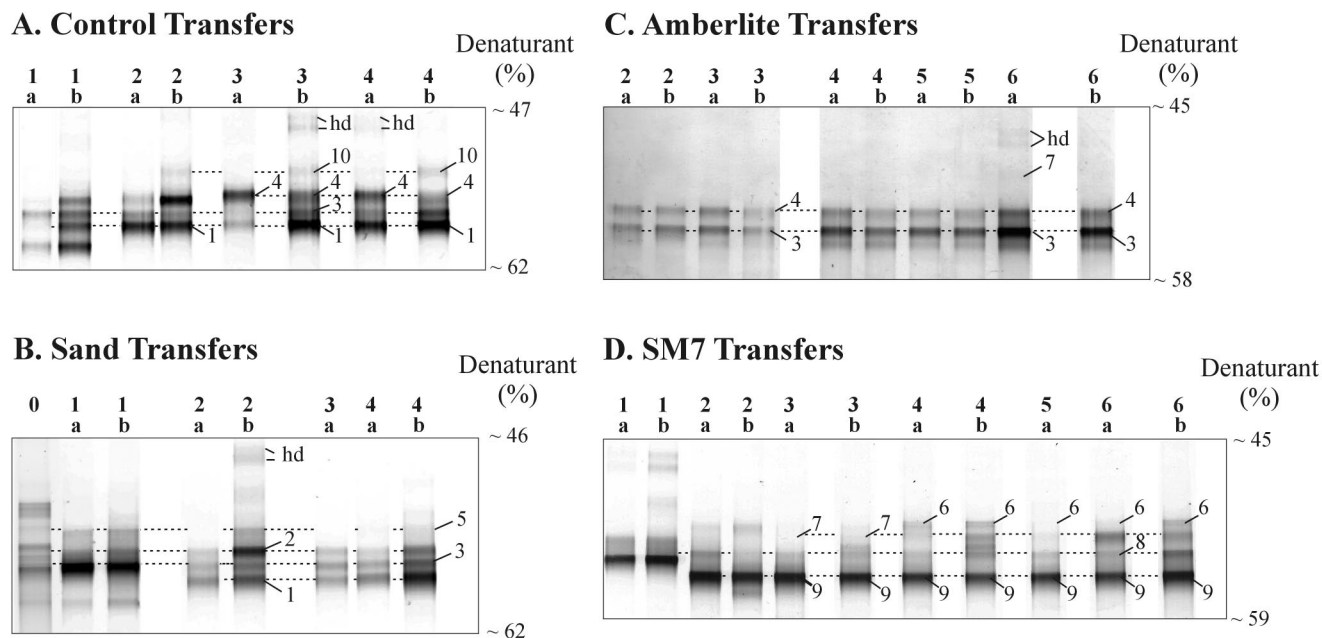†Present address: NRMRL, US EPA, Cincinnati, OH 45268.

FIG. 1. DGGE analysis of PCR-amplified 16S rRNA gene segments from replicates (labeled a and b) after sequential transfers (numbers above the lanes) of enrichment cultures inoculated with Dover, Ohio, soil. (A) Control. (B) Sand. (C) AMB. (D) SM7. The band numbers correspond to those in Tables 1 and 2. The dashed lines are included to help visualize comigrating bands. hd, heteroduplex bands.

threne, we also attempted to cultivate the populations present in the enrichment cultures (9a). Molecular analyses, as well as cultivation, revealed differences in the species compositions of enrichment cultures with different phenanthrene bioavailabilities, especially where bioavailability was most reduced. The ecological relevance of contaminant availability for selection of specialized microbial populations is discussed below.

<center>MATERIALS AND METHODS</center>

Enrichments in the presence of model organic phases. Microorganisms from hydrocarbon-contaminated coal gasification plant (Dover, Ohio) and creosote-contaminated (Libby, Mont.) soils were enriched on [9-^{14}C]phenanthrene presorbed to model organic phases AMB and SM7, as described in detail in the accompanying paper (9a). Parallel control enrichment cultures contained equivalent amounts of [^{14}C]phenanthrene and either sand or no sorptive phase. Conversion of [^{14}C]phenanthrene to $^{14}CO_2$ was monitored, and enrichment cultures were transferred when $^{14}CO_2$ evolution began to reach a plateau. Samples (50 ml) of enrichment cultures were used for cultivation or were frozen, and subsequently they were used for molecular characterization.

DNA extraction. Frozen samples of enrichment cultures or pure cultures obtained from them (9a) were quickly thawed in a water bath at 30°C and immediately placed on ice. Samples (2 ml) containing model solids and mineral medium were transferred to 2-ml screw-cap tubes. Cells and beads were separated from the medium by centrifugation for 5 min at $14,000 \times g$. Subsequently, cells were lysed with an FP120 FastPrep cell disruptor (Savant Instruments Inc., Farmingdale, N.Y.). Between 1 and 1.8 g of oven-baked 0.1-mm-diameter zirconium beads, 800 μl of 120 mM sodium phosphate buffer (pH 8.0), and 260 μl of 0.5 M Tris-HCl (pH 8.0)–0.1 M NaCl–10% sodium dodecyl sulfate were added prior to bead beating at 6.5 m s^{-1} for 45 s. After centrifugation for 5 min at $14,000 \times g$, 700 μl of supernatant was removed, and the DNA was purified by ammonium acetate precipitation (14), followed by standard isopropanol precipitation (0.7 volume) for 30 min. The DNA was dissolved in 100 μl of distilled H$_2$O and analyzed by standard agarose gel electrophoresis. Samples from earlier transfers containing larger amounts of soil inoculum were subjected to a spin column purification step (Qiamp blood kit; Qiagen Inc., Chatsworth, Calif.) according to the manufacturer's instructions for crude cell lysates.

PCR, DGGE, and sequencing of DGGE bands and pure-culture 16S rRNA genes. Prior to DGGE analysis, PCR was carried out as described previously (4). Briefly, the 16S rDNA gene was amplified between positions 1055 and 1406 (*Escherichia coli* numbering), a segment which included some hypervariable regions. It has been shown that the primers which we utilized (primers 1070F and 1392RGC) recover 16S rRNA genes from diverse members of the domain *Bacteria* under the PCR conditions used in this analysis (32). For pure cultures the almost complete 16S rRNA gene was amplified with primers 27F and 1492R (32). To obtain better band resolution in DGGE gels, 0.75-mm gels (35 to 80% denaturant solution) were employed. For sensitive band detection the gels were stained with SYBR green (Molecular Probes, Eugene, Oreg.) as recommended by the manufacturer and photographed. The photographs of DGGE gels were scanned and converted to negative images. Samples were obtained from individual DGGE bands by removing a small gel core with a sterile 200-μl pipette tip; the core was transferred to a tube containing 150 μl of sterile H$_2$O and incubated overnight at 4°C to allow diffusion of the PCR product out of the gel core. A 0.5- to 1-μl portion of supernatant was used to reamplify the DGGE bands with primers 1070F and 1392RGC, and subsequently the PCR products were reanalyzed by DGGE to verify that bands were pure. Pure DGGE bands and PCR products from pure cultures were sequenced with either an ABI 373A sequencer (Applied Biosystems, Foster City, Calif.) at the Murdock Molecular Biology Facility (University of Montana, Missoula) by using primers 1114F and 1368R, as described elsewhere (5), or an ABI 377 sequencer at Medigenomix Sequencing Service (Martinsried, Germany) by using primers 27F and 1492R. Band sequences were considered unique only if there was unambiguous evidence of sequence difference.

Sequences were compared with sequences in the Ribosomal Database Project (RDP) (http://www.cme.msu.edu/RDP/) 16S rDNA database (release 7.0, 15 July 1998) by using the Similarity_Rank and Check_Chimera software (12) and with GenBank sequences by using BLAST software (2). Our 16S rDNA DGGE band and pure-culture sequences were aligned with closely related 16S rDNA sequences from the RDP and GenBank databases by using the Genetic Data Environment or the ARB software package (version 2.5b; O. Strunk and W. Ludwig, Technische Universität München, Munich, Germany; http://www.biol .chemie.tu-muenchen.de/pub/ARB/), and percent similarity to other sequences was determined.

<center>RESULTS</center>

Stabilization of enrichment culture DGGE patterns. When contaminated soils were analyzed directly by DGGE, they typically produced a smear, which we interpreted as a high level of biodiversity. In contrast, DGGE analysis of enrichment cultures resulted in less complex patterns even after just one transfer, as shown in Fig. 1. Relatively stable DGGE band patterns were observed with subsequent transfers, with only minor band position and intensity differences between duplicates and transfers. This indicated the development of a stable and less diverse set of populations. For instance, for the control

enrichment culture (Fig. 1A) the DGGE band patterns changed somewhat in early transfers, but by the third or fourth transfer, four distinct bands (bands 1, 3, 4, and 10) were consistently detected. Similarly, for the sand enrichment culture (Fig. 1B) three distinct bands (bands 1 through 3) were consistently detected after two to four transfers, and a fourth band (band 5) was sometimes observed. For the AMB enrichment culture (Fig. 1C), the patterns were stable after only two transfers, and bands 3 and 4 were consistently detected. For the SM7 enrichment culture (Fig. 1D) the patterns were stable after four to six transfers, and bands 6 through 9 were consistently detected. The labeled bands are those that were actually purified and sequenced; the dashed lines in Fig. 1 indicate the band positions relative to the positions of comigrating bands that were not sequenced and also emphasize that in most cases different band patterns were obtained for early and late transfers. Identical sequences were obtained for comigrating bands after various transfers, increasing our confidence that comigrating bands were likely to have the same sequences. The bands were not numbered consecutively because the numbers reflect the phylogenetic organization of sequences observed in DGGE bands obtained with various enrichment cultures (Table 1) (see below). A few DGGE bands (bands hd) were identified as heteroduplex artifacts based on the fact that reamplification yielded four products, two of which migrated very high in the gels and two of which comigrated with other bands that migrated farther in the gels (4). This was probably the result of a very high template concentration combined with a high sequence similarity of the two bands.

Figure 2 shows a comparison of DGGE patterns of different enrichment cultures after a number of transfers sufficient to achieve stable assemblages. Small differences between comparable lanes in Fig. 1 and Fig. 2 were due to the fact that the data shown in Fig. 2 resulted from independent gel analyses. The band patterns of the control and sand enrichment cultures were similar, though there were differences in both band composition and intensity. Although the AMB enrichment culture appeared to produce bands that comigrated with some of those produced by the control and sand enrichment cultures (e.g., bands 3 and 4), there was an obvious difference in the most intense band (band 3), band 1 was absent, and a unique band (band 7) was detected. The SM7 enrichment culture produced three unique bands (bands 6, 8, and 9) and was most obviously different from the other enrichment cultures.

Sequences of DGGE bands in stabilized enrichment cultures inoculated with Dover soil. Because all DGGE bands from all enrichment cultures migrated to a narrow section of the denaturing gradient gel (47 to 55% denaturant), even on gels with narrower gradients, it was difficult to determine whether bands actually comigrated. Moreover, different sequences can migrate to the same location on a DGGE gel (see below). Therefore, we sequenced individual bands purified from denaturing gradient gels for a precise determination and comparison of the 16S rRNA genes. For most DGGE bands, the PCR products had to be subjected to multiple purification cycles (extraction from the denaturing gradient gel, PCR, DGGE) to obtain pure bands. We successfully sequenced all DGGE bands that were detected in stabilized enrichment cultures; the sequences were identified in terms of their closest database relatives, and closely related sequences were compared to each other (Table 1).

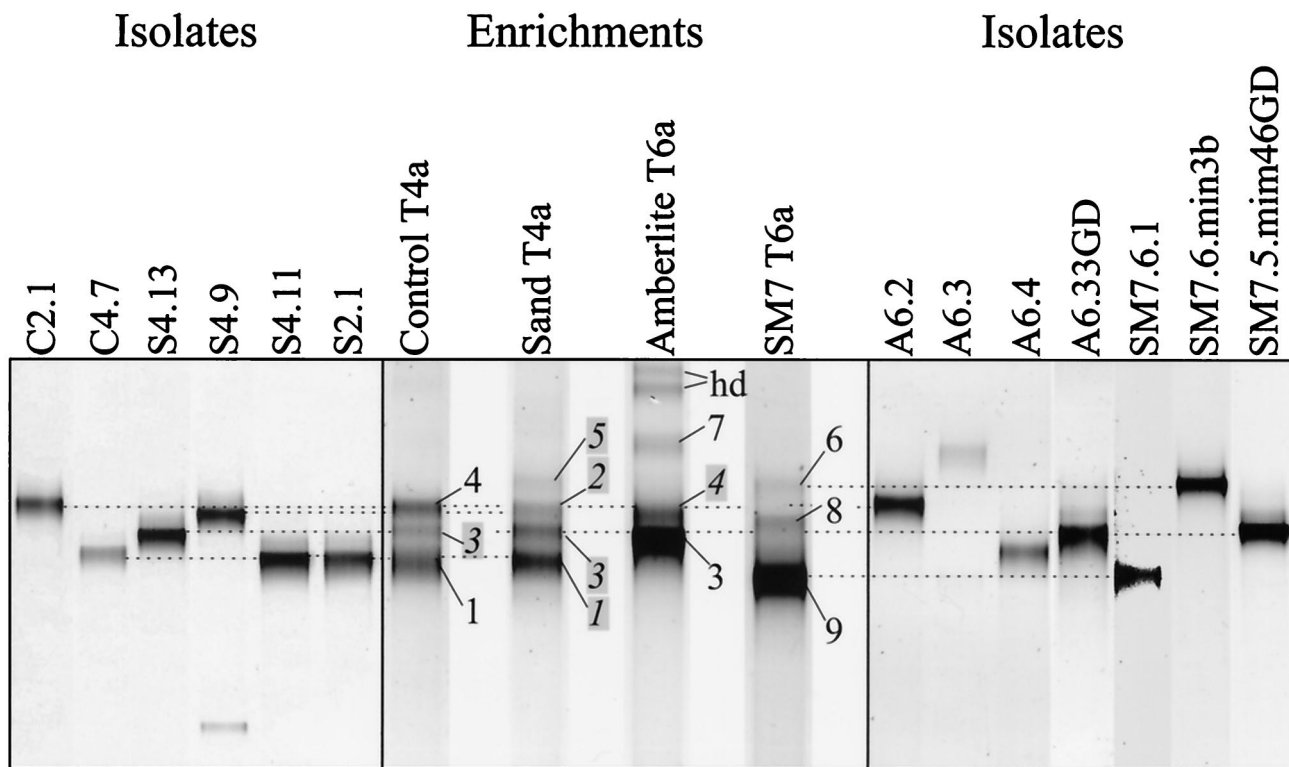Bands 1, 2, and 3, which were commonly obtained with the

FIG. 2. Comparison of the DGGE profiles of PCR-amplified 16S rDNA segments from isolates to those obtained in the enrichment cultures from which the isolates were cultivated. The band numbers correspond to those in Fig. 1 and Tables 1 and 2. The dashed lines indicate possible comigration. The positions of bands whose numbers are highlighted and italicized were inferred based on comigration with bands in earlier transfer preparations that were actually sequenced (Fig. 1).

TABLE 1. Phylogenetic analysis of 16S rRNA sequences of DGGE bands detected in stable enrichment cultures

DGGE band[a]	Closest RDP/GenBank relative[b]		% Similarity to closest relative[c]	% Sequence similarity to[c]:					
	Phylogenetic group	Strain or species		DGGE band 1	DGGE band 2	DGGE band 3	DGGE band 4	DGGE band 5	DGGE band 6
1	β-*Proteobacteria*	*Burkholderia* sp. strain N3P2	99.6						
2	β-*Proteobacteria*	*B. glathei*	99.4	96.7					
3	β-*Proteobacteria*	*Burkholderia* sp. strains N3P2 and N2P5	100	99.6	97.1				
4	β-*Proteobacteria*	*B. cepacia*	98.1	96.3	94.7	96.7			
5	β-*Proteobacteria*	*R. solanacearum*	99.2	92.6	93.4	93.0	94.7		
6	β-*Proteobacteria*	*R. solanacearum*	100	91.8	93.4	92.2	93.9	99.2	
7	β-*Proteobacteria*	*M. methylotrophus*	96.3	88.1	89.8	88.5	89.8	88.5	87.7
8	α-*Proteobacteria*	*A. lipoferum*	98.8						
9	Gram-positive bacteria	*M. gilvum*[d]	100						
10	*Chlamydiales*	*Chlamydia* sp.	96.0						

[a] The band numbers correspond to DGGE band numbers in the figures. The GenBank accession numbers for the DGGE band nucleotide sequences are AF247476 to AF247485, respectively.

[b] The GenBank accession numbers for the closest database relatives are as follows: *Burkholderia* sp. strain N3P2, U37344; *Burkholderia* sp. strain N2P5, U37342; *B. glathei*, Y17052; *B. cepacia*, M22518; *R. solanacearum*, X67035; *M. methylotrophus*, L15475, *A. lipoferum*, Z29619; *M. gilvum*, X55599; and *Chlamydia* sp. Y07556.

[c] DGGE band sequences were compared by using *E. coli* 16S rRNA positions 1115 to 1367.

[d] *M. gilvum* has a 16S rRNA sequence identical to that of *M. chitae* and *M. smegmatis* in the region used for DGGE analysis.

stabilized control, sand, and AMB enrichment cultures (Fig. 2), had sequences that were ≥96.7% similar to each other in the region analyzed (Table 1). These sequences were closely related or identical in this region to the sequences of several members of the β subclass of the class *Proteobacteria* (β-*Proteobacteria*), including *Burkholderia* sp. strains N3P2 and N2P5, which are polycyclic aromatic hydrocarbon (PAH)-mineralizing isolates from creosote-contaminated Norwegian soils and have identical sequences in the region analyzed by DGGE (15), and *Burkholderia glathei*, an isolate from vertisol microaggregates (1). The sequence of band 4, which was also obtained with control and AMB enrichment cultures, was slightly less closely related to the sequences of bands 1, 2, and 3 and was closely related to the sequence of *Burkholderia cepacia*. Although there was just one unambiguous base difference between the *Burkholderia* sp. strain N3P2- and N2P5-like sequences of bands 1 and 3 in the region analyzed (verified by sequences from more than one DGGE band that migrated to the same position [Fig. 1 and 2]), there was a difference in the distribution of these bands among stable enrichment cultures. Band 3 was the most intense band detected in the AMB enrichment culture, whereas band 1 (which was not detected in the AMB enrichment culture) was the most intense band detected in the control and sand enrichment cultures (Fig. 2). The lack of consistent co-occurrence of bands 1 and 3 is important, as it suggests a difference in selective pressure between the AMB and control or sand enrichments for unique *Burkholderia* sp. populations. The difference cannot be attributed to changes in the expression of different 16S rRNA operons within one organismic population (19), because we analyzed genes and not the 16S rRNA itself (see below).

SM7 enrichments provided the strongest evidence of population selection. The most intense band (band 9) had a sequence identical to those obtained for the gram-positive bacteria *Mycobacterium gilvum*, *Mycobacterium chitae*, and *Mycobacterium smegmatis*. These three species have 16S rRNA sequences that are ≥96.5% similar overall but are identical in the region used for DGGE analysis.

Several other less intense bands were obtained only in AMB and/or SM7 enrichment cultures, providing further evidence of population selection. These bands had sequences closely related to those of other β-*Proteobacteria* (*Ralstonia solanacearum* [bands 5 and 6] and *Methylophilus methylotrophus* [band 7]) and α-*Proteobacteria* (*Azospirillum lipoferum* [band 8]). Interestingly, the *R. solanacearum*-like sequence (band 5) obtained only in sand enrichment cultures was different (two unambiguous base differences) from that obtained in SM7 enrichment cultures (band 6). The control enrichment culture produced one unique band (band 10) (Fig. 1), with a sequence related to that of a *Chlamydia* sp.

Bacteria isolated from enrichment cultures. We cultivated bacteria from the various enrichment cultures by using standard techniques (9a) in an attempt to link the 16S rRNA gene segments identified as DGGE bands with the abilities of the populations contributing these genes to metabolize phenanthrene. As shown in Fig. 2, many isolates exhibited DGGE bands that comigrated with bands detected in the enrichment cultures. Table 2 shows the sequence identity between 16S rRNA sequences of isolates and DGGE bands and indicates whether an isolate was capable of phenanthrene degradation. The closest RDP/GenBank relative shown in Table 2 is not always identical to the closest relative based on the corresponding DGGE band in Table 1 because nearly full-length sequence data were used to prepare Table 2. Nearly full-length sequence data also permitted a higher-resolution comparative analysis of closely related strains.

Based on both comigration and sequence identity data, the most prominent DGGE bands detected in the enrichment cultures were associated with phenanthrene-oxidizing bacterial isolates obtained from high-dilution platings of the enrichment cultures. For instance, a band produced by isolate SM7.6.1, a close relative of *Mycobacterium* sp. strain HE-5, a bacterium that degrades the heterocyclic xenobiotic compound morpholine (25), corresponded to DGGE band 9, the most prominent band detected in SM7 enrichment cultures. Similarly, AMB isolate A6.33GD, which was identical in the region analyzed to *Burkholderia* sp. strain N2P5, corresponded to DGGE band 3, the most prominent band detected in the AMB enrichment culture. The situation was more complex with respect to DGGE band 1, the most intense band detected in control and sand enrichment cultures. The DGGE bands of control isolate C4.7 and sand isolate S4.11 both comigrated with and had sequences identical to that of *Burkholderia* sp. strain N3P2-like band 1. However, these isolates had sequences that were 1.5% different due to 20 unambiguous nucleotide differences outside the region analyzed by DGGE (Table 2). Isolate C4.7 was most closely related to *Burkholderia caryophylli* MCII-8, whereas isolate S4.11 most closely resembled *Burkholderia* sp. strain

TABLE 2. Phenanthrene utilization and phylogenetic properties of isolates and correlation with DGGE bands

Representative isolate[a]	Phenanthrene use[b]	Identical DGGE band[c]	Closest RDP/GenBank relative[d]		% Similarity to closest relative[e]	% Sequence similarity to[e]:				
			Phylogenetic group	Strain or species		C4.7	S4.11	S4.9	A6.33 GD	S2.1
C4.7	+	1	β-*Proteobacteria*	*B. caryophylli* MCII-8	98.7					
S4.11	+	1	β-*Proteobacteria*	*Burkholderia* sp. strain N2P5	98.5	98.5				
S4.9	−	2	β-*Proteobacteria*	*B. glathei*	97.6	96.5	96.5			
A6.33GD	+	3[f]	β-*Proteobacteria*	*Burkholderia* sp. strain N2P5	100	97.6	98.5	96.3		
S2.1	+		β-*Proteobacteria*	*Burkholderia* sp. strain DhA54	97.5	96.9	96.8	96.4	97.2	
A6.2	−	4[g]	β-*Proteobacteria*	*B. caryophylli* ATCC 25418	97.7	95.8	95.9	96.4	96.6	97.8
SM7.6.min.3b	−	6	β-*Proteobacteria*	*R. solanacearum*	100					
A6.4	−		γ-*Proteobacteria*	*F. aurantia*	97.0					
SM7.6.1	+	9	Gram-positive bacteria	*Mycobacterium* sp. strain HE-5	99.1					
A6.3	−		Gram-positive bacteria	*B. megaterium*	99.4					

[a] C, isolate from a stabilized control enrichment culture; S, isolate from a sand enrichment culture; A, isolate from an AMB enrichment culture; SM7, isolate from an SM7 enrichment culture. The number after an initial letter is the transfer number. The number after the period is the isolate number. GD, isolate obtained from ground beads by using SSE as described in the accompanying paper (9a); min, isolate obtained from an SSE agar plate sprayed with phenanthrene. The GenBank accession numbers for the isolates are as follows: C4.7, AF247493; S4.11, AF247495; S4.9, AF247496; A6.33GD, AF247492; S2.1, AF247494; A6.2, AF247491; SM7.6.min3b, AF247498; A6.4, AF247489; SM7.6.1, AF247497; and A6.3, AF247490.

[b] +, isolate grew on phenanthrene in SSE and/or converted [^{14}C]phenanthrene to ^{14}CO$_2$; −, isolate did not grow on phenanthrene in SSE or convert [^{14}C]phenanthrene to ^{14}CO$_2$.

[c] The band numbers correspond to labeled DGGE band numbers in all figures and Table 1.

[d] GenBank accession numbers for the closest database relatives are as follows: *Burkholderia* sp. strain N2P5, U37342; *Burkholderia* sp. strain DhA-54, AJ011508; *B. glathei*, Y17052; *B. caryophylli* MCII-8, U91570; *B. caryophylli* ATCC 25418, X67039; *R. solanacearum*, X67035; *F. aurantia*, AJ010481; *Mycobacterium* sp. strain HE-5, AJ012738; and *B. megaterium*, D16273.

[e] Determined by using nearly full-length sequences (1413 to 1451 bases).

[f] Other isolates whose sequences corresponded to DGGE band 3 were obtained from sand and SM7 enrichment cultures (e.g., S4.13 [GenBank accession no. AF247487] and SM7.5.min46GD [GenBank accession no. AF247486] [Fig. 2]).

[g] Other isolates whose sequences corresponded to DGGE band 4 were obtained from the control enrichment culture (e.g., C2.1 [GenBank accession no. AF247488] [Fig. 2]).

N2P5. Furthermore, the DGGE band of another phenanthrene-degrading *Burkholderia* sp. strain DhA54-like isolate, S2.1, comigrated with band 1, even though its sequence did not match that of band 1 (Table 2).

Several isolates which were unable to degrade phenanthrene and which were obtained from low-dilution platings had 16S rRNAs corresponding to less intense DGGE bands obtained with enrichment cultures. For instance, the 16S rRNA of *B. glathei*-like sand isolate S4.9 corresponded to DGGE band 2 detected in sand enrichment cultures, the 16S rRNA of *B. caryophylli*-like AMB isolate A6.2 corresponded to DGGE band 4 obtained with control and AMB enrichment cultures, and the 16S rRNA of *R. solanacearum*-like SM7 isolate SM7.6.min.3b corresponded to DGGE band 6 detected in the SM7 enrichment culture. Two AMB isolates that did not degrade phenanthrene, *Frateuria aurantia*-like isolate A6.4 and *Bacillus megaterium*-like isolate A6.3, exhibited DGGE band patterns that did not match those of enrichment cultures (Fig. 2).

The six *Burkholderia* isolates exhibited ≥95.8% similarity in their nearly full-length 16S rRNA sequences (Table 2), despite differences in their abilities to metabolize phenanthrene and in their distribution among the various enrichment cultures.

Comparison of enrichment cultures inoculated with Libby and Dover soils. A DGGE analysis of enrichment cultures inoculated with Libby soil resulted in intense DGGE bands with mobilities similar to those of bands produced by Dover soil enrichment cultures (Fig. 3). AMB enrichment cultures obtained with both soils resulted in intense comigrating bands with identical sequences most closely related to those of *Burkholderia* sp. strains N3P2 and N2P5 (i.e., identical to the sequence of DGGE band 3). SM7 enrichment cultures obtained with both soils resulted in intense comigrating bands with sequences identical to the sequences of *M. smegmatis*, *M. chitae*, and *M. gilvum* (i.e., identical to the sequence of DGGE band 9).

DISCUSSION

In the accompanying paper (9a), we describe model enrichment cultures used to evaluate the selection of phenanthrene-utilizing bacteria under different phenanthrene bioavailability conditions. Model organic solids, specifically AMB, a polystyrene-based weak cation exchanger with carboxylic acid functionality, and SM7, a polyacrylic acid ester, were used to successively reduce bioavailability compared to controls that contained sand or no sorbing phase. Other conditions that might have affected selection (e.g., soil inoculum, medium, and incubation conditions) were held constant. The correspondence between molecular and cultivation methods used to analyze bacteria present in the enrichment cultures was reasonable considering the usual incongruence of these approaches when they are applied to natural samples (29). This correspondence was presumably due to direct plating from enrichment cultures, which must have eliminated competitors present in
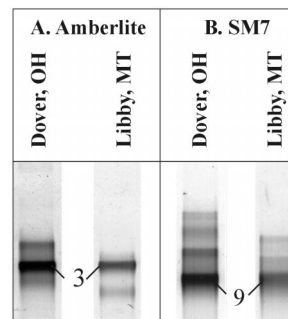

FIG. 3. Comparison of DGGE profiles of PCR-amplified 16S rRNA gene segments from stabilized AMB (A) and SM7 (B) enrichment cultures inoculated with soil from Dover, Ohio, or Libby, Mont. The band numbers correspond to those in Tables 1 and 2.

the soil that might have otherwise dominated our culture collection. The combined molecular and cultivation results suggested that the conditions which we used to reduce phenanthrene availability resulted in selection of phenanthrene-utilizing bacteria different from those found in controls.

The most obvious example of selection occurred in the SM7 treatment, which enriched for a mycobacterial population (DGGE band 9) that was capable of phenanthrene metabolism. In contrast, AMB, sand, and control enrichment cultures selected mostly for *Burkholderia* sp.-like phenanthrene-utilizing populations. An SM7 mycobacterial isolate (SM7.6.1) representative of DGGE band 9 exhibited 5- to 7.5-fold-greater relative rates of metabolism of phenanthrene bound to SM7 than did *Burkholderia* sp. isolates C4.7 and S2.1, which were representative of DGGE band 1 and dominated control and sand enrichment cultures (9a). This suggests that enrichment under low-bioavailability conditions selected for isolates that are better able to metabolize phenanthrene when its bioavailability has been reduced by sorption to organic solids.

The 16S rRNA sequences of our mycobacterial isolates from SM7 enrichment cultures were 98.6 and 96.8% similar to the 16S rRNA sequences of PAH-degrading mycobacteria obtained previously from other contaminated soils and sediments, respectively, such as *Mycobacterium* sp. strain PAH 135 or *Mycobacterium* sp. strain PYR-1(9). Despite these relatively high levels of similarity, we must leave open the possibility that the phenanthrene-degrading mycobacteria which we selected might be unique species with adaptations to low-bioavailability microenvironments. Differentiation of mycobacterial species by means of comparative 16S rDNA analysis has proven difficult even with identical or nearly identical full-length sequence data (21, 22, 31). We (29) and others (6, 20) have found that populations with closely related or even identical 16S rRNA sequences may be ecologically unique and may actually be unique species (27, 29).

Selection also occurred in the AMB enrichment cultures, which exhibited a level of phenanthrene bioavailability between those of SM7 cultures and control or sand enrichment cultures (9a). The most intense DGGE band produced by AMB enrichment cultures (band 3) represented a *Burkholderia* sp. strain N3P2- and N2P5-like population. Because of possible PCR biases, band intensity may (3) or may not (5) indicate that a population is dominant. However, the fact that we were able to cultivate a phenanthrene-oxidizing *Burkholderia* sp. population with the same sequence from high dilutions of AMB enrichment cultures suggests that this population was the dominant phenanthrene-metabolizing population enriched under these conditions. Isolates with this sequence were also recovered from sand and SM7 enrichment cultures but from lower dilutions, consistent with the weaker or undetectable band 3 produced by these enrichment cultures (Fig. 2). The most intense band produced by control and sand enrichment cultures (band 1, whose mobility and sequence were different from those of band 3) was also *Burkholderia* sp. strain N3P2-like. This band might have indicated that any or all of three different *Burkholderia* sp. isolates cultivated from control and/or sand enrichment cultures were present. Two of these isolates had sequences that matched that of DGGE band 1, while one sequence that did not match the DGGE band 1 sequence comigrated with band 1. This observation highlights two problems associated with DGGE analysis that may lead to underestimation of genetic diversity: (i) relatively small, identical, conserved sequence domains may be present in molecules with different full-length 16S rRNA gene sequences, and (ii) DGGE bands with different sequences may comigrate. In

our study it was necessary to cultivate phenanthrene-degrading bacteria in order to reveal limitations of DGGE.

The partial 16S rRNA sequences of bands 1 and 3 were highly related (Table 1), but the unique mobilities in DGGE and the larger differences in nearly full-length sequences of isolates with bands that matched bands 1 and 3 (Table 2) supported the hypothesis that the populations were different. As mentioned above, ecologically unique populations (i.e., species) may exhibit close phylogenetic relationships. The ecological differences among *Burkholderia* sp. populations which we observed may be reflected by their differential distributions and abundances under different bioavailability conditions and by the abilities of the populations to metabolize phenanthrene (i.e., isolates with a band that corresponded to band 2 did not oxidize phenanthrene). *Burkholderia* spp. known for their ability to degrade PAHs have been frequently isolated from soils (15). However, low bioavailability was not considered part of the isolation strategy, and abundance was considered in only a few studies (10). Our evidence of closely related yet ecologically distinct populations forced us to consider the possibility that, like mycobacterial isolates, our *Burkholderia* sp. isolates, even the ones that were 100% similar in the region analyzed to a previously described isolate (e.g., the nearly full-length sequence of our isolate A6.33GD was 100% similar to the sequence of isolate N2P5 of Mueller et al. [15]), could be unique with regard to utilization of phenanthrene under moderately low-bioavailability conditions.

The use of a small segment of a highly conserved genetic marker may also have limited our ability to observe differences among populations in soils from geographically distant locations. Hence, even though identical DGGE band sequences were detected under the same selection conditions when two distinct soils were used, we cannot eliminate the possibility that such differences might exist and might be detected by using a higher-resolution genetic approach. Mueller et al. (15), for instance, detected minor differences in nearly full-length 16S rRNA sequences of phenanthrene-degrading *Burkholderia* sp. isolates from different Florida and Norwegian sites; the Norwegian strains formed a separate phylogenetic (possibly geographic) cluster. The DGGE approach which we used did reveal that selection conditions, more than geographic location, controlled the enrichment of either mycobacterial or *Burkholderia*-like phenanthrene-degrading bacteria, which were obviously present in both Ohio and Montana soils. This suggests that there must be some general adaptive differences between these two very different types of microorganisms that could control their distribution and activity. In our enrichment cultures, selection must have been based on the different properties of SM7 compared to AMB, sand, or no sorptive phase.

The solids used to achieve variation in phenanthrene bioavailability differed not only in their sorption characteristics but also in their surface properties. Hence, we concluded that our results are consistent with selection for reduced bioavailability, as selection controlled by surface properties might also explain our findings. For example, the more hydrophobic surface of SM7 could have favored selection for mycobacteria, which are known for their hydrophobic cell surfaces. A recent observation supports the hypothesis that the basis of selection was reduced bioavailability (26). A phenanthrene-oxidizing bacterium that was selected in the presence of phenanthrene sorbed to SM7 was shown to have a greater propensity to degrade phenanthrene associated with sediments than a phenanthrene-oxidizing bacterium selected in the presence of nonsorbed phenanthrene. However, the isolate's phylogenetic type was not determined. As mentioned above, we found similar evidence of such selection in our companion study (9a).

Further work will be necessary to determine whether selection is based on phenanthrene availability and/or surface properties of the model organic phase utilized.

Whether selection is based on reduced bioavailability, surface properties, or both, the ecological significance is that phenanthrene-degrading microorganisms appear to be adapted to different features of the microenvironment. Even in our simple enrichment environments there must have been some niche diversity. For instance, some enrichment cultures contained more than one phenanthrene-degrading population. This might be explained by the simultaneous presence of different types of phenanthrene (e.g., dissolved, solid associated, and perhaps surfactant associated). All enrichment cultures also contained bacteria that do not use phenanthrene, suggesting that the phenanthrene degraders themselves may have increased niche diversity through metabolism of the primary carbon and energy source to other compounds.

In a contaminated soil many more factors must influence the structure of the microbial community responsible for contaminant biodegradation. Microbial populations may, of course, also be specialized with respect to other noncontaminant resources (e.g., oxygen or other nutrient types and concentrations) or other environmental conditions (e.g., temperature, moisture, etc.). However, even when only contaminant partitioning is considered, it is possible to envisage diverse niches. Most hydrocarbon- or creosote-contaminated systems consist of a complex mixture of pollutants at different concentrations rather than a single compound at a single concentration, as in our study. Furthermore, the composition of NOM is more complex and diverse than the uniform model organic phases tested in our study. Different nonionic contaminants may exhibit different degrees of sorption to different solids and, depending on the type and extent of contamination, may also partition into non-aqueous-phase liquids. Such factors constitute the real microenvironmental features that have controlled the evolutionary trajectories of contaminant-degrading bacteria, leading to their present diversity. The existence of different niches in soil could permit the coexistence of different contaminant degraders. The effects of different niches on contaminant distribution and availability could control the relative abundances and distributions of these contaminant degraders, as well as the contributions which they make to contaminant bioremediation. Given the ubiquity of NOM in soils and sediments and its propensity to sorb nonpolar organic solutes, bacteria adapted to degrade NOM-sorbed contaminants may have special relevance. The present study demonstrates the importance of recognizing and understanding microbial adaptations to such conditions if we are to obtain a predictive knowledge of how to use microorganisms to achieve contaminant removal in situ.

ACKNOWLEDGMENTS

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