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Horizontal Transfer of *phnAc* Dioxygenase Genes within One of Two Phenotypically and Genotypically Distinctive Naphthalene-Degrading Guilds from Adjacent Soil Environments

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Several distinct naphthalene dioxygenases have been characterized to date, which provides the opportunity to investigate the ecological significance, relative distribution, and transmission modes of the different analogs. In this study, we showed that a group of naphthalene-degrading isolates from a polycyclic aromatic hydrocarbon (PAH)-contaminated hillside soil were phenotypically and genotypically distinct from naphthalene-degrading organisms isolated from adjacent, more highly contaminated seep sediments. Mineralization of ^{14}C-labeled naphthalene by soil slurries suggested that the in situ seep community was more acclimated to PAHs than was the in situ hillside community. *phnAc*-like genes were present in diverse naphthalene-degrading isolates cultured from the hillside soil, while *nahAc*-like genes were found only among isolates cultured from the seep sediments. The presence of a highly conserved *nahAc* allele among gram-negative isolates from the coal tar-contaminated seep area provided evidence for in situ horizontal gene transfer and was reported previously (J. B. Herrick, K. G. Stuart-Keil, W. C. Ghiorse, and E. L. Madsen, Appl. Environ. Microbiol. 63:2330–2337, 1997). Natural horizontal transfer of the *phnAc* sequence was also suggested by a comparison of the *phnAc* and 16S ribosomal DNA sequences of the hillside isolates. Analysis of metabolites produced by cell suspensions and patterns of amplicons produced by PCR analysis suggested both genetic and metabolic diversity among the naphthalene-degrading isolates of the contaminated hillside. These results provide new insights into the distribution, diversity, and transfer of *phnAc* alleles and increase our understanding of the acclimation of microbial communities to pollutants.

The biochemical, enzymatic, and genetic details of microbial naphthalene degradation have been examined extensively since the early description in 1964 of a naphthalene metabolic pathway in *Pseudomonas* (9). Studies of naphthalene degradation are significant for at least four reasons: (i) naphthalene's aromatic character poses unique biochemical challenges for enzymatic attack (24, 67); (ii) naphthalene is a common pollutant that serves as a chemical model for the degradation of polycyclic aromatic hydrocarbons (PAHs) (56), which are often carcinogenic (49); (iii) insights are provided into the behavior of catabolic plasmids, the lateral transfer of genetic information among bacteria, and the evolution of oxygenase genes and enzymes (19, 22, 51, 59, 60, 63); and (iv) our abilities to effectively manage and treat polluted environments and to engineer novel enzymes for new technologies are increased (15, 48).

In all pure cultures of naphthalene-mineralizing bacteria that have been examined, the aerobic metabolism of naphthalene is initiated by a multicomponent enzyme system called naphthalene dioxygenase (NDO) (EC 1.14.12.12) (56). The NDO of *Pseudomonas putida* NCIB 9816-4 has been studied comprehensively and shown to be a three-component dioxygenase. Electrons from NAD(P)H are transferred via reductase (encoded by *nahAa*) and ferredoxin (encoded by *nahAb*) components to the $\alpha 3\beta 3$ catalytic oxygenase. The large subunits of this oxygenase (encoded by *nahAc*) each contain a Rieske [^{2}Fe-^{2}S] center and mononuclear nonheme iron (26) and are thought to confer substrate specificity (46). The ability to degrade naphthalene has been demonstrated for a wide range of bacterial genera, and a number of distinct NDO large subunits have been characterized and shown to have various degrees of nucleotide and amino acid sequence similarities to the canonical genes from *Pseudomonas* spp. (1, 5, 10, 12, 13, 17, 20, 27, 28, 31, 32, 42, 53, 57, 58).

The characterization of these distinct NDO analogs allows researchers to address questions concerning the ecological significance, relative distribution, and transmission of the different dioxygenases. A relevant example is *phnAc*, which was first described in 1999 when *Burkholderia* sp. strain RP007 was isolated from a PAH-contaminated site in New Zealand by using phenanthrene as a substrate (32). The *phnAc* gene was subsequently amplified from DNA extracted from pristine and contaminated soils in New Zealand, but it was not detected in a group of PAH-degrading bacteria isolated from those same soils (34), which suggested that it may be difficult to culture the *phnAc*-carrying organisms. Quantitative studies using competitive PCR showed that *phnAc* was present at equivalent or greater concentrations than was *nahAc* in contaminated New Zealand soils and that the addition of naphthalene to a pristine soil enriched it for *phnAc* but not for *nahAc* (33). In that study, *phnAc* was also shown to be present in pristine soils from Siberia, the Antarctic, and New Zealand. Subsequently, a group of PAH-degrading bacteria isolated from pristine soils in

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Japan and petroleum-contaminated soil in Indonesia were shown to contain *phnAc* alleles (62).

In this study, our objective was to compare and contrast, at the organism and gene levels, naphthalene-degrading bacterial guilds from adjacent but physically distinct areas of the same field site in upstate New York. Surprisingly, we found that culturable naphthalene-degrading bacteria from the adjacent sites were phenotypically and taxonomically distinct and that the *nahAc* allele was distributed only among isolates from the highly contaminated seep sediments while the *phnAc* allele was distributed only among taxonomically divergent hosts isolated from the less-contaminated hillside soil.

MATERIALS AND METHODS

Site description, sampling, and sample characterization. Samples were obtained from a coal tar-contaminated area located in South Glens Falls in upstate New York. This site, known as site 24, has been studied intensively, and details, including the site history and sample characteristics, have been published previously (2, 22, 35, 65, 66). Sampling was done at four locations near the seep area, which was located at the foot of a hillside approximately 400 m from the original coal tar deposit. The seep sample was taken aseptically from saturated naphthalene-contaminated sediments immediately within the contaminated portion of the seep. The hillside soil sample was obtained within 3 m of the seep on an adjacent hillside down gradient from the contaminated source that was approximately 1.5 m higher in elevation than the area where the seep sample was taken. Vegetation on the hillside included pine trees. The upstream sample was obtained from saturated sediments 25 m upstream in a northerly direction from the seep in a shallow drainage that emptied into the seep area. The adjacent sample was taken from saturated sediments in a drainage that was expected to contain no or low contamination and was parallel to, and 50 m east of, the seep. Sediment samples were obtained aseptically 10 cm below the surface, while the hillside soil sample was taken at a depth of 2 cm. All samples (25 to 30 g [wet weight]) were placed on ice in the field and maintained at 4°C until subsampling on the following day for mineralization, plating, and characterization.

Sample characterization, strain isolation, and reference cultures. All chemicals and reagents were of the highest available quality and were purchased from Sigma Chemical Co. (St. Louis, Mo.) unless otherwise noted. Soil and sediment samples were tested for mineralization of ^{14}C-labeled naphthalene in sealed flasks as previously described (35). Preparation and counting of total bacteria by acridine orange fluorescent-direct counting were also done as described previously (41) but with results reported as the means for two smears, 15 fields counted per smear. For dilution plating, 10 g of each sample was suspended in 90 ml of 0.1% sodium pyrophosphate (pH 7.0), further diluted in a 10-fold series in phosphate-buffered solution (0.07% K_2HPO_4, 0.03% KH_2PO_4 [pH 7.0]), plated in three replicates per dilution on Difco (Detroit, Mich.) R2A agar medium (for total counts) and on Stanier's mineral salts medium (54) solidified with 1.5% Noble agar (Difco), and incubated in the presence of naphthalene vapor. For each sample, 20 to 30 colonies with diameters greater than 1 mm were selected randomly from replicate naphthalene dilution plates that had between 20 and 200 well-defined colonies. Presumptive growth on naphthalene as a sole carbon source was confirmed by plating on mineral salts medium with and without naphthalene vapor. Isolation and characterization of strains were done as previously described (22).

P. putida G7 was a gift of G. Sayler, University of Tennessee. *P. putida* NCIB 9816-4 and *Comamonas testosteroni* GZ42 were gifts from G. Zylstra, Rutgers University.

PCR assays. Genomic DNA was obtained from bacterial isolates as previously reported (64). The presence of an amplifiable NDO large-subunit gene was determined by using PCR assays with a range of different PCR primers, primer combinations, and protocols. These included primers targeting *nahAc* from *P. putida* G7 (primers nahAc1 and nahAc3 andprotocol as described previously [21]); degenerate primers targeting a range of NDO sequences, including *nahAc* from *P. putida* G7 and *nahAc1* from *C. testosteroni* GZ42 (forward primers Ab248F, Ac114F, and Ac307F, reverse primers Ac596R, Ac893R, and Ac1095R, and protocol as described previously [65]); and primers targeting *phnAc* of *Burkholderia* sp. strain RP007 (forward primers 6897F and 8073F, reverse primers 8420R and 9047R, and protocol as described previously [32] except that the temperature cycling program was modified to 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 60 s). As positive controls, PCR amplifications using 16S rRNA primers 16SP-5 and 16SP-3 (21) were performed with each isolate. Amplification of the 16S rRNA genes from the hillside isolates was performed as previously described by a modification of the method described by Herrick et al. (22), using the eubacterial primers 27f and 1492r (30). PCR amplification was performed with a 50-μl volume with a primer concentration of 1 μM and a cycling regime of 94°C for 5 min (1 cycle); 94°C for 1 min, 56°C for 45 s, and 72°C for 45 s (35 cycles); and 72°C for 10 min (1 cycle). The amplicons were analyzed by electrophoresis on a 1% agarose gel and ligated into the pCR2.1 vector (Invitrogen, Carlsbad, Calif.). The constructed plasmids were introduced into *Escherichia coli* INV-F competent cells (One Shot; Invitrogen), and sequencing of the resulting white clones was completed on an ABI Prism 377XL instrument (Applied Biosystem Instruments, Foster, Calif.) by using M13 forward and/or reverse primers.

DNA hybridization. Digoxigenin-labeled probes were prepared by using PCR as described previously (21). Two probes were used: a 702-bp *nahAc* probe was prepared from *P. putida* G7 by using primers nahAc1 and nahAc3, and a 482-bp *nahAc* probe was prepared from *C. testosteroni* GZ42 by using primers Ac114F and Ac596R. Five micrograms of DNA from each isolate was denatured in 0.1 volume of a solution containing 4 M NaOH plus 0.1 M Na EDTA and blotted onto a Magna Graph (Micron Separations Inc., Westborough, Mass.) nylon membrane by using a vacuum-blotter (Bio-Rad, Hercules, Calif.) and baked at 65°C according to the manufacturer's (MSI) instructions. Prehybridization and hybridization were carried out in accordance with the instructions from the manufacturer for the Genius system (Boehringer-Mannheim, Indianapolis, Ind.). Hybridization and prehybridization were carried out at 65°C for the G7 probe and at 55°C for the GZ42 probe. Percent mismatch for a given wash temperature (T_m) and SSC concentration ([Na^+]; 1×SSC is 0.15 M NaCl plus 0.015 M sodium citrate) was calculated by using the formula $T_m = [81.5 + 16.6 (\log \text{concentration of } Na^+) + 0.41(\%G+C) - 600/l] - \%$ mismatch, where l is the length of the hybrid in base pairs (38, 50). The bound probe was detected by using the Genius kit and Lumiphos with chemiluminescent exposure of X-ray film (G7) or by using Nitro Blue Tetrazolium and X-phosphate (GZ42) according to the manufacturer's instructions (Boehringer-Mannheim).

Cloning, sequencing, and analysis of PCR amplicons from hillside isolates. Cloning of the PCR products was performed with the pGEM-T Easy Vector System I (Promega Corporation, Madison, Wis.) according to the manufacturer's instructions. Plasmid preparations were performed with the Promega Wizard-Plus SV Minipreps kit. Sequencing reactions were performed with the Thermo Sequenase fluorescence-labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Life Science, Piscataway, N.J.) and analyzed on an IR2 Long ReadIR2 automated DNA sequencer (Li-Cor, Inc, Lincoln, Nebr.). The nucleotide sequences were determined in both directions. The *phnAc* sequences were aligned and the percent identities were calculated by using the CLUSTALW and CLUSTALDIST programs available at San Diego Supercomputing Center's The Biology Workbench (http://workbench.sdsc.edu). 16S rRNA sequences were aligned and analyzed by using sequence match and similarity matrix programs available at the Ribosomal Database Project II (37).

Metabolite production by pure cultures. Cultures were grown on liquid Stanier's mineral salts medium supplemented with naphthalene at 24°C in the dark with shaking at 225 rpm, harvested by centrifugation, washed twice in an equal volume of 50 mM KH_2PO_4 (pH 7.25) at 4°C, and resuspended in 10 ml of the KH_2PO_4 buffer. Fifty microliters of 200 mM substrate (naphthalene or naphthalene which was uniformly labeled with deuterium) in *N,N*-dimethylformamide was added to 5 ml of the concentrated cell suspension, and metabolism was allowed to proceed for 5 min. Suspensions were extracted twice with 7.5 ml of neutralized ethyl acetate (64), which was then dried by using anhydrous Na_2SO_4 and concentrated under an atmosphere of N_2 to a volume of 100 μl. Extracts were derivatized with 10 μl of bis(trimethylsilyl)trifluoroacetimide (Supelco, Bellefonte, Pa.) for 2 to 5 min prior to gas chromatography-mass spectrometry (GC-MS) analysis. Negative controls included washed cell suspensions with no added substrate, KH_2PO_4 buffer (no cells) plus naphthalene, and in some cases uninduced cell suspensions (grown on yeast extract-peptone rather than on naphthalene). Studies were also performed with *E. coli* JM109(pDTG601), which carries the cloned toluene dioxygenase genes from *P. putida* F1 (68). Studies designed to determine whether a strain used a dioxygenase-mediated attack in the initial oxidation of naphthalene were performed with 10-ml volumes of cell suspensions in 25-ml serum bottles with crimp tops. To remove ambient dissolved $^{16}O_2$ from the cell suspension, N_2 was passed through a 0.22-μm-pore-size filter and gently bubbled through the liquid for 5 min. Stripped gases were allowed to exit via an 18-gauge syringe needle piercing the seal. A measured amount of $^{18}O_2$ (Cambridge Isotope Laboratories, Andover, Mass.) or a mixture of $^{18}O_2$ and $^{16}O_2$ was introduced with a syringe, after which the bottle was inverted and shaken vigorously to dissolve the headspace gas. The isotope composition of the oxygen in the headspace was measured by sampling with a

TABLE 1. Chemical and microbiological characteristics of samples[a]

Sample designation[b]	Type of sample	Water content (%)	Naphthalene conc ($\mu g \cdot cm^{3-1}$)	Acridine orange microscopic count of total bacteria [$(\pm SD) \cdot 10^9 \cdot gdw^{-1}$]	Total no. of cultured heterotrophs[c] [$(\pm SD) \cdot 10^6 \cdot gdw^{-1}$]	Total no. of cultured naphthalene-degrading bacteria[d] [$(\pm SD) \cdot 10^4 \cdot gdw^{-1}$]
Seep	Sediment	61.7	22.00	4.98 (±0.87)	1.6 (±0.3)	4.6 (±1.6)
Hillside	Soil	36.3	0.80	3.37 (±0.10)	1.8 (±0.5)	3.2 (±0.7)
Adjacent	Sediment	51.1	0.17	4.01 (±0.39)	1.1 (±0.6)	Below detection
Upstream	Sediment	61.3	0.70	5.23 (±0.46)	0.54 (±0.25)	Below detection

[a] gdw, gram (dry weight) of cells.
[b] See Materials and Methods for a description of sampling locations.
[c] Grown on R2A medium.
[d] Only large colonies (~3 mm in diameter) that responded to naphthalene vapor were counted. See Materials and Methods for details.

gas-tight syringe and analysis via GC-MS. The ratio of $^{18}O_2$ to $^{16}O_2$ was adjusted by the addition of further gas, if necessary, and reanalyzed prior to the addition of naphthalene and subsequent assay of metabolite production. The cell suspension assays were performed three times, and the identity of detected compounds was verified by comparison with the retention times and mass spectra of authentic standards.

GC-MS analysis. To determine the amount of naphthalene in sediment and soil samples, 4 g (wet weight) of soil or 5 g (wet weight) of sediment was mixed with 3 ml of a 9:1 mixture of hexane-butanol and shaken for 5 days in Teflon-sealed glass vials as previously described (36). After the mixtures settled, 0.9 μl of the organic phase was analyzed as outlined below. Metabolites produced by washed-cell suspensions were analyzed by injecting 9 μl of the derivatizeded ethyl acetate extract into a Hewlett-Packard (H-P) model 5890 Series II gas chromatograph equipped with an H-P 5 (5% phenyl methyl silicone) fused-silica capillary column (30 m by 0.25 mm with a 0.25-mm film thickness) connected to an H-P model 5971A quadrupole mass selective detector operated at an electron energy of 70 eV and a detector voltage of 1,600 to 3,000 V. A splitless injection was used, with a 1-min delay before purge. Helium was the carrier gas at a linear gas velocity of 25 cm/s. The injector and transfer line were maintained at 250 and 300°C, respectively. The ion source pressure was maintained at 1.0×10^{-5} torr. The GC temperature profile was 40°C for 1 min followed by increases of 10°C/min to 250°C.

Nucleotide sequence accession numbers. The *phnAc* and 16S rRNA gene sequences are available under GenBank accession numbers AY154358 to AY154379.

RESULTS

Characterization of samples. The chemical and microbiological characteristics of the four samples are presented in Table 1. The seep area lies in a direct line of the main flow of contaminated groundwater at the site. As expected, the naphthalene concentration was near saturation in the seep sediments but considerably lower in the other three samples. All samples were equivalent with regard to total microscopic counts and viable counts of bacteria. The seep sediment rapidly mineralized naphthalene with no apparent lag phase (Fig. 1). By comparison, the mineralization patterns by the nonseep (less-contaminated) samples displayed a clear lag phase, lower rates, and lesser extents of [^{14}C]naphthalene respiration. In contrast to the naphthalene mineralization results, mineralization of ^{14}C-labeled *p*-hydroxybenzoate was rapid and showed no noticeable lag in any of the samples (data not shown). *Para*-hydroxybenzoate serves as a useful positive control for aromatic hydrocarbon degradation because it is easily metabolized by many heterotrophic bacteria and because it is structurally similar to many naturally occurring phenolic compounds (14, 35).

Isolation and enumeration of naphthalene-degrading bacterial strains. Naphthalene-degrading bacteria were readily isolated from both the seep and hillside samples without prior enrichment. Dilution plates containing bacteria from the contaminated seep and hillside samples were incubated in the presence of naphthalene vapor and yielded robust colonies (>3 mm in diameter after 3 days of incubation at 20°C) at a population density of ca. 10^4 cells/g (Table 1). By using relative growth with and without naphthalene vapor as a presumptive criterion for naphthalene catabolic capability, 18 presumptive naphthalene-degrading bacterial isolates were picked and purified from the contaminated seep plates and 23 were picked from the hillside sample plates. Of these, 17 seep and 22 hillside isolates (Table 2) mineralized [^{14}C]naphthalene to $^{14}CO_2$ after 2 days of incubation at 25°C.

Identical enumeration and isolation procedures were applied to samples adjacent to and upstream from the seep area. For these samples, however, colony sizes were not larger in the presence of naphthalene vapor; thus, initial counts were deemed to be below the level of detection (Table 1). Naphthalene-degrading bacterial strains were obtained from these

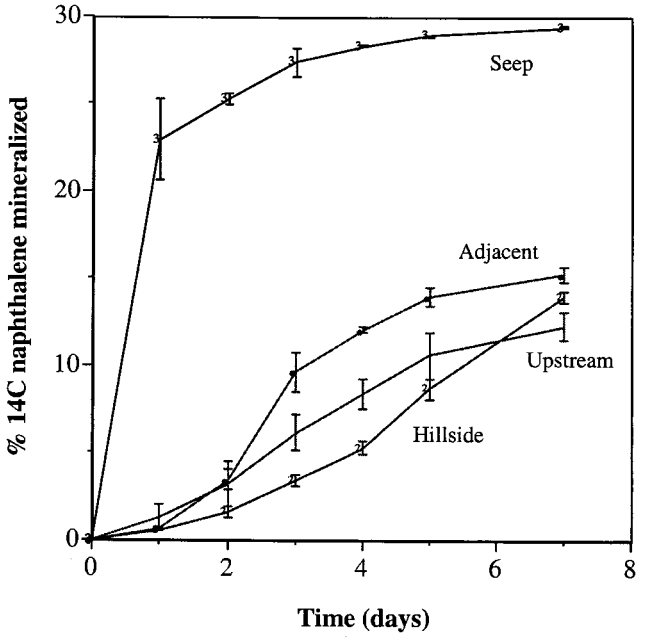

FIG. 1. Cumulative $^{14}CO_2$ production (mineralization) from ^{14}C-labeled naphthalene by samples from the study site. Points represent the means of results for triplicate samples relative to values for poisoned controls. Error bars indicate 1 standard deviation greater than and less than the mean.

TABLE 2. Characteristics of naphthalene-mineralizing bacterial isolates from adjacent site habitats, seep sediment and hillside soil

Habitat/strain	Gram stain/ morphology[a]	Oxidase	Closest taxon identified by[b]:		16S rRNA (% identity)
			API-NFT	BIOLOG	
Seep					
Cg1	Neg./rod	+	*Pseudomonas putida*	*Pseudomonas putida* A1	*Pseudomonas gramini* (100)
Cg2	Neg./rod	+	*Pseudomonas fluorescens*	*Pseudomonas fluorescens* G	*Pseudomonas azotoformans* (99.7)
Cg3	Pos./rod	−	ND	*Micrococcus diversus*	ND[c]
Cg4	Neg./rod	+	No ID	No ID (*Pseudomonas fulva*)	*Pseudomonas amygdali* (99.4)
Cg5	Neg./rod	+	(*Pseudomonas fluorescens*)	*Pseudomonas fluorescens G*	*Pseudomonas gramini* (100)
Cg7	Neg./rod	+	No ID	(*Pseudomonas fluorescens* C)	*Pseudomonas azotoformans* (99.7)
Cg8	Neg./rod	+	*Pseudomonas fluorescens*	No ID (*Pseudomonas asplenii*)	ND
Cg9	Neg./rod	+	No ID	*Pseudomonas fluorescens* G	ND
Cg11	Neg./rod	+	*Ralstonia picketii*	*Pseudomonas mendocina*	*Pseudomonas amygdali* (99.4)
Cg12	Neg./rod	+	*Pseudomonas fluorescens*	(*Pseudomonas fluorescens* G)	ND
Cg13	Pos./rod	−	ND	ND	ND
Cg14	Pos./rod	−	ND	ND (*Arthrobacter)*[d]	ND
Cg15	Neg./rod	+	*Ralstonia picketii*	No ID (*Pseudomonas cichorii*)	*Pseudomonas amygdali* (99.4)
Cg16	Neg./rod	+	(*Pseudomonas aureofaciens*)	(*Pseudomonas corrugata*)	ND
Cg18	Pos./rod	−	ND	*Micrococcus diversus*	ND
Cg20	Pos./rod	−	ND	ND	ND
Cg21	Neg./rod	−	*Sphingomonas paucimobilis*	*Sphingobacterium mizutaii*	*Pseudomonas azotoformans* (100)
Hillside					
Hg1	Neg./rod	−	No ID	(*Chryseomonas luteola*)	*Herbaspirillum seropedicae* (96.6)
Hg2	Neg./rod	−	No ID	(*Burkholderia glathei*)	*Burkholderia glatheii* (97.7)
Hg3	Neg./rod	−	No ID	*Burkholderia glathei*	*Burkholderia glatheii* (97.8)
Hg4	Neg./rod	−	No ID	*Burkholderia glathei*	*Burkholderia glatheii* (97.9)
Hg5	Neg./rod	−	No ID	*Burkholderia glathei*	*Burkholderia glatheii* (98.0)
Hg6	Neg./rod	−	*Burkholderia cepacia*	(*Burkholderia glathei*)	ND
Hg7	Neg./coccus	−	No ID	(*Burkholderia glathei*)	*Burkholderia glatheii* (97.3)
Hg8	Neg./rod	−	*Burkholderia cepacia*	*Burkholderia gladioli*	*Burkholderia phenazinium* (97.7)
Hg9	Neg./rod	−	No ID	*Burkholderia* sp. (*B. gladioli*)	ND
Hg10	Neg./rod	−	*Burkholderia cepacia*	*Burkholderia gladioli*	*Burkholderia phenazinium* (97.8)
Hg11	Neg./rod	−	No ID	No ID (*B. glathei*)	*Burkholderia glathei* (98.1)
Hg12	Neg./rod	−	*Burkholderia cepacia*	No ID (*B. gladioli*)	ND
Hg13	Neg./rod	−	No ID	*Burkholderia* sp. (*B. gladioli*)	ND
Hg14	Neg./rod	−	No ID	(*Pseudomonas fluorescens* G)	*Burkholderia phenazinium* (97.9)
Hg15	Neg./rod	−	No ID	*Burkholderia glathei*	ND
Hg16	Neg./rod	−	No ID	*Burkholderia glathei*	*Burkholderia phenazinium* (97.5)
Hg17	Neg./rod	−	*Burkholderia cepacia*	*Burkholderia gladtoli*	*Burkholderia glathei* (98.1)
Hg18	Neg./rod	+	No ID	*Burkholderia glathei*	*Burkholderia glathei* (98.0)
Hg19	Neg./rod	−	No ID	No ID (*B. glathei*)	*Burkholderia glathei* (98.1)
Hg20	Neg./rod	−	No ID	*Xanthomonas maltophilia*	ND
Hg21	Neg./rod	−	No ID	No ID (*Burkholderia glathei*)	ND
Hg22	Neg./rod	−	*Burkholderia cepacia*	*Burkholderia gladioli*	ND

[a] Neg., negative; Pos., positive.
[b] Names in parentheses indicate the database taxon closest to the unidentified genus or species. ND, not determined; ID, identification. The API-NFT test kit is designed for gram-negative bacteria only.
[c] ND, not done
[d] Based on Biolog characterization and microscopic observation of coccus/irregular rod cycling

samples after seven or more days of incubation in a minimal broth medium plus naphthalene, indicating that naphthalene-catabolizing bacteria were present and could be enriched (data not shown).

Characterization of naphthalene-degrading isolates and comparison of hillside and seep isolates. All isolates were characterized and, when possible, identified by using standard microbiological methods and the API Rapid-NFT and Biolog test kits (Table 2). 16S rRNA gene sequences (1,454 nucleotides, corresponding to nucleotides 28 through 1491 of rRNA from *E. coli*) for 14 of the hillside isolates were compared to rRNA sequences of characterized organisms. Partial 16S rRNA gene sequences (305 nucleotides, corresponding to nucleotides 23 through 328 of rRNA from *E. coli*) were obtained for a subset of the seep isolates as part of a previous study (22). The two sets of naphthalene-degrading isolates, one from the contaminated seep and the other from the hillside, were taxonomically and phenotypically distinct. Cluster analysis of the gram-negative isolates based on 95 carbon source utilization tests in the Biolog system revealed seven well-defined phenetic groups (data not show). Members of each of the phenetic groups were homogeneous with respect to the source of the sample from which they were isolated. Three of the groups consisted entirely of seep isolates, while four groups were composed entirely of isolates from the hillside sample. Isolates from the hillside sample were all gram-negative, oxidase-negative, mainly rod-shaped organisms. Most of these isolates were identified by substrate utilization profiles as members of the genus *Burkholderia* (Table 2). Partial 16S rRNA gene sequences supported this taxonomic affiliation, with most of the

TABLE 3. Distance matrix of *phnAc* and 16S rRNA gene sequences from hillside isolates

Strain	Dissimilarity[a] to:							
	Hg2	Hg4	Hg8	Hg10	Hg11	Hg14	Hg16	RP007
Hg1	**0.002**[a]	**0.001**	**0.005**	**0.006**	**0.005**	**0.008**	**0.003**	**0.099**
	0.104	*0.102*	*0.101*	*0.102*	*0.097*	*0.103*	*0.107*	*0.097*
Hg2		**0.003**	**0.003**	**0.008**	**0.003**	**0.005**	**0.005**	**0.038**
		0.006	*0.040*	*0.037*	*0.008*	*0.039*	*0.043*	*0.039*
Hg4			**0.006**	**0.005**	**0.006**	**0.008**	**0.002**	**0.036**
			0.038	*0.035*	*0.006*	*0.036*	*0.041*	*0.036*
Hg8				**0.005**	**0.000**	**0.009**	**0.008**	**0.030**
				0.003	*0.034*	*0.004*	*0.008*	*0.031*
Hg10					**0.005**	**0.013**	**0.007**	**0.029**
					0.032	*0.002*	*0.006*	*0.029*
Hg11						**0.009**	**0.008**	**0.034**
						0.033	*0.037*	*0.034*
Hg14							**0.005**	**0.027**
							0.006	*0.028*
Hg16								**0.033**
								0.033

[a] Dissimilarity of *phnAc* sequences is indicated in bold, white dissimilarity of 16S rRNA sequences is indicated in italics.

sequences matching (ca. 98% identity) that of one of three *Burkholderia* species: *Burkholderia glathei*, *Burkholderia gladioli*, or *Burkholderia phenazinium*. Strain Hg20 was identified by Biolog as *Xanthomonas maltophilia*, a member of the gammaproteobacteria. No matches were found in the Biolog database for Hg1, a strain whose 16S rRNA gene sequence was found to share more than 96% identity with that of *Herbaspirillum seropedicae*. In contrast, the naphthalene-degrading bacteria from the contaminated seep samples were phenotypically and taxonomically more diverse. The seep guild was comprised of both gram-positive and -negative and oxidase-positive and -negative isolates (Table 2). The gram-negative isolates had substrate utilization profiles and 16S rRNA gene sequences that placed them within the rRNA group 1 (45) of the genus *Pseudomonas.* None of the hillside isolates fell within this genus.

Detection of *nahAc* homologs by PCR analysis and Southern hybridization. As previously reported, all of the gram-negative seep isolates were found to possess a highly conserved allele of *nahAc* located on a pDTG1-like plasmid (22, 55). Previously optimized PCR primers and protocols (21, 65) and Southern hybridization were used to screen the hillside isolates for the presence of *nahAc* homologs. *nahAc* amplification products were not produced from the hillside strains nor from the gram-positive strains from the seep, and these strains did not hybridize with a 702-bp *nahAc* probe under nonstringent (~13% mismatch) conditions. Under even less stringent conditions (~25% mismatch), 11 of the 22 strains hybridized with the *nahAc* probe (Hg4, Hg7, Hg10, Hg11, Hg14, and Hg16 to Hg22). None of the seep gram-positive strains hybridized, even at conditions approximating 40% mismatch. A second PCR screen that targeted a wider array of genes encoding dioxygenase iron-sulfur protein large subunits was performed. This assay used a suite of degenerate primers shown to amplify a range of diverse large-subunit sequences, including *nahAc2* from *C. testosteroni* GZ42, which is ~80% identical at the nucleotide level to *nahAc* from *P. putida* G7 and to *ndoB* from *P. putida* NCIB 9816-4 (65). However, none of the hillside isolates produced a PCR product by this alternate PCR assay. Lack of amplification was probably not due to the unavailability of template DNA, since PCR amplification of the 16S rRNA gene for all of the isolates was successful (data not shown).

Amplification, sequencing, and analysis of *phnAc* homologs. The hillside isolates were then screened for the presence of homologs of *phnAc* from *Burkholderia* sp. strain RP007 by using PCR primers that had been developed for studies of that gene (32, 33). Strain RP007 was isolated on phenanthrene from a PAH-contaminated soil from New Zealand and shown to possess a set of naphthalene-catabolic genes that were highly divergent from those that had previously been described. Using primers phn8073F and phn9047R, we were able to amplify a product of the expected size from all 14 of the Hg strains tested (Hg1 to Hg5, Hg7, Hg8, Hg10, Hg11, Hg14, and Hg16 to Hg19) (data not shown). These strains represented five of the six phenotypic clusters identified from carbon-substrate-utilization profiles. Hg20, the only member of the sixth cluster, could not be recovered from frozen stocks and therefore was not included in this analysis. The PCR results indicated that these strains contained *phnAc* homologs. This result was verified by cloning and sequencing the phn8073F-phn9047R amplicons from eight of the isolates and comparing the resulting sequences (992 nucleotides) to the sequence from *Burkholderia* sp. strain RP007. The sequences were aligned and the uncorrected percentages of dissimilarity were calculated by using ClustalW and ClustalDist programs at San Diego Super-Computing Center's The Biology Workbench (http://workbench.sdsc.edu) (Table 3). The *phnAc* nucleotide sequences from the hillside isolates were ca. 98% identical to that from *Burkholderia* sp. strain RP007. The new sequences were even more closely related to one another; in all but one case, they were more than 99% identical to one another. The sequences from strains Hg8 and Hg11 were 100% identical.

The natural horizontal transfer of genes has been inferred by showing that a highly conserved gene is shared by a group of taxonomically diverse hosts (22, 29). To examine whether lateral transfer of the *phnAc* allele might be taking place among the hillside microorganisms, we compared the *phnAc* sequence

TABLE 4. Potential metabolites detected in extracts of cell suspensions incubated with naphthalene

Strain	Potential metabolite detected[a]						% of 1,2-DHDN labeled with the indicated no. of ^{18}O atoms[b]		
	Catechol	Gentisate	Salicylate	Salicylald.	1,2-DHN	1,2-DHDN	0[c]	1[d]	2[e]
P. putida G7	+	BD	+	+	+	+	66	11	22
C. testosteroni GZ42	BD	+	+	+	+	+	—		
Hg1	BD	BD	+	BD	+	+	46	9	45
Hg2	+	BD	+	BD	+	+	55	9	36
Hg3	+	BD	+	BD	+	+	—		
Hg4	+	BD	+	+	+	+	45	9	46
Hg6	+	BD	+	+	+	+	—		
Hg8	+	BD	+	+	+	+	55	10	35
Hg11	BD	+	+	+	+	+	—		
Hg12	+	BD	+	BD	+	+	62	10	28

[a] +, detection; BD, below detection. Salicylald., salicyladehyde; 1,2-DHN, 1,2-dihydroxynaphthalene.
[b] Mass spectrometry determined the distribution of ^{18}O within 1,2-DHDN parent ions produced under an atmosphere of 50% $^{18}O_2$–50% $^{16}O_2$. —, not determined.
[c] 1,2-DHDN mass contained two ^{16}O atoms. Molecular ion = M.
[d] 1,2-DHDN mass contained one ^{16}O atoms and one ^{18}O atom. Molecular ion = M + 2.
[e] 1,2-DHDN mass contained zero ^{16}O atoms and two ^{18}O atoms. Molecular ion = M + 4.

divergence with the16S rRNA gene sequence divergence of the same strains. rRNA gene sequences (1,454 nucleotides) were aligned and uncorrected percentages of dissimilarity were calculated by using the Similarity Matrix program at the Ribosomal Database Project II (37), excluding gaps and regions that could not be unambiguously aligned (Table 3). For several of the strains, the 16S rRNA gene sequences were markedly different while the *phnAc* sequences were identical or nearly identical. For example, the *phnAc* sequences of Hg1 and Hg4 differed by a single nucleotide in the region sequenced, but the 16S rRNA sequences of these strains shared only 90% identity. Similarly, the *phnAc* sequences of Hg8 and Hg11 were identical, but the 16S rRNA gene sequences of these strains shared only 97% identity.

The sequencing results suggested that diverse *phnAc* alleles may be present in the different hillside isolates. To investigate whether these isolates possessed a similar gene arrangement to that found in *Burkholderia* sp. strain RP007, other PCR primers targeted at regions of DNA flanking *phnAc* (32) were used to screen the isolates. The four possible combinations of two forward PCR primers (phn6897F and phn8073F) and two reverse primers (phn8420R and phn9047R) were used to screen for the presence of diverse *phnAc* homologs in several of the hillside isolates. All strains tested produced amplicons of the expected size with each of the four primer combinations, except Hg14, which produced amplicons of the expected size with all the primer combinations except phn6897F-phn9047R.

Metabolite production by pure cultures of *phnAc*-containing strains. The cloned *phn* genes of *Burkholderia* sp. strain RP007 have been shown to produce 1,2-dihydroxynaphthalene and salicylate from naphthalene, and two distinct catechol 2,3-dioxygenase genes have been cloned from this strain and characterized (32). To examine the metabolic diversity of naphthalene catabolism in the *phnAc*-containing hillside isolates, metabolite production by pure cultures was examined by using GC-MS analysis. Seven of the hillside strains were chosen to represent the subgroups that were identified by carbon substrate utilization profiles. Characterized strains *P. putida* G7 and *C. testosteroni* GZ42 were included as controls. These control strains metabolize naphthalene to salicylate; *P. putida* G7 oxidizes salicylate to catechol by using a salicylate-1-hydroxylase (56), while it is thought that *C. testosteroni* GZ42 converts salicylate to gentisate, presumably via a salicylate-5-hydroxylase (16).

Metabolites produced from washed cell suspensions that were incubated briefly with naphthalene are included in Table 4. Parallel experiments in which deuterium-labeled naphthalene was substituted for the unlabeled substrate were performed to verify that these compounds originated from the added naphthalene substrate. Some variability was detected among the pathways for naphthalene catabolism in the hillside (Hg) strains (Table 4). When *P. putida* G7 cells were assayed, all of the expected metabolites from the catechol pathway were detected. Strains Hg4, Hg6, and Hg8 also produced all of the compounds expected from the catechol pathway, while Hg2 and Hg12 produced all of the compounds expected from the catechol pathway except for salicylaldehyde. *C. testosteroni* GZ42 produced all of the metabolites present in the gentisate pathway, as expected. Hg11 cell suspensions also produced all of the metabolites of the gentisate pathway. In Hg1 cell suspensions, only three of the previously reported metabolites were detected: 1,2-dihydroxy-1,2-dihydronaphthalene (1,2-DHDN); 1,2-dihydroxynaphthalene, and salicylate.

Three possible mechanisms can be envisioned for the enzymatic formation of 1,2-DHDN from naphthalene, and these three mechanisms can be distinguished by examining the patterns of ^{18}O-labeled products formed under atmospheres containing 50/50 mixtures of $^{18}O_2$ and $^{16}O_2$. The first mechanism is the dioxygenase-mediated single-step addition of both atoms of molecular oxygen (O_2) to adjacent carbons of a naphthalene molecule (56). Alternatively, a two-step attack — initial monooxygenase followed by the action of an epoxide hydrolase — would result in the addition of one atom of O that originated from O_2 and one atom of O from water (6). Finally, two mono-oxygenations, a mechanism that we could find no reports of in the literature, would proceed via the sequential addition of two O atoms that each originated from the pool of O_2 molecules. Thus, the predicted distributions of labeled products formed by the different mechanisms under atmospheres containing 50/50 mixtures of $^{18}O_2$ and $^{16}O_2$ areas follows: for a

dioxygenase, two equal pools of 1,2-DHDN differing by 4 atomic mass units; for a mono-oxygenase followed by an epoxide hydrolase, two equal pools of 1,2-DHDN differing by 2 atomic mass units; and for sequential mono-oxygenations, three pools of 1,2-DHDN, in ratios of 1:2:1, each differing by 2 atomic mass units. Cell suspensions of all strains, when incubated in the presence of a mixture of $^{18}O_2$ and $^{16}O_2$, produced labeling patterns in 1,2-DHDN that deviated from theoretical ratios (discussed above) but conformed most closely with that of an initial dioxygenase attack (Table 4).

DISCUSSION

Several distinct NDOs have been characterized to date, but the ecological significance, relative distribution, and transmission of the different NDO analogs are poorly investigated. The *phn* genes have been described only recently, and it has been suggested several times that these genes were not discovered earlier because organisms with *phn* genotypes are more difficult to culture than are organisms with *nah* genotypes (32, 34). However, in this study, only *phn*-containing organisms were isolated from a hillside soil, which appears to conflict with the suggestion that culturing these organisms is problematic. Additionally, a recent study of PAH-degrading bacteria isolated from pristine and contaminated soils in Kuwait, Indonesia, Thailand, and Japan found that 6 out of 19 isolates hybridized to a *phnAc* probe (62). A likely explanation is that *phnAc*-utilizing organisms have been cultured routinely in the past but were not recognized as such because the *phn* genes had not been described. Several studies have reported the isolation of naphthalene-degrading organisms that did not hybridize to probes derived from *nahAc* of *P. putida* G7 and *ndoB* of *P. putida* NCIB 9816-4 (4, 7, 18, 39, 42, 47, 62). Studies of PAH dioxygenase gene distribution need to be updated by using the suite of recently described PAH dioxygenase genes as hybridization targets.

It also appears that the restriction of *nahAc* alleles to seep isolates and that of *phnAc* alleles to hillside isolates in this study are consistent with natural selective enrichment of the different genotypes in the two environments. While retrospective identification of selective factors is necessarily speculative, possible selective factors can be divided into two groups: those that relate to the PAH-degrading phenotype and those that are independent of this phenotype. With regard to the first group, there may have been selection for the *nah* genotype in the seep because the concentration of PAHs was higher and the *phn* genotype was inhibited, the mixture of PAHs in the seep was more susceptible to attack by the *nah* genotype, lateral transfer of the *nah* genotype occurred to a greater extent in the seep environment, or the *nah* genotype allowed for more rapid growth in the seep environment. Selective enrichment of microbial populations in response to pollutant contamination was observed by Ka et al. (25), who found that certain groups of 2,4-dichlorophenoxyacetic acid degraders which predominated after long exposure were found only at low frequencies initially, while others were dominant at first but isolated only rarely after prolonged contamination. Also, Ogunseitan et al. (43) found that seeding soils with salicylate, an inducer of naphthalene degradation, caused an increase in the proportion of *nahAB*-hybridizing strains in soil microcosms, while Laurie and Lloyd-Jones (33) found enrichment of *phnAc* but not *nahAc* after the addition of naphthalene to an uncontaminated soil.

Alternatively, a selective factor could be independent of the PAH-degrading phenotype. That is, there could have been selection for different taxonomic groups in the two environments, and the different NDO genotypes are coincidentally associated with those different taxonomic groups. Environmental factors that might selectively enrich for different taxonomic groups are diverse (e.g., moisture content or anaerobiosis), but an examination of the phylogenetic identity of the hillside isolates suggests one particular factor, vegetation. Hillside organisms primarily belonged to several species of *Burkholderia*, including *B. glathei*, *B. gladioli*, and *B. phenazinium*. All of these species are common soil inhabitants that have been found to reach much higher densities in the rhizosphere, representing half the culturable bacteria in some rhizosphere studies (61). Hg1 and Hg20 were the only hillside isolates that were not among these *Burkholderia* species. Hg1 was most closely related to *H. seropedicae*, which has been found only intimately associated with plant tissues and roots (3, 44), and Hg20 was most closely related to *X. maltophilia*, a common plant pathogen. Thus, it may be that the hillside community represented naphthalene degraders that are selected for in the rhizosphere environment. This interpretation leads to the suggestion that difficulties reported by other investigators in culturing *phn*-containing strains reflect problems with culturing *Burkholderia* on nonselective media from nonrhizosphere soils and sediments. However, this interpretation is tentative because we did not actively attempt to sample the rhizosphere, for example, by washing and crushing roots to sample endophytic bacteria. Furthermore, even efforts to plate rhizosphere samples onto selective media are not always particularly effective at recovering *Burkholderia* species (40). In addition, *Pseudomonas* species, including those that hybridize with *nahAc* probes, have been isolated from rhizosphere (7) and endophyte (52) samples.

An alternative explanation for the irregular distribution of NDO genes among site isolates is that the communities were limited by the genetic material available to them. This possibility seems unlikely given the widespread distribution of both alleles in pristine as well as contaminated soils (33, 34) and the likelihood of cotransport of PAHs and PAH-degrading bacteria (36). It would not be surprising if additional sampling produced some *nahAc* alleles in hillside isolates or *phnAc* alleles in seep isolates. However, it is unlikely that the trend described here would disappear with further sampling.

We previously reported evidence indicating that natural horizontal transfer of diverse *nahAc*-containing plasmids had taken place among the microbial inhabitants of the seep. An identical *nahAc* allele was shared by seven different seep isolates whose 16S rRNA gene sequences differed by as much as 7.9% (22). The sequence divergence of the highly conserved 16S rRNA gene, coupled with the lack of divergence of the *nahAc* allele, was taken as evidence that the *nahAc* allele had been recently transferred among the seep isolates. Large plasmids were identified in all of these isolates, and a 407-bp *nahAc* probe hybridized to plasmids in all but one (22). Filter matings between naphthalene-degrading bacterial isolates and their cured progeny revealed that the naphthalene-catabolic plas-

mids were self-transmissible, and distinct naphthalene-catabolic plasmids were retrieved directly from the microbial community indigenous to the contaminated site in filter matings by using a cured, rifampin-resistant, site-derived isolate as the recipient (23, 55). We initiated similar studies to investigate whether lateral transfer of *phnAc* alleles might be taking place among the members of the hillside community.

Partial *phnAc* sequences (992 nucleotides) and 16S sequences (1,454 nucleotides) were obtained from eight isolates in five of the six phenotypic clusters identified from carbon substrate-utilization profiles. Comparison of the *phnAc* sequences revealed greater diversity than was seen with the *nahAc* allele in the seep study (22), but the *phnAc* sequences were all closely related. Although only two strains had identical *phnAc* alleles (Hg8 and Hg11), the most dissimilar strains still shared 98.7% identity (Hg10 and Hg14). Comparison of the 16S rRNA gene sequences, however, revealed much greater divergence than the comparison of *phnAc* sequences. For example, the 16S rRNA gene sequences of Hg1 and Hg4 are only 90% identical, but the *phnAc* sequences differ by merely a single nucleotide in the 992-nucleotide region sequenced. Similarly, the 16S rRNA gene sequences of Hg8 and Hg11 share identical *phnAc* sequences but have 16S sequences that are only 97% identical. We interpret these results, which show that a highly conserved gene is shared by a group of taxonomically diverse hosts, as evidence that horizontal transfer of the *phnAc* allele has taken place among the members of the hillside community. That the hosts are in fact taxonomically diverse is further supported by the substrate-utilization profiles (Table 4).

There were two important differences between the results of this study and those obtained in the *nahAc* study (22). In this study, there were multiple alleles of *phnAc* present and in some pair-wise comparisons the *phnAc* sequences of the isolates were more highly diverged than were the 16S rRNA gene sequences. The divergence observed among the *phnAc* alleles of the hillside isolates, although slight, is interesting in light of the fact that a single *nahAc* allele was recovered from the seep isolates. This divergence may reflect lateral transfer that took place prior to, or over a longer period of time than, that of the *nahAc* allele. Alternatively, it may indicate transfer of multiple, dissimilar *phn*-carrying mobile elements among bacteria in the hillside environment. Genetic diversity within the *phn* genes was also suggested when Hg14 did not produce an amplification product with primers phn6897F and phn9047R. Although this is a negative result and therefore problematic to interpret, it may indicate a different gene order in Hg14 or it may reflect sequence divergence of the *phnC* gene at the site where phn6897F binds. Further studies, including the characterization of large plasmids (if present), should provide greater understanding of the means by which the *phn* genes are disseminated at the site.

We also examined whether the hillside isolates were metabolically diverse. By using GC-MS analysis of culture extracts, these isolates were tested to determine what metabolites they produced, their mechanism of initial ring attack, and the source of oxygen atoms added to the ring. Both the metabolites themselves and their deuterated and ^{18}O-labeled analogs indicated uniformly that the mechanism of initial ring attack was dioxygenase mediated. Two distinct pathways for subsequent naphthalene catabolism have been characterized, one in which salicylate is oxidized to catechol and one in which salicylate is converted to gentisate, and evidence for each of these pathways was found in different subsets of the hillside isolates. Interestingly, evidence for the catechol pathway was demonstrated in one strain (Hg8) that had a *phnAc* allele identical to that of an isolate that may be using the gentisate pathway (Hg11). In addition to being the first indication that a *phn*-utilizing organism may catabolize naphthalene through the gentisate pathway, this result further supports the notion that multiple *phn* mobile elements may have been transferred at the site. In Hg1 cell suspensions, only three of the previously reported metabolites were detected. The inability of this assay to detect additional compounds in extracts from this strain could reflect differences in the rate at which it uses certain metabolites, or it could reflect the existence of further pathway variation. The detection of a compound in supernatant fluids of naphthalene-grown cells, or the demonstration that it is produced from naphthalene by induced cells, does not prove that the compound is an intermediary metabolite of a naphthalene-catabolic process. Compounds might be produced as dead-end metabolites, as side reactions, or as artifacts of the extraction and analysis procedure (8). A better approach for designating a compound as an intermediary metabolite can result from studies of enzymes isolated from an organism. Genetic approaches, such as that utilized by Eaton and Chapman to study the ring cleavage of 1,2-dihydroxynaphthalene (11), are more stringent and less likely to suffer from these difficulties. However, the methods used in this study are helpful for screening a large number of isolates to identify which organisms should be more intensively studied.

These results provide further evidence that lateral transfer is important in the biodegradation of contaminants in polluted field sites. They extend the known geographic distribution of *phnAc* alleles, expand the range of microbial genera known to contain *phnAc* alleles, and suggest previously unidentified metabolic diversity among *phnAc*-containing bacteria. Genetic investigations of the site-derived strains that were examined in this study and field studies targeting rhizosphere versus nonrhizosphere communities may be useful in furthering the understanding of PAH-degradative processes at this and other contaminated field sites. Greater understanding of these processes can assist with the development of new technologies and management strategies for use in contaminated field sites and can improve the knowledge of the evolution of natural bacterial populations.

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