Applied and Environmental Microbiology, May 2009, p. 2613–2620 Vol. 75, No. 9
0099-2240/09/$08.00+0 doi:10.1128/AEM.01955-08

Characterization of a Polycyclic Aromatic Hydrocarbon Degradation Gene Cluster in a Phenanthrene-Degrading *Acidovorax* Strain[▽]

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Received 21 August 2008/Accepted 21 February 2009

***Acidovorax* sp. strain NA3 was isolated from polycyclic aromatic hydrocarbon (PAH)-contaminated soil that had been treated in a bioreactor and enriched with phenanthrene. The 16S rRNA gene of the isolate possessed 99.8 to 99.9% similarity to the dominant sequences recovered during a previous stable-isotope probing experiment with [U-^{13}C]phenanthrene on the same soil (D. R. Singleton, S. N. Powell, R. Sangaiah, A. Gold, L. M. Ball, and M. D. Aitken, Appl. Environ. Microbiol. 71:1202–1209, 2005). The strain grew on phenanthrene as a sole carbon and energy source and could mineralize ^{14}C from a number of partially labeled PAHs, including naphthalene, phenanthrene, chrysene, benz[*a*]anthracene, and benzo[*a*]pyrene, but not pyrene or fluoranthene. Southern hybridizations of a genomic fosmid library with a fragment of the large subunit of the ring-hydroxylating dioxygenase gene from a naphthalene-degrading *Pseudomonas* strain detected the presence of PAH degradation genes subsequently determined to be highly similar in both nucleotide sequence and gene organization to an uncharacterized *Alcaligenes faecalis* gene cluster. The genes were localized to the chromosome of strain NA3. To test for gene induction by selected compounds, RNA was extracted from amended cultures and reverse transcribed, and cDNA associated with the enzymes involved in the first three steps of phenanthrene degradation was quantified by quantitative real-time PCR. Expression of each of the genes was induced most strongly by phenanthene and to a lesser extent by naphthalene, but other tested PAHs and PAH metabolites had negligible effects on gene transcript levels.**

Increasingly, stable-isotope probing (SIP) has been used to identify environmentally relevant bacteria capable of expressing a particular phenotype prior to directed efforts to cultivate those organisms. For instance, RNA-based SIP with [$^{13}C_6$]benzene was used to identify *Azoarcus* strains in gasoline-contaminated groundwater before targeted cultivation attempts (19). In another example, a *Burkholderia* strain isolated from soil was matched to the dominant terminal restriction fragment length polymorphism band from heavy DNA resulting from incubation with [^{13}C]benzoic acid (40). The bacterium *Polaromonas naphthalenivorans* CJ2 was isolated from coal-tar-contaminated sediments after having been identified as a dominant member of clone libraries in SIP experiments with ^{13}C-labeled naphthalene (17). That strain has since been the focus of additional studies, one of which resulted in the discovery of a unique ring-hydroxylating dioxygenase (RHD) (18).

In prior research on polycyclic aromatic hydrocarbon (PAH) degradation, we performed DNA-SIP with uniformly labeled naphthalene, phenanthrene, and pyrene to identify the dominant degraders of those compounds in a bioreactor treating PAH-contaminated soil (45, 46). In those experiments, the most frequently encountered sequences in ^{13}C-enriched DNA fractions from incubations with labeled phenanthrene were associated with the *Acidovorax* genus (45). Members of that genus have previously been found in high abundance in soils containing PAHs (9, 22) or have been implicated in PAH degradation (12), particularly phenanthrene degradation (7, 35, 42, 45). However, little is known about the genetic determinants for PAH degradation among *Acidovorax* strains.

There exists wide genetic diversity among organisms capable of PAH degradation. Members of the *Pseudomonas* genus generally contain the well-studied and commonly encountered *nah*-type genes and are typically associated with the degradation of naphthalene (14, 56). Other organisms contain homologous but significantly different genes. Examples of these include the *nag* genes from *Polaromonas naphthalenivorans* (16) and *Ralstonia* sp. strain U2 (58), the *phn* genes of *Burkholderia* sp. strain RP007 (29), and the *nid* genes of *Mycobacterium vanbaalenii* (20). Multiple attempts have been made by researchers to design specific PCR primer sets for the detection of the RHD (or initial dioxygenase) gene, whose product catalyzes the first reaction in the aerobic bacterial metabolism of PAHs (5, 30, 33, 36, 54). More recently, degenerate primers based on the known diversity of dioxygenase sequences from both gram-negative and gram-positive organisms have been designed (8). Yet, despite their frequent association with PAH degradation, *Acidovorax* sequences do not appear in environmental surveys using these primer sets due to the lack of reference sequences from isolates in the genus.

In this study we describe the isolation and PAH-degradative properties of a phenanthrene-degrading *Acidovorax* isolate representative of those members of the genus previously detected by SIP in the same soil. We identified and sequenced the putative upper-pathway genes of phenanthrene degradation

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▽ Published ahead of print on 6 March 2009.

and determined compounds that induce expression of those genes.

MATERIALS AND METHODS

Chemicals. PAHs, their purities, and vendors were as follows: naphthalene (99+%; Aldrich, Milwaukee, WI), phenanthrene (>96%; Sigma, St. Louis, MO), anthracene (scintillation grade; Kodak, Rochester, NY), pyrene (product P-2146; Sigma), fluoranthene (98%; Aldrich), chrysene (98%; Aldrich), benz[*a*]anthracene (1,2-benzanthracene) (99%; Aldrich), and benzo[*a*]pyrene (97%; Aldrich). Salicylic acid (>99%) was obtained from Aldrich and phthalic acid (99%) from Acros (NJ). ^{14}C-labeled compounds used for mineralization experiments, their purities, specific activities, and vendors were as follows: [5,6-^{14}C]benz[*a*]anthracene (>98%, 54.6 mCi/mmol; Chemsyn, Lenexa, KA), [7-^{14}C]benzo[*a*]pyrene (≥98%, 26.6 mCi/mmol; Sigma), [5,6,11,12-^{14}C]chrysene (≥98%, 47.6 mCi/mmol; Chemsyn), [3-^{14}C]fluoranthene (≥98%, 45 mCi/mmol; Sigma), [benzene-UL-^{14}C]naphthalene (>98%, 17.8 mCi/mmol; Sigma), [9-^{14}C]phenanthrene (>98%, 8.3 mCi/mmol; Sigma), [ring-UL-^{14}C]phthalic acid (>98%, 12.7 mCi/mmol; Sigma), [4,5,9,10-^{14}C]pyrene (~95%, 61 mCi/mmol; Sigma), and [ring-UL-^{14}C]salicylic acid (>98%, 10 mCi/mmol; Sigma).

Isolation and growth media. Contaminated soil from the site of a former manufactured gas plant, which had been treated in a lab-scale, aerobic bioreactor (45), was enriched for phenanthrene degraders by adding crystalline phenanthrene in excess of its aqueous solubility to a flask containing slurry and incubating the vessel for 2 weeks. Strain NA3 was isolated by plating serial dilutions of the enriched slurry on Difco nutrient agar plates (pH 7.0) (Becton, Dickinson, and Co., Sparks, MA). The identity of the isolated organism was confirmed by 16S rRNA gene sequencing (see below), and it was maintained on nutrient agar or in Difco nutrient broth (NB) (pH 7.0) (Difco Laboratories, Detroit, MI). To test for indigo production, indole (99+%; Aldrich) was added to nutrient agar at a final concentration of 1 mM. For testing of growth substrates, strain NA3 was grown in liquid MM2 medium (24) containing 2 g · liter^{-1} of the carbon source added in solid form postautoclaving. Substrates for these tests included naphthalene, phenanthrene, anthracene, fluoranthene, pyrene, phthalate, and salicylate.

Mineralization. Strain NA3 was grown to high turbidity in 300 ml of NB (pH 7.0) at 30°C. The volume was divided in half and each half centrifuged at 3,000 × *g* for 5 min to pellet cells. The liquid was discarded and the pellets resuspended in a total of 75 ml of fresh M9 medium (43). One of those volumes was then treated with 85% phosphoric acid to a pH of <2 as a killed control. The experimental and acidified suspensions were allow to sit at room temperature for 1 h prior to dispensing 2.5 ml (approximately 2.4 × 10^9 cells) into sterile 40-ml amber-glass EPA vials. The number of cells in the live sample was determined by plating serial dilutions of cells on nutrient agar plates. An additional 2.5 ml of M9 medium was added to each vial to bring the total volume to 5 ml. Resuspended and acid-killed cells were created in triplicate for each of the tested compounds. Approximately 20,000 dpm of each test compound was then added directly to the cell suspension. A sterile 12- by 75-mm glass test tube containing a piece of filter paper saturated with 60 μl of 2 M KOH to act as a CO_2 trap was placed in each vial. The vial was sealed with an aluminum foil-covered, Teflon-seal cap and incubated at 30°C with gentle agitation for 12 h. At the end of the incubation, the filter paper was removed and the captured ^{14}C counted on a Packard (Meriden, CT) Tri-Carb liquid scintillation analyzer, model 1900 TR. Differences in captured ^{14}C between live and acidified controls were tested for significance using Student's *t* test (ProStat for Windows, v.4.02; Poly Software International, Inc., Pearl River, NY). To determine the percentage of added ^{14}C mineralized, the mean of the values for the acidified controls for each compound was subtracted from each of the triplicate experimental values and divided by the total dpm of ^{14}C added.

Genomic fosmid library construction and analysis. A genomic library was constructed from strain NA3 DNA using the CopyControl fosmid library production kit with the pCC1FOS vector and the phage T1-resistant EPI300 *Escherichia coli* plating strain (Epicentre, Madison, WI). Briefly, high-molecular-weight DNA was isolated from cells grown in NB using a Wizard genomic DNA purification kit (Promega, Madison, WI) and sheared to ~40 kb by pipetting. The fragments were end repaired, ligated into the fosmid vector, and packaged, and the host cells were grown overnight following the kit instructions. Colonies were selected and transferred to LB plates containing chloramphenicol.

Colonies on plates were then tested for the presence of genes homologous to the naphthalene RHD gene of *Pseudomonas putida* G7. A digoxigenin (DIG)-labeled nucleotide probe was generated by PCR using as the template DNA extracted from *Pseudomonas putida* G7 and with primers specific for a portion of the *nahAc* gene (primers NAH-F and NAH-R) (5).

Colonies were transferred to nylon membranes (Roche, Indianapolis, IN) per the manufacturer's instructions. Hybridizations with the DIG-labeled *Pseudomonas nahAc* amplicon were carried out per the manufacturer's instructions using DIG Easy Hyb (Roche) and DIG Wash and Block buffer sets (Roche) with final stringency washes at 45°C. *Pseudomonas putida* G7 DNA was added to each membrane as a positive control. The presence of DNA that bound the probe was detected by enzyme immunoassay and enzyme-catalyzed color reaction with nitroblue tetrazolium chloride–5-bromo-4-chloro-3-indolyl phosphate as part of the DIG nucleic acids detection kit (Roche). Positive colonies were transferred to LB broth containing chloramphenicol and induced to high copy number with the CopyControl induction solution (Epicentre) before fosmid isolation with the FosmidMAX DNA purification kit (Epicentre).

Molecular analyses. The 16S rRNA gene of strain NA3 was amplified by PCR with primers 8f (10) and 1492r (27). The resulting product was then cloned into the plasmid PCR4-TOPO using the TOPO-TA cloning kit for sequencing (Invitrogen, Carlsbad, CA). The insert was sequenced with primers M13R, M13F, and 341F (37); 338R (2); 907F (28); and 939R (4) at the University of North Carolina Genome Analysis Facility. Sequences were assembled using the program Sequencher 4.8 (Gene Codes Corp., Ann Arbor, MI).

An isolated fosmid which hybridized the *nahAc* gene of *Pseudomonas* (designated phnNA3.1) was digested with BamHI (New England Biolabs, Ipswich, MA), Sau3A1, and a combination of the two enzymes. The resulting fragments were separated by agarose gel electrophoresis and the DNA transferred to a membrane and analyzed by Southern hybridization as described above. A new fosmid digest was performed solely with BamHI, the resulting fragments were ligated into plasmid pCR2.1 (Invitrogen) using standard methods (43), inserts were screened by PCR with M13F and M13R, and one insert of approximately 800 bp was sequenced with primer M13R. Subsequent cloning and sequencing efforts employed a combination of primer walking and PCR/sequencing primer design based on GenBank entry AB024945 (*Alcaligenes faecalis* phenanthrene degradative gene cluster) to recover the *Acidovorax* gene sequences of interest. To avoid the introduction of erroneous base calls from the *Alcaligenes* sequence, all primer sequences were trimmed from the recovered sequences prior to assembly of the fragments in Sequencher.

For comparisons of gene organization, open reading frames (ORFs) from NA3 and other organisms whose sequences were recovered from GenBank were visualized with the frames program of the GCG suite of programs (Accelrys Inc., San Diego, CA). ORFs were identified and labeled according to the results of BLAST searches (1) and GenBank entry header information. The resulting images were then scaled based on the size of the RHD large-subunit gene and aligned according to the same.

To generate phylogenetic trees, sequences were first aligned with the Pileup program of the GCG suite of programs prior to constructing neighbor-joining trees with the ClustalW program (48). Trees were bootstrapped 1,000 times, and gaps in the alignment were ignored.

Pulsed-field gel electrophoresis (PFGE) was combined with Southern blotting to determine whether the identified genes were located on a chromosome or plasmid. Agarose-embedded NA3 DNA was prepared according to the instructions provided in the CHEF-DR III pulsed-field electrophoresis systems (Bio-Rad, Hercules, CA) manual. S1 nuclease treatment was performed according to the method of Basta et al. (6). Gels were run under the following conditions on the CHEF-DR III pulsed-field electrophoresis system: (i) separation of high-molecular-weight DNA, with *Saccharomyces cerevisiae* chromosomal standards (Bio-Rad), 1% agarose gel, 60- to 120-s switch, 20 h, 6 V/cm, 105° angle, and 0.5× Tris-borate-EDTA running buffer, or (ii) separation of mid-range DNA, with a λ concatemer ladder and same conditions as above except for a 120° angle. Transfer of DNA for Southern hybridizations was performed as described in the CHEF-DR III instruction manual, and hybridizations were performed as described above using a DNA fragment spanning the *phnAcd* genes of strain NA3 as a probe.

Primer sets suitable for quantitative real-time PCR (qPCR) were designed to amplify fragments of the *phnAc*, *phnB*, and *phnC* genes of *Acidovorax* sp. strain NA3 (Table 1). Standard curves for the quantification of *phn* genes were created by digesting fosmid phnNA3.1 with SmaI (New England Biolabs), quantifying the amount of DNA using a NanoDrop ND-3300 fluorospectrometer (Thermo Fisher Scientific, Wilmington, DE), and performing qPCR with serial dilutions of the digested fosmid. Standard curves for the quantification of *Acidovorax* 16S rRNA genes were generated with BamHI-digested 16S rRNA gene clone PHE7d8 (45) as described previously (44). Amplification efficiencies (39) of the primer sets for *phnAc*, *phnB*, *phnC*, and *Acidovorax* 16S rRNA genes were 1.92, 1.92, 1.94, and 1.90, respectively. The 16S rRNA and *phnAc* reactions were run with an annealing temperature of 55°C, and the *phnB* and *phnC* reactions were run at 60°C.

TABLE 1. Quantitative real-time PCR primers

Primer	Target	Sequence (5′→3′)	Amplicon size (bp)	Annealing temp (°C)	Reference
phnAc-F phnAc-R	Ring-hydroxylating dioxygenase gene, *phnAc*	GAC AGC TTG ATT CCG TGC AAC TGA ACG CAC	171	55	This study
phnB-F phnB-R	Dihydrodiol dehydrogenase gene, *phnB*	TGT CCC CCT TGT CGA CC TAT AGA GCA CGC CGC CG	187	60	This study
phnC-F phnC-R	Ring cleavage dioxygenase gene, *phnC*	CAT TCT GCG ATC CGT AGA CC CCA CGG AAT GCT CAC GG	148	60	This study
AcidF AcidR	*Acidovorax* 16S rRNA gene	TAA CGG AGC GAA AGC TT GTC CGC GCA AGG CCT T	75	55	44

Fosmid phnNA3.1 was used as a template to test whether degenerate PCR primers for the amplification of the initial RHD would be successful for NA3 using the methods of Cébron et al. (8).

***phn* gene induction.** Strain NA3 was grown overnight in NB (pH 7.0) at 30°C with shaking at 225 rpm. To 5 ml of fresh NB was added 100 μl of the overnight culture in triplicate for each compound tested. Cultures were allowed to grow several hours at 30°C with shaking to an optical density at 600 nm of approximately 0.12 before 100 μl of a methanol solution containing a given compound was added. Each PAH was added at or slightly above its aqueous solubility (32). The compounds and their nominal concentrations in the medium were as follows: naphthalene,21 mg/liter; phenanthrene, 1 mg/liter; pyrene, 0.13 mg/liter; fluoranthene, 0.2 mg/liter; chrysene, 0.002 mg/liter; benz[*a*]anthracene, 0.01 mg/liter; benzo[*a*]pyrene, 0.003 mg/liter; salicylate, 100 mg/liter; and phthalate, 100 mg/liter. Additionally, methanol-only (100 μl) and no-carbon-added controls were also performed in triplicate. After addition of each compound, tubes were returned to the shaker for 10 min before 500 μl of each culture was removed and added to 1 ml of RNAprotect Bacteria Reagent (Qiagen, Valencia, CA). Each sample was vortexed, centrifuged, and stored at −20°C as suggested by the manufacturer (41).

Total RNA was extracted and purified from each cell pellet individually according to protocols 4 and 7 of reference 41 using an RNeasy Mini Kit (Qiagen). RNA was eluted in a total of 30 μl of RNase-free water (Fisher Scientific, Pittsburgh, PA). For removal of genomic DNA and creation of cDNA, 12 μl of extracted total RNA from each cell pellet was first treated with genomic DNA wipeout buffer before addition of reverse transcriptase, buffer, and the RT primer mix included with the QuantiTect reverse transcription kit (Qiagen), per the manufacturer's instructions.

The transcript number was quantified with a SmartCycler (Cepheid, Sunnydale, CA) and QuantiTect SYBR green PCR kit (Qiagen). For the template, 1 μl of the cDNA solution was used for each reaction containing primers for the *phn* transcripts, and 1 μl of cDNA diluted 1:1,000 was used in the qPCR for 16S rRNA transcripts. All qPCR reactions were run at least in duplicate. The quantity of *phn* transcripts for each sample was normalized to 10^8 16S rRNA transcripts per μl of cDNA to allow direct comparison.

Nucleotide sequence accession numbers. Sequences generated in this study were deposited in GenBank with the accession numbers EU910093 and EU910094.

RESULTS

Growth. Strain NA3 was isolated on nutrient agar from phenanthrene-enriched PAH-contaminated soil (44). The addition of indole to nutrient agar plates resulted in purple colonies from the production of indigo and was indicative of an active dioxygenase (11). In MM2 medium, strain NA3 grew on phenanthrene as a sole source of carbon and energy after 6 days but did not display visibly turbid growth with naphthalene, anthracene, fluoranthene, pyrene, phthalic acid, or salicylic acid.

Phylogeny. The nearly complete 16S rRNA gene sequence of strain NA3 indicated that the bacterium was a member of the *Acidovorax* genus (Fig. 1). It was most similar to two sequences from the same bioreactor-treated, PAH-contaminated soil recovered during an SIP experiment with [U-^{13}C]phenanthrene (45), with 99.93% sequence similarity to clone PHE7d7 and 99.80% similarity to clone PHE7d8. It also shared similarity to clone sequences from Siberian deep-well groundwater (clone S15A-MN11) (unpublished), BTEX contaminated groundwater (clone W18i3) (unpublished), and a tar-oil-impacted aquifer BTEX plume (clone D12_21) (55).

Mineralization. Cells of NA3 in M9 medium were incubated in the presence of ^{14}C-labeled PAHs and PAH metabolites. After 12 h of incubation, significant levels of CO_2 production from labeled substrates compared to acidified controls (Student's *t* test, $P < 0.05$) were observed for naphthalene (5.9% ± 0.2% of added ^{14}C mineralized), phenanthrene (23.7% ± 10.7%), chrysene (3.9% ± 2.1%), benz[*a*]anthracene (6.2% ± 0.5%), benzo[*a*]pyrene (0.2% ± 0.01%), and phthalic acid (0.05% ± 0.01%). Strain NA3 did not mineralize pyrene, fluoranthene, or salicylate to a greater extent than acidified controls. In separate experiments, longer incubations of labeled benzo[*a*]pyrene and phthalic acid resulted in higher levels of $^{14}CO_2$ production than observed during this test (data not shown).

Genetics. Southern hybridizations using the *nahAc* gene of *Pseudomonas putida* G7 as a probe successfully identified a fosmid clone from the genomic library (designated phnNA3.1) containing a homologous gene. Digestion of the fosmid and subcloning and sequencing of an approximately 800-bp piece of DNA identified a gene fragment with nearly 100% nucleotide identity to the *phnC* gene of *Alcaligenes faecalis* AFK2 (GenBank accession number AB024945). The sequences of the putative large and small subunits of the RHD (*phnAcd*), dihydrodiol dehydrogenase (*phnB*), and 3,4-dihydroxyphenanthrene dioxygenase (*phnC*) genes were specifically targeted and determined through a combination of primer walking from the *phnC* gene fragment and use of the AFK2 operon as a template to design specific sequencing primers for recovering the other genes of interest. A total of 8,286 nucleotides in three discontinuous regions of 4343, 1344, and 2599 bases were sequenced, and all of the recovered sequences were present on fosmid phnNA3.1. The arrangement of recovered genes and gene fragments suggests that the *phn* operon of *Acidovorax* strain NA3 is organized similarly to that of *Alcaligenes faecalis* AFK2 (Fig. 2). Partial sequences of other genes from NA3

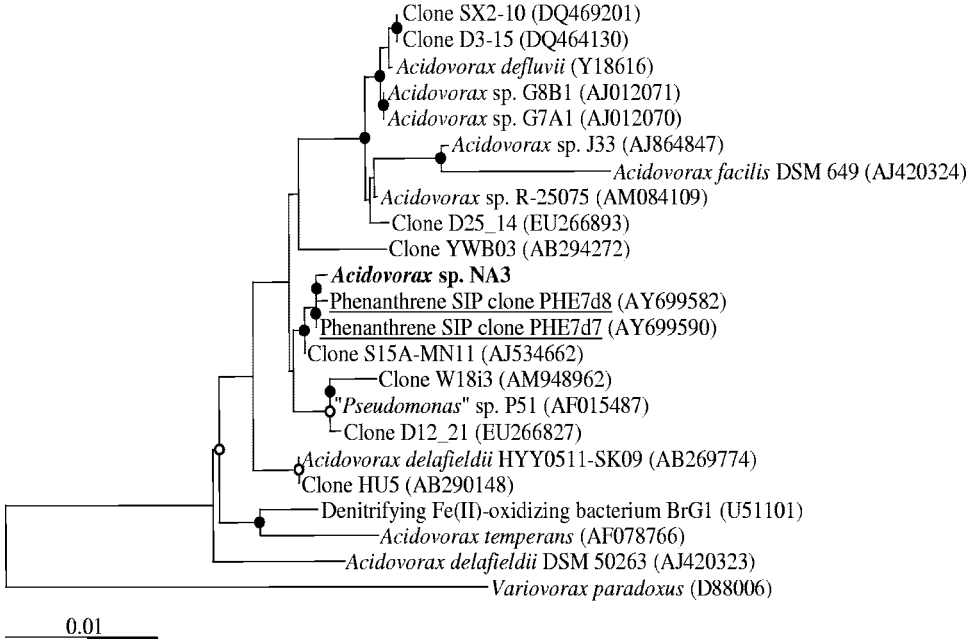

FIG. 1. Neighbor-joining phylogenetic tree of 16S rRNA gene sequences showing the relationship of strain NA3 to highly similar clone and isolate sequences. Strain NA3 is shown in bold, while clonal sequences from the same environmental sample are underlined. The tree was constructed from 1,285 aligned bases. Open and closed circles at nodes represent >50 and >95% bootstrap support, respectively. GenBank accession numbers are indicated in parentheses. The scale bar indicates the number of substitutions per position. The chlorinated aromatic compound degrader *Pseudomonas* sp. strain P51 was originally classified as a pseudomonad based on physiological data (51). However, based on the 16S rRNA gene sequence of the organism, strain P51 appears to be a member of the *Acidovorax* genus (47).

(*phnH*, *phnG*, and *phnF*) supported this finding as well, and although the sequences between the three fragments were not determined, PCR analyses confirmed their arrangement as presented in Fig. 2 (data not shown). This organization of genes in strains NA3 and AFK2 differs from that of other well-known PAH degradation operons. Notably, the putative dihydrodiol dehydrogenase gene appears upstream of the large subunit of the initial RHD, and the ring cleavage dioxygenase (*phnC*) is separated from the other genes of the upper pathway.

The large subunit of the RHD is the most well studied of the PAH degradation genes. While the predicted protein sequence of the large subunit of the RHD in NA3 was similar to that of *Alcaligenes faecalis* AFK2, the protein sequences with the highest similarity were found in several recently cultivated *Burkholderia* strains (Fig. 3).

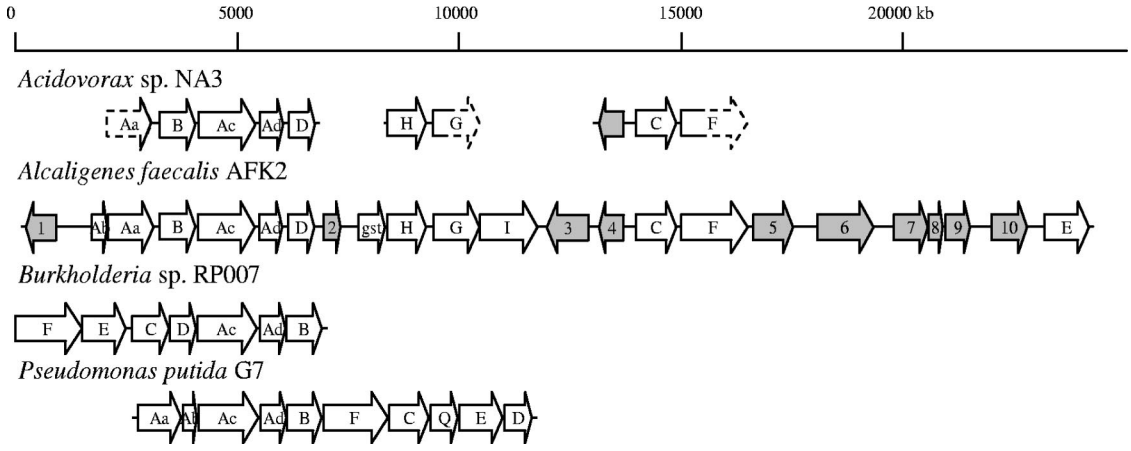

FIG. 2. Organization of sequenced genes from *Acidovorax* strain NA3 aligned to homologous genes from *Alcaligenes faecalis* AFK2 (GenBank accession number AB024945), *Pseudomonas putida* G7 (AB237655), and *Burkholderia* sp. strain RP007 (AF061751). Letters indicate the gene designations for each PAH degradation operon (gst, glutathione *S*-transferase). Numbered or shaded ORFs are presumably unaffiliated with PAH degradation. Sequenced ORFs for NA3 are shown with solid arrows, while segments of genes for which the complete sequence was not obtained are indicated with dashed lines. Unsequenced areas of the NA3 operon lack a horizontal line. Operons were aligned by the RHD large subunit (Ac).

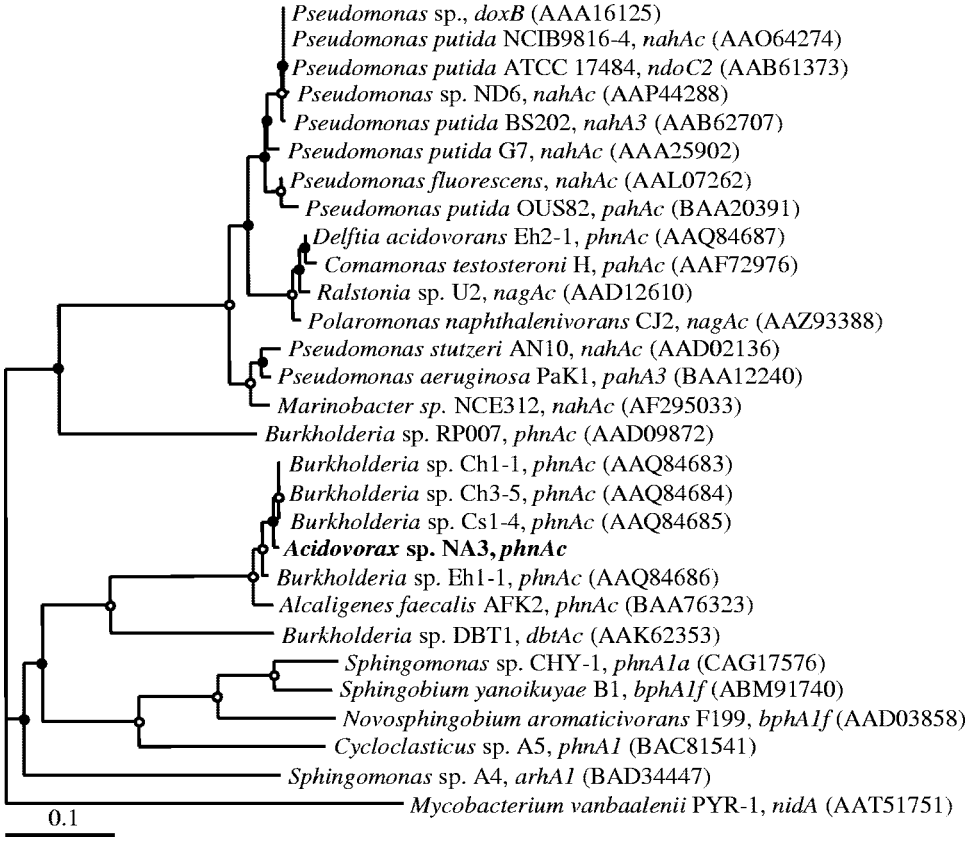

FIG. 3. Neighbor-joining phylogenetic tree based on predicted amino acid sequences of the large subunit of the RHD for *Acidovorax* strain NA3 and other gram-negative bacteria. The tree was based on 189 aligned amino acids. The *nidA* gene of *Mycobacterium vanbaalenii* was used as an outgroup. Other notation is as described in Fig. 1.

After the identification of the *phn* genes in strain NA3, fosmid phnNA3.1 was used to test whether degenerate PCR primers for the RHD gene designed by Cébron et al. could amplify the *Acidovorax phnAc* gene (8). Despite significant predicted mismatches (4 of 24 bases and 8 of 28 bases for the forward and reverse gram-negative organism RHD primers, respectively), a weak PCR amplicon of the expected size was produced (data not shown).

Localization of *phn* genes. PFGE was used to determine whether the detected *phn* genes were located on the chromosome or on a plasmid. No low-molecular-weight DNA that might be indicative of a plasmid was detected in any PFGE runs, and Southern hybridization with a DIG-labeled *phnAcd* fragment showed hybridization only with very high-molecular-weight DNA in or near the wells (Fig. 4 and data not shown), suggesting that the genes were located on the chromosome.

Induction. To test the effect of compound addition on the expression of three genes putatively involved in PAH degradation, selected PAHs were added at or near their aqueous solubility limits to NA3 cells grown in NB. Two PAH metabolites, salicylate and phthalate, were also added at a 100-mg/liter final concentration. Of the compounds tested only two, naphthalene and phenanthrene, strongly induced the expression of the *phnAc*, *phnB*, and *phnC* genes compared to unamended controls (Table 2). All other compounds, including methanol (the carrier solvent for the addition of test compounds), pyrene, chrysene, fluoranthene, benz[*a*]anthracene, benzo[*a*]pyrene, salicylate, and phthalate, produced much smaller or no responses. For the two compounds that did elicit a pronounced response, the levels of induction were similar for the *phnAc* and *phnB* genes, and although the levels of *phnC* induction were lower, they were still significantly higher than basal levels of expression as determined by the quantification of transcripts in unamended cultures.

DISCUSSION

Members of the *Acidovorax* genus are frequently encountered in association with PAH degradation, notably that of phenanthrene, but prior to this work little was known of the underlying genetic determinants behind the phenotype. The particular *Acidovorax* strain used in this study has genes very similar in sequence and arrangement to those in *Alcaligenes faecalis* AFK2, and the initial dioxygenase sequence is also highly similar to those in several *Burkholderia* strains, although not to that of *Burkholderia* sp. strain RP007. These observations suggest that this particular genotype may be widespread among some PAH-degrading members of the order *Burkholderiales*.

Unfortunately for comparative purposes, while the genes determined in this study bear significant resemblance to the *Alcaligenes faecalis* AFK2 sequences deposited in GenBank,

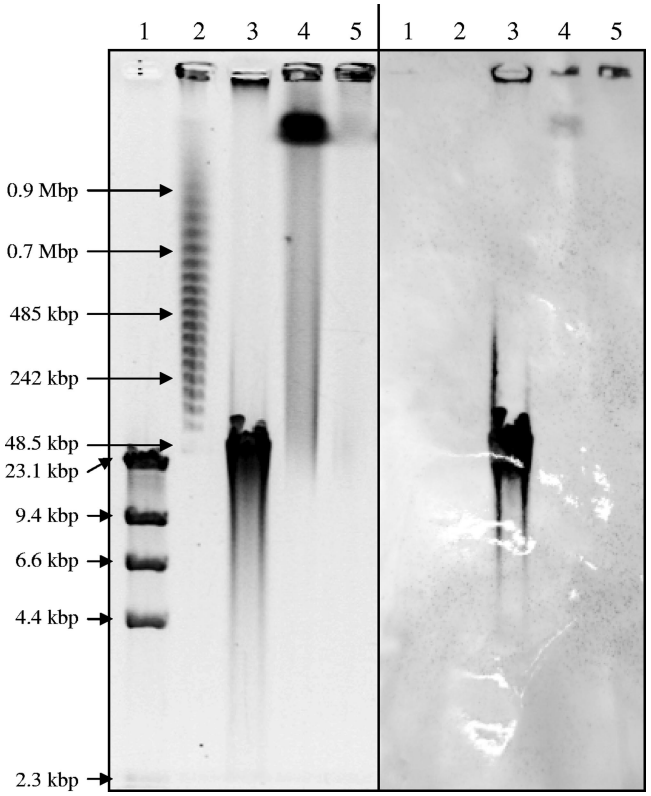

FIG. 4. (Left) Pulsed field electrophoresis gel of λHindIII digest markers (lane 1), λ concatemer marker (lane 2), S1 nuclease-digested fosmid phnNA3.1 (lane 3), S1 nuclease-digested NA3 genomic DNA (lane 4), and undigested NA3 genomic DNA (5). (Right) Southern blot of the same gel using the *phnAcd* genes of NA3 as a probe. Sizes of the markers are indicated on the left.

there does not appear to be a publication associated with the entry (GenBank accession number AB024945). However, other previously published research on AFK2 revealed the presence of two plasmids, the larger of which (named pHK2, 42.5 kb) conferred the phenanthrene degradation phenotype to a *Pseudomonas* strain (25). Presumably the deposited gene sequences originated from that plasmid. In contrast to the case for *Alcaligenes faecalis* AFK2, the phenanthrene degradation genes of *Acidovorax* strain NA3 appear to be located on the chromosome, as no evidence for plasmids was found during this study. However, this appears to be one of few significant differences between the clusters, as even intergenic regions and genes unaffiliated with phenanthrene degradation in NA3 are highly similar to equivalent regions of the AFK2 sequence deposited in GenBank.

Due to the high similarity of the *phn* operon between the NA3 and AFK2 strains, it might be presumed that *Alcaligenes faecalis* AFK2 may actually be a member of the *Acidovorax* genus. The *Acidovorax* genus was created in 1990 (53), well after AFK2 was first described in 1982 (23). Not only did the newly created *Acidovorax* genus include organisms previously characterized as *Alcaligenes* strains, but many traits used to define AFK2 as an *Alcaligenes* species (e.g., flagellated, oxidase positive, and catalase positive) were also shared by the *Acidovorax* genus (15, 53). In support of its current classification, however, AFK2 was reported as being unable to grow on D-glucose (23), a ubiquitous trait in *Acidovorax* strains (53). Additionally, numerous other examples of PAH-degrading *Alcaligenes* species, including some associated with phenanthrene degradation, have been reported recently (see, e.g., references 3, 49, and 52). Unfortunately, the ribosomal sequences of AFK2 are not available in public databases, and the organism does not appear to be available in either the ATCC or DSMZ culture collections in order to verify its genus affiliation.

Organisms containing the type of *phn* genes present in NA3 and other related strains appear to be associated primarily with the degradation of three-ring PAHs (notably phenanthrene). Of the compounds tested, only phenanthrene supported the growth of NA3, and the highest level of mineralization was observed for phenanthrene. Similarly, the highest levels of gene induction were observed when phenanthrene was added to the culture medium. *Alcaligenes faecalis* AFK2 was described as a phenanthrene- and anthracene-degrading strain (23), and the *Burkholderia* isolates with *phnAc* genes highly similar to that in NA3 were isolated on humic acid-absorbed phenanthrene (50). Additional support for this apparent association comes from a recent survey of RHDs from PAH-contaminated sediments that found only AFK2-type or closely related *phnAc* genes in samples containing solely phenanthrene (31).

It was expected that transcription of the *phnAc*, *phnB*, and *phnC* genes in *Acidovorax* strain NA3 would be induced by the addition of phenanthrene to the culture media. There is evidence for increased protein expression of RHDs in *Mycobacterium* strains when exposed to various PAHs (21, 26), and phenanthrene has been shown to increase the expression of the *nahAc* gene in *Pseudomonas putida* G7 (34). In environmental samples, increases in both *nidA* and *nahAc* transcripts could be detected in sediments when PAHs were added (57). The nearly equivalent levels of increased expression of both the *phnAc* and *phnB* genes in NA3 when exposed to either phenanthrene or naphthalene suggest that the genes are cotranscribed. The *phnAabcd* genes encode the four subunits of the initial RHD, and the *phnB* gene is located in the middle of that cluster, so it is likely that the parent compound induces the expression of both *phnAc* and *phnB*. However, in both *Acidovorax* strain

TABLE 2. Increases in expression of strain NA3 *phn* genes in response to addition of PAH or PAH metabolites compared to unamended controls[a]

Compound	Fold increase in expression		
	phnAc	*phnB*	*phnC*
Methanol	0	0	1
Naphthalene	18	17	8
Phenanthrene	34	34	12
Pyrene	1	1	0
Chrysene	0	0	0
Fluoranthene	1	1	0
Benz[*a*]anthracene	0	0	0
Benzo[*a*]pyrene	−1	0	0
Salicylate	0	0	0
Phthalate	0	0	0

[a] Calculated levels in the unamended controls for *phnAc*, *phnB*, and *phnC* transcripts per 10^8 16S rRNA transcripts were $1.2 \times 10^4 \pm 4.0 \times 10^3$, $2.8 \times 10^4 \pm 5.8 \times 10^3$, and $2.4 \times 10^4 \pm 3.1 \times 10^3$, respectively.

NA3 and *Alcaligenes faecalis* AFK2, the gene encoding the third protein in the upper pathway of PAH degradation (*phnC*) is distinct from the *phnABD* cluster and is likely controlled by separate transcriptional regulators. For both phenanthrene and naphthalene, the level of induction for *phnC* was lower than that for *phnAc* and *phnB*, and it is uncertain whether the expression of *phnC* is influenced by the parent compound or a metabolite such as 3,4-dihydroxyphenanthrene. It was also interesting that the presence of naphthalene could induce the expression of *phnAc*, *phnB*, and *phnC* in NA3, because the organism could not utilize naphthalene as a sole source of carbon and energy in the defined media tested and *Acidovorax* sequences did not appear in ^{13}C-enriched DNA fractions during previous SIP experiments with naphthalene (45).

Phenanthrene is typically degraded through a pathway that utilizes either salicylate or phthalate as an intermediate (13). While the pathway utilized by NA3 was not explicitly tested during this experiment, we found it incapable of growth on either compound under the conditions tested, and neither salicylate nor phthalate induced expression of the upper pathway *phn* genes. Additionally, it did not mineralize salicylate but was capable of weakly mineralizing phthalate. Based solely on these results, it would be difficult to speculate which pathway NA3 might utilize in the metabolism of phenanthrene. However, there are indications that *Alcaligenes faecalis* AFK2 utilizes the phthalate pathway (23, 38), and given the similarities in the *phn* genes of NA3 and AFK2 and the weak mineralization of phthalate by NA3, metabolism of phenanthrene through phthalate is probably the case for this *Acidovorax* strain as well.

ACKNOWLEDGMENTS

Liza Guzmán Ramirez was supported by a grant from the National Institute of General Medical Sciences' Bridges to the Doctorate Program. This work was supported by the National Institute of Environmental Health Sciences (grant 5 P42 ES05948).

We thank Beatriz Zayas, Alberto Rivera, and Donald Fox for their contributions to the summer internship program.

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