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Molecular Characterization and Substrate Preference of a Polycyclic Aromatic Hydrocarbon Dioxygenase from *Cycloclasticus* sp. Strain A5

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***Cycloclasticus* sp. strain A5 is able to grow with petroleum polycyclic aromatic hydrocarbons (PAHs), including unsubstituted and substituted naphthalenes, dibenzothiophenes, phenanthrenes, and fluorenes. A set of genes responsible for the degradation of petroleum PAHs was isolated by using the ability of the organism to oxidize indole to indigo. This 10.5-kb DNA fragment was sequenced and found to contain 10 open reading frames (ORFs). Seven ORFs showed homology to previously characterized genes for PAH degradation and were designated *phn* genes, although the sequence and order of these *phn* genes were significantly different from the sequence and order of the known PAH-degrading genes. The *phnA1*, *phnA2*, *phnA3*, and *phnA4* genes, which encode the α and β subunits of an iron-sulfur protein, a ferredoxin, and a ferredoxin reductase, respectively, were identified as the genes coding for PAH dioxygenase. The *phnA4A3* gene cluster was located 3.7 kb downstream of the *phnA2* gene. PhnA1 and PhnA2 exhibited moderate (less than 62%) sequence identity to the α and β subunits of other aromatic ring-hydroxylating dioxygenases, but motifs such as the Fe(II)-binding site and the [2Fe-2S] cluster ligands were conserved. *Escherichia coli* cells possessing the *phnA1A2A3A4* genes were able to convert phenanthrene, naphthalene, and methylnaphthalene in addition to the tricyclic heterocycles dibenzofuran and dibenzothiophene to their hydroxylated forms. Significantly, the *E. coli* cells also transformed biphenyl and diphenylmethane, which are ordinarily the substrates of biphenyl dioxygenases.**

Polycyclic (fused) aromatic hydrocarbons (PAHs) are a group of hydrocarbons composed of two or more fused benzene rings that occur mostly as a result of fossil fuel combustion and as other by-products of industrial processing. PAHs are released into the marine environment by processes such as accidental discharges during the transport, use, and disposal of petroleum products, marine seepage, and forest and grass fires. PAHs are concentrated in urban marine sediments by industrial processes. Some PAHs are cytotoxic, genotoxic, and carcinogenic to marine organisms and may be transferred to humans through seafood consumption (33, 36, 54).

One group of bacteria capable of degrading aromatic compounds in marine environments belongs to the genus *Cycloclasticus*. These bacteria utilize a limited number of organic compounds as sole carbon sources, including aromatic hydrocarbons such as toluene, xylene, biphenyl, naphthalene, and phenanthrene (8, 14). It has recently been reported that *Cycloclasticus* bacteria play an important role in the degradation of petroleum PAHs in a marine environment (24). Also, four *Cycloclasticus* strains have been isolated from seawater by using phenanthrene as the sole carbon and energy source, and the role of these organisms in the biodegradation of petroleum PAHs in a marine environment has been investigated (24). These strains could grow on petroleum and degraded unsubstituted and substituted PAHs, such as phenanthrene, naphthalene, methylnaphthalene, fluorene, and dibenzothiophene (24).

The results of a previous study suggested that obligate marine bacteria may be more significant PAH degraders in a coastal marine environment than bacteria from terrestrial habitats, such as *Pseudomonas* sp. (51, 52). However, the PAH degradation mechanisms of obligate marine bacteria have been only partially studied (18, 19, 20, 21, 46, 59). In contrast, the enzymes involved in PAH degradation and genetic regulation have been extensively characterized in terrestrial isolates, including isolates of *Pseudomonas* and *Sphingomonas* species (6, 7, 11, 13, 25, 26, 30, 35, 55, 57).

In the present study, we isolated a set of genes that are for degradation of naphthalene, methylnaphthalene, phenanthrene, and dibenzothiophene from the marine bacterium *Cycloclasticus* sp. strain A5 and analyzed the structure and function of the genes and the enzymes which they encode.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Cycloclasticus* sp. strain A5 was grown in an ONR7a medium (8) with naphthalene or phenanthrene at 25°C. *Escherichia coli* XL1-Blue was used for routine genetic manipulation. *E. coli* cells containing recombinant plasmids were maintained on Luria-Bertani (LB) agar plates (49) supplemented with appropriate antibiotics. The concentration of the antibiotics used was 50 μg/ml for both ampicillin and kanamycin.

Molecular techniques. Standard procedures were used for plasmid DNA preparation and manipulation and for agarose gel electrophoresis (49). Total genomic DNA of *Cycloclasticus* sp. strain A5 was isolated by the method of Marmur (34). Plasmid DNA for sequencing was isolated with a QIAprep Spin Miniprep kit (Qiagen), and the cloned sequences were determined by using a *Taq* DyeDeoxy terminator cycle sequencing kit and a model 377 sequencer

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or phenotype	Reference or source
Strains		
Cycloclasticus sp. strain A5	Degrades PAHs. such as naphthalene. phenanthrene. biphenyl. etc.	24
E. coli JM109	*recA1 relA1 thi-1* Δ(*lac-proAB*) *gyrA96 hsdR17 endA1 supE44* (r_k^- m_k^-) [F7 *traD36 proAB lacI*^{r} *Z*ΔM15]	60
E. coli XL1-Blue	*hsdR17 supE44 recA1 endA1 gyrA46 thi-1 relA1 lac*/F′ [*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15::Tn*10* (Tet^r)]	Stratagene
E. coli XL1-Blue MRA	Δ(*mcrA*) *183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA96 gyrA1 relA1 lac*	Stratagene
Plasmids		
Lorist6	Km^r: 5.28-kb cosmid vector	15
pBluescript II SK+	*lacZ* Ap^r: 2.96-kb cloning vector	Stratagene
pH1a	Km^r Lorist6::11-kb *Sau*3A1 fragment from A5 containing *phnA1A2A3A4*	This study
pH1b	Km^r Lorist6::11-kb *Sau*3A1 fragment from A5 containing *phnA1A2A3A4*	This study
pPhnA	Ap^r: pBluescript:: ring-hydroxylating dioxygenase gene *phnA1A2A3A4* from A5	This study
pPhnC	Ap^r: pBluescript:: extradiol dioxygenase gene *phnC* from A5	This study

(Applied Biosystems). A nucleotide sequence analysis, translation, and alignment with related genes and proteins were carried out by using the SeqED (Applied Biosystems) and GENETYX computer programs. A search of the GenBank nucleotide library for sequences similar to the sequences determined was performed by using BLAST (1) through the National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov/BLAST/). A phylogenetic analysis was performed by using Clustal W, version 1.7 (58), and phylogenetic trees were constructed from evolutionary distance data (28) by the neighbor-joining method (47) by comparing closely related protein sequences. Recombinant plasmids in *E. coli* XL1-Blue were induced with isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h for protein analysis. Crude cell extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by using the method of Laemmli (31).

Construction of the genomic library for *Cycloclasticus* sp. strain A5. Total genomic DNA of *Cycloclasticus* sp. strain A5 was partially digested with restriction endonuclease *Sau*3AI. After separation in a 0.6% agarose gel, the fraction containing fragments in the size range from 10 to 20 kb was recovered. A calf intestinal alkaline phosphatase-treated and *Not*I-digested Lorist6 vector (15) was ligated with the digested *Cycloclasticus* DNA fragments. The ligated DNA was packaged in bacteriophage λ by using a Gigapack III Gold packaging extract in accordance with the manufacturer's instructions (Stratagene, La Jolla, Calif.). The phages were transferred into *E. coli* strain XL1-Blue MRA and plated for colony formation. The library was replica plated onto LB agar plates with kanamycin, and the plates were screened for clones that turned purple or blue-gray as a result of the formation of indigo from indole due to the dioxygenase activity expressed by the clones (10).

Construction of plasmids for overexpression of the enzymes. To express dioxygenase genes under control of the *lac* promoter in *E. coli*, pPhnA and pPhnC were constructed as follows. A 2,723-bp *Pst*I-*Sal*I fragment containing the *phnA1* and *phnA2* genes from cosmid clone pH1a was subcloned into pBluescript II SK+ (Stratagene) digested with *Pst*I-*Sal*I, and the plasmid was designated pISP. The fragment containing ferredoxin and ferredoxin reductase genes was amplified by PCR by using PCR primers pF-Kp (5′-GGGGTACCAATCTTAGCTA ATCTTATTC) and pFr-Xh (5′-TCCCTCGAGTAGGTTAACAATGTTTG AAT) (the introduced *Kpn*I and *Xho*I restriction sites are underlined). The 1,497-bp PCR product was separated by electrophoresis on a 0.8% (wt/vol) low-melting-point agarose gel and purified with a QIAquick gel extraction kit (Qiagen). The amplified DNA was subsequently digested with *Kpn*I and *Xho*I and ligated into *Kpn*I-*Xho*I-digested pISP. In the resulting construct (pPhnA), the *phnA1A2A3A4* genes were oriented so that they could be expressed under the control of the *lac* promoter.

A 2,305-bp *Hin*dIII-*Eco*RV fragment containing the *phnC* gene from cosmid clone pH1a was subcloned into pBluescript II SK+ digested with *Hin*dIII-*Eco*RV, and the resulting plasmid was designated pPhnC.

Whole-cell biotransformations. *E. coli* JM109 harboring pPhnA or *E. coli* JM109 harboring pBluescript II SK+ was grown in LB medium containing 150 μg of ampicillin per ml at 28°C with reciprocal shaking at 175 rpm for 8 h. Four milliliters of this culture was inoculated into 70 ml of M9 medium (49) containing 150 μg of ampicillin per ml, 10 mg of thiamine per ml, and 0.4% (wt/vol) glucose in an Erlenmeyer flask and grown at 28°C with reciprocal shaking at 175 rpm for 16 to 17 h, until the absorbance at 600 nm reached approximately 1. The cells were collected by centrifugation, washed once with the M9 medium, resuspended in 70 ml of fresh M9 medium containing 150 μg of ampicillin per ml, 10 mg of thiamine per ml, 0.4% (wt/vol) glucose, 1 mM (final concentration) IPTG, and each aromatic compound listed in Table 2 at a final concentration of 1 mM, and cultivated in an Erlenmeyer flask at 28°C with reciprocal shaking at 175 rpm for 2 to 3 days. Three independent biotransformation experiments were conducted.

TABLE 2. Substrate specificity of PhnA dioxygenase

Substrate	Product[a]	Conversion rate (%)
Phenanthrene	3,4-Dihydrophenanthrene-3,4-diol (1)	33
Anthracene	ND[b]	
Naphthalene	1,2-Dihydronaphthalene-1,2-diol (2)	71
1-Methylnaphthalene	8-Methyl-1,2-dihydronaphthalene-1,2-diol (3)	93
2-Methylnaphthalene	7-Methyl-1,2-dihydronaphthalene-1,2-diol (4)	56
Dibenzofuran	2-Hydroxydibenzofuran (5)	57
Dibenzothiophene	2-Hydroxydibenzothiophene (6)	21
Pyrene	ND	
Benzo[a]pyrene	ND	
Toluene	ND	
Biphenyl	*o*-Biphenylol (2-phenylphenol) (7)	12
	m-biphenylol (3-phenylphenol) (8)	4
Diphenylmethane	2-Benzylphenol (9)	14
2-Phenylbenzoxazole	ND	

[a] The numbers in parentheses corresponds to the numbers in Fig. 4.
[b] ND, no product detected.

Extraction and HPLC analysis of the converted products. To extract the converted products and the substrates, the same volume of methanol (MeOH) as the volume of the culture medium was added to the culture of the transformed *E. coli* cells and mixed for 30 min. After centrifugation to remove the cells, the culture supernatant was subjected to high-pressure liquid chromatography (HPLC) or to further purification of the converted products.

The liquid phase (80 μl) was passed through an XTerra C_{18} HPLC column (4.6 by 250 mm; Waters) equipped with a photodiode array detector (model 2996; Waters). The column was developed at a flow rate of 1 ml/min with solvent A (H_2O-MeOH, 1:1) for 5 min and then with a no. 3 gradient (Waters) from solvent A to solvent B (MeOH–2-propanol, 6:4) for 15 min, and the maximum absorbance in the 230- to 350-nm range was monitored.

Purification and identification of the converted products. The culture supernatant (700 to 1,400 ml) obtained by the procedure described above was concentrated in vacuo and then extracted twice with 500 ml of ethyl acetate (EtOAc). The resulting organic layer was concentrated in vacuo and analyzed by thin-layer chromatography on silica gel (0.25-mm Silica Gel 60; Merck) developed with the following solvent systems: phenanthrene, hexane-EtOAc (1:1), naphthalene, hexane-EtOAc (1:1), 1-methylnaphthalene, hexane-EtOAc (4:1), 2-methylnaphthalene, hexane-EtOAc (4:1), dibenzofuran, hexane-EtOAc (5:1), dibenzothiophene, hexane-EtOAc (3:1), biphenyl, hexane-EtOAc (10:1), diphenylmethane, and hexane-EtOAc (2:1). The transformed products, as well as the substrates which were in the organic phase, were applied to a silica gel chromatography column (20 by 250 mm; Silica Gel 60; Merck) that was developed with the following solvent systems: phenanthrene, hexane-EtOAc (1:1), naphthalene, hexane-EtOAc (1:1), 1-methylnaphthalene, hexane-EtOAc (4:1), 2-methylnaphthalene, hexane-EtOAc (4:1, stepwise), dibenzofuran, hexane-EtOAc (5:1), dibenzothiophene, hexane-EtOAc (3:1), biphenyl, hexane-EtOAc (15:1), diphenylmethane, and hexane-EtOAc (4:1).

The structure of each transformed product was analyzed by mass spectrometry with a JEOL JMS-AX505W and by nuclear magnetic resonance with a BRUKER AMX400. Tetramethylsilane was used as the internal standard.

Assay of recombinant PAH extradiol dioxygenase. The extradiol dioxygenase gene (*phnC*) was cloned into the pBluescript SK+ vector (Stratagene) and expressed in *E. coli* XL1-Blue. After growth of 20-ml cultures to an absorbance at 600 nm of 0.2, IPTG was added to a concentration of 1 mM, and incubation was continued for 3 h to induce *phnC*. *E. coli* cells were harvested, washed with 0.1 M sodium phosphate buffer (pH 7.5) containing 10% (vol/vol) acetone, and resuspended in 10 ml of the same buffer. The cells were disrupted by sonication, the cellular debris was removed by centrifugation at 20,000 × *g* for 30 min, and the resulting supernatant was used as the crude cell extract. The protein concentration of each crude extract was estimated with a protein assay kit (Bio-Rad Laboratories). The activity against 1,2-dihydroxynaphthalene (1,2-DHN) was determined spectrophotometrically by the method of Kuhm et al. (29); the reaction mixture contained a 50 mM acetic acid–NaOH buffer (pH 5.5) and 1 to 50 μg of protein. The reaction was started by adding 0.4 μmol of 1,2-DHN in 10 μl of tetrahydrofuran, and the initial rate of decrease in the absorbance at 331 nm was measured. A molar extinction coefficient of 2,600 M^{-1} cm^{-1} for 1,2-DHN at 331 nm, as calculated by Kuhm et al. (29), was used. The extradiol dioxygenase activities with other substrates were assayed by measuring formation of the corresponding ring fission products. The absorbance maxima and extinction coefficients used for the ring fission products of the substrates were as follows: 375 nm and 36,000 M^{-1} cm^{-1}, respectively, for catechol (41); 388 nm and 15,000 M^{-1} cm^{-1}, respectively, for 3-methylcatechol; 382 nm and 31,500 M^{-1} cm^{-1}, respectively, for 4-methylcatechol (32); and 434 nm and 22,000 $M^{-1}cm^{-1}$, respectively, for 2,3-dihydroxybiphenyl (56). Each reaction mixture contained 0.1 M sodium phosphate (pH 7.5), 10% (vol/vol) acetone, and 100 μM 2,3-dihydroxybiphenyl, 400 μM 3-methylcatechol, 400 μM 4-methylcatechol, or 400 μM catechol.

Chemicals and reagents. All the chemicals used in this study were the highest purity commercially available. PAHs were purchased from Wako Pure Chemical Industries, and the other chemicals were purchased from Wako Pure Chemical Industries, Difco, Aldrich Chemicals, and Gibco BRL. The enzymes and reagents used for nucleic acid manipulation were purchased from Takara Shuzo.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have been deposited in the DDBJ, EMBL, and GenBank libraries under accession number AB102786.

RESULTS

Cloning and sequencing of the PAH catabolic genes from strain A5. To clone the genes responsible for the early stage of PAH degradation, genomic DNA from *Cycloclasticus* sp. strain A5 was partially digested with restriction endonuclease *Sau*3AI and ligated into *Not*I-digested Lorist6 to generate a genomic library. Two of the resulting constructs, which were designated pH1a and pH1b, had the ability to oxidize indole to indigo, which is indicative of the presence of an aromatic oxygenase gene (10). Restriction mapping of these clones revealed that they had the same 10.5-kb *Sau*3AI fragment.

Analysis of the pH1a nucleotide sequence. The 10.5-kb nucleotide sequence of pH1a was determined, and sequence analysis revealed the presence of nine complete open reading frames (ORFs) and one partial ORF (Fig. 1). Each ORF was initiated by the canonical ATG start codon and was preceded by a ribosomal binding site.

The functions of individual ORFs were deduced from sequence homology to previously described genes and the corresponding amino acid sequences (Table 3). The predicted polypeptide sequences of seven ORFs exhibited high degrees of similarity with the polypeptide sequences used for catabolism of PAHs. The seven ORFs, ORFs 2 to 5 and 8 to 10, were designated the *phnA1b*, *phnA1*, *phnA2*, *phnC*, *phnA4*, *phnA3*, and *phnD* genes, respectively (genes for the degradation of PAHs isolated from the phenanthrene degrader). The order of these *phn* genes, which were not continuously clustered, is different from that previously reported for aromatic ring dioxygenase genes (55, 61).

The predicted amino acid sequence of PhnA1b exhibited 60 to 48% sequence identity with the amino acid sequences of the iron-sulfur protein (ISP) α subunits of aromatic oxygenases from *Sphingomonas* sp. strain P2 (AhdA1d) (43), *Sphingomonas aromaticivorans* strain F199 (BphA1d) (45), and *Sphingomonas* sp. strain P2 (AhdA1c) (43). The phylogenetic analysis revealed that PhnA1b clustered with the large oxygenase subunits that catalyzed the hydroxylation of salicylate and substituted salicylate (Fig. 2A). Both the Reiske-type [2Fe-2S] cluster binding site motif (Cys-X_1-His-X_{17}-Cys-X_2-His) and the His-213, His-218, and Asp-361 residues that may coordinate the mononuclear nonheme iron active site (42) were conserved in the deduced amino acid sequence encoded by *phnA1b*. The gene homologs for large and small oxygenase subunits are usually located in pairs; however, no small-subunit gene was found in the flanking region of *phnA1b*.

The amino acid sequence of PhnA1 exhibited 51 to 62% identity with the amino acid sequences of the α subunits of PAH dioxygenases from *S. aromaticivorans* strain F199 (BphA1f) (45), *Burkholderia* sp. strain DBT1 (DbtAc) (accession no. AF380367), and *Alcaligenes faecalis* strain AFK2 (PhnAc) (accession no. BAA76323), whereas the other known α subunits exhibited levels of sequence identity lower than 45%. The phylogenetic analysis of the ISP α subunits of the oxygenase components of aromatic ring dioxygenases revealed that PhnA1 was divergent from *nah-nod-dox-pah* group (Fig. 2A). The ISP α subunit has both the Cys-X_1-His-X_{17}-Cys-X_2-His Rieske-type [2Fe-2S] cluster binding motif (35) and the potential mononuclear nonheme iron coordinate site consensus sequence consisting of His-207, His-211, and Asp-360 (42).

The *phnA2* gene was found downstream of the *phnA1* gene. PhnA2 exhibited moderate amino acid sequence homology to the ISP β subunits of aromatic ring dioxygenases. The β subunit of PAH dioxygenase from *S. aromaticivorans* (*bphA2f*)

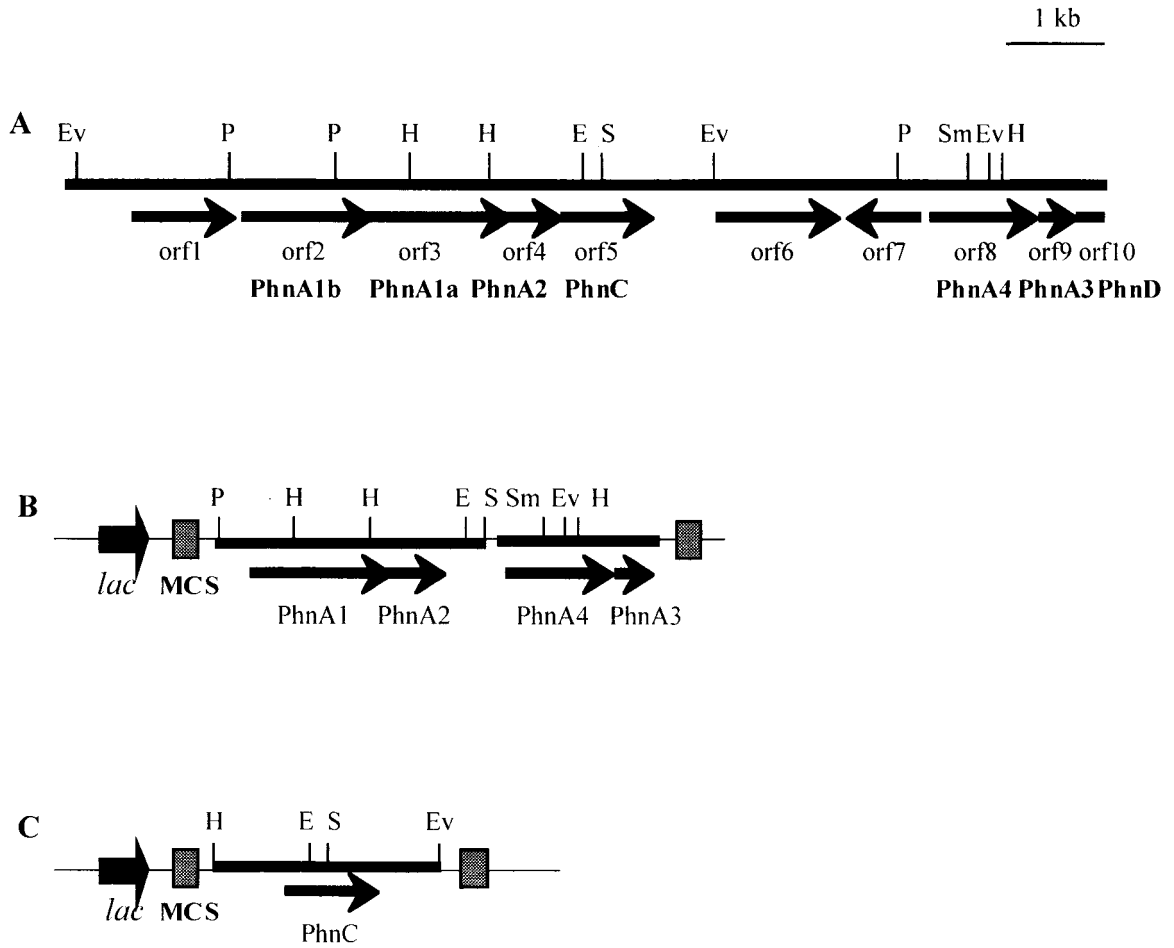


FIG. 1. Physical map and genetic organization of the PAH degradation genes (A) and structure of the plasmids constructed, pPhnA (B) and pPhnC (C). ORFs are indicated by arrows, and the direction of reading is indicated by the arrowheads. The gene designation of each ORF is indicated, as are the sites of the common restriction enzymes. Subclones pPhnA and pPhnC contain the *phnA1A2A3A4* genes and the *phnC* gene, respectively, under the control of the *lac* promoter of pBluescript II SK+. Restriction sites: E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; V, *Eco*RV.

(45) exhibited relatively high sequence identity to PhnA2 (52%). The other known β subunits exhibited levels of sequence identity less than 39%. Figure 2B shows a dendrogram of the ISP β subunits of aromatic ring dioxygenases.

The *phnC* gene was located downstream of the *phnA2* gene, and the amino acid sequence of PhnC exhibited 60 to 66% identity with the amino acid sequences of the 2,3-dihydroxybiphenyl 1,2-dioxygenases from *Sphingomonas paucimobilis* (BphC) (56), *S. aromaticivorans* (BphC) (45), and *Rhodococcus* sp. strain RHA1 (EtbC) (17). Figure 3 shows a dendrogram resulting from a comparison of two-domain extradiol dioxygenases of the I.3 family which preferentially cleave bicyclic substrates (12). The members of the PhnC cluster in group 2 of the I.3.E subfamily, which have more diverse sequences, and enzymes belonging to this group have exhibited high activity with different substrates (2).

The *phnA4* gene was located 2.8 kb downstream of the *phn*C gene. The amino acid sequence of PhnA4 exhibited 47 to 51% identity with the amino acid sequences of the NADH-ferredoxin oxidoreductase components of mono- and dioxygenase systems from *Pseudomonas stutzeri* (TouF) (accession no. AJ438269), *A. faecalis* (PhnAa) (accession no. AB024945), and *Sphingopyxis macrogoltabida* (ThnA4) (accession no. AF157565). PhnA4 possesses an N-terminal region similar to that of the chloroplast-type ferredoxins, in which four Cys residues (Cys-39, -44, -47, and -78) involved in the coordination of two iron atoms of the [2Fe-2S] cluster are conserved. The flavin adenine dinucleotide-binding domain (amino acids 106 to 196) and NAD-binding domain (amino acids 206 to 318) were found in the C-terminal region of PhnA4.

The *phnA3* gene was located downstream of the *phnA4* gene, and the gene product exhibited 63% sequence identity with the Rieske-type [2Fe-2S] ferredoxin components of known dioxygenases.

The partial *phnD* gene was located downstream of the *phnA4* gene, and the predicted amino acid sequence of the *phnD* product exhibited 58 to 60% identity with the amino acid sequences of 2-hydroxychromene-2-carboxylate isomerase from *Burkholderia* sp. strain DBT1 (*orf8*) (accession no. AAK96187), *Sphingomonas* sp. strain BN6 (*nsaD*) (accession no. AAD45416), and *S. aromaticivorans* strain F199 (*nahD*) (45).

TABLE 3. Properties of the *phn* genes identified on the 10.5-kb fragment of A5

Gene	Function	Deduced molecular mass (kDa)	Protein with similar sequence	% Identity (no. of residues)	Organism	Accession no.
orf1	PdxA homolog	34	Orf1158	56 (319)	*Sphingomonas aromaticivorans* F199	NP_049207
			PdxA	35 (328)	*Bacillus halodurans C*-125	NP_241670
			PdxA	34 (322)	*Fusobacterium nucleatum* ATCC 25586	NP_603133
phnA1b	ISP α subunit	48	AhdA1d	60 (424)	*Sphingomonas* sp. strain P2	BAC65433
			BphA1d	60 (426)	*Sphingomonas aromaticivorans* F199	NP_049206
			AhdA1c	48 (420)	*Sphingomonas* sp. strain P2	BAC65426
			PhnA1	22 (460)	*Cycloclasticus* sp. strain A5	AB102786
phnA1	ISP α subunit	52	BphA1f	64 (452)	*Sphingomonas aromaticivorans* F199	NP_049062
			DbtAc	51 (443)	*Burkholderia* sp. strain DBT1	AAK62353
			PhnAc	52 (416)	*Alcaligenes faecalis* AFK2	BAA76323
phnA2	ISP β subunit	21	BphA2f	52 (173)	*Sphingomonas aromaticivorans* F199	NP_049061
			ThnA2	39 (177)	*Sphingopyxis macrogoltabida* TFA	AAN26444
			BphA2	33 (171)	*Comamononas testosteroni* TK 102	BAC01053
phnC	Extradiol dioxygenase	34	BphC	66 (289)	*Sphingomonas paucimobilis* Q1	P11122
			DmdC	64 (289)	*Sphingomonas paucimobilis* TZS-7	BAB07894
			BphC	64 (289)	*Sphingomonas aromaticivorans* F199	NP_049210
trpB	Tryptophan synthase β subunit	42	TrpB	57 (388)	*Thermoanaerobacter tengcongensis* MB4T	NP_623178
			TrpB-2	56 (380)	*Pyrococcus furtosus* DSM 3638	NP_579435
			TrpB	56 (391)	*Brucella suis* 1330	NP_699085
orf7	Unknown	26	No obvious homolog			
phnA4	Reductase	38	TouF	50 (313)	*Pseudomonas stutzeri* OX1	CAA06659
			PhnAa	47 (338)	*Alcaligenes faecalis* AFK2	BAA76321
			ThnA4	47 (342)	*Sphingopyxis macrogoliabida* TFA	AAN26446
phnA3	Ferredoxin	11	BphA3	63 (104)	*Sphingomonas aromaticivorans* F199	NP_049211
			PhnR	63 (104)	*Sphingomonas chungbukensis*	AAC95320
			NsaA3	59 (103)	*Sphingomonas* sp. strain BN6	AAB06726
phnD	Isomerase		Orf8	64 (115)	*Burkholderia* sp. strain DBT1	AF96187
			NsaD	61 (115)	*Sphingomonas* sp. strain BN6	AAD45416
			NahD	58 (115)	*Sphingomonas aromaticivorans* F199	NP_049214

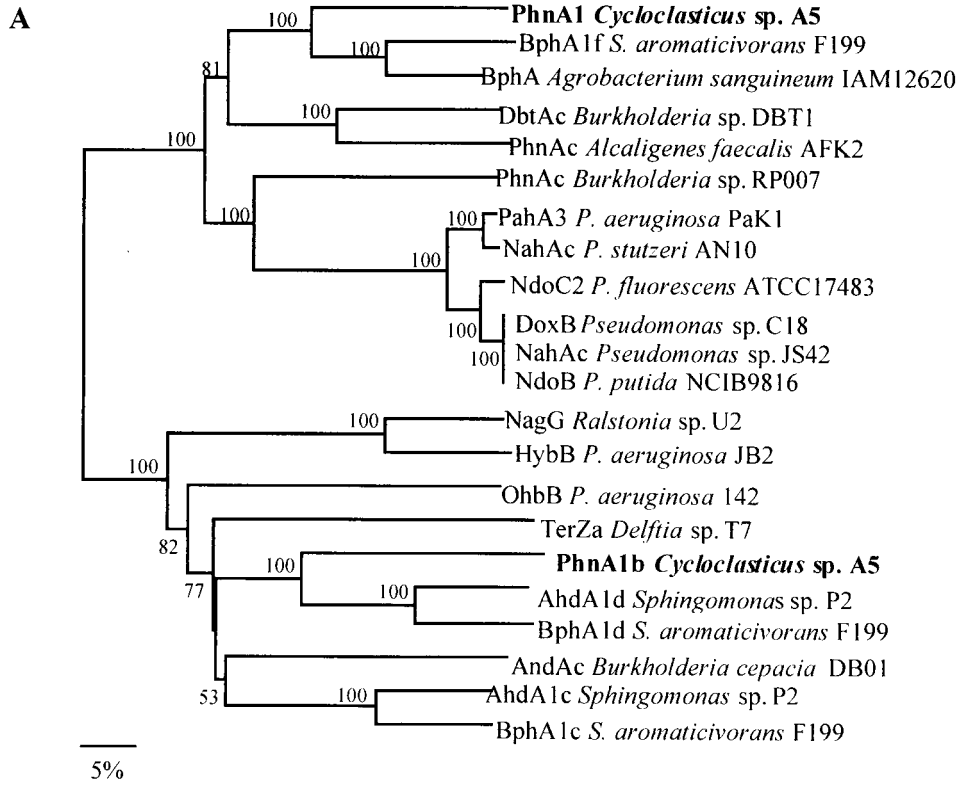
Expression of the *phnA1A2A3A4* genes in *E. coli*. To determine whether the *phnA1A2A3A4* genes actually encode the holoenzyme of an aromatic ring-hydroxylating dioxygenase, the genes were introduced into *E. coli* and expressed. The 2.7-kb *Pst*I-*Sal*I DNA fragment of pH1a, which contained the *phnA1* and *phnA2* genes, was cloned into the same site of pBluescript II SK+ to obtain pISP. The DNA fragment containing the *phnA3* and *phnA4* genes into which the *Xho*I and *Kpn*I sites had been introduced by PCR amplification was cloned into the *Xho*I-*Kpn*I site of pISP to obtain pPhnA (Fig. 1B).

E. coli carrying pPhnA and pBluescript II SK+ (negative control) was inoculated into LB medium containing ampicillin and induced by 1 mM IPTG. The recombinant proteins encoded by the *phnA1*, *phnA2*, *phnA3*, and *phnA4* genes were overexpressed and separated by SDS-PAGE (data not shown). The sizes estimated by SDS-PAGE (52.5, 23.3, 38, and 12.5 kDa) agreed well with the predicted sizes of PhnA1 (52 kDa), PhnA2 (23 kDa), PhnA4 (38 kDa), and PhnA3 (11 kDa).

Biotransformation of various aromatic compounds with recombinant dioxygenase. To examine the substrate range of aromatic ring-hydroxylating dioxygenase, we conducted three independent biotransformation (bioconversion) experiments with the various aromatic compound substrates listed in Table 2 using the recombinant *E. coli* cells expressing the *phnA1A2A3A4* genes (pPhnA). After the substrates and cells had been cultured for 2 to 3 days, an HPLC analysis showed that *E. coli* possessing pPhnA transformed phenanthrene, naphthalene, 1-methylnaphthalene, 2-methylnaphthlene, biphenyl, and diphenylmethane, in addition to the tricyclic (fused) heterocycles dibenzofuran and dibenzothiophene, while it was not able to transform anthracene, pyrene, benzo[a]pyrene, toluene, and 2-phenylbenzoxazole (Table 2).

Structural analysis of the products converted by *E. coli* expressing *phnA1A2A3A4*. The crude products obtained with the recombinant *E. coli* strain from phenanthrene, naphthalene, 1-methylnaphthalene, 2-methylnaphthlene, dibenzofuran, dibenzothiophene, biphenyl, and diphenylmethane were determined by HPLC and thin-layer chromatography, and the products detected were purified by silica gel column chromatography. Table 2 shows the hydroxylated products which were identified by comparison with the previously reported spectral data (mass spectrometry and nuclear magnetic resonance data) (5, 22, 23, 38, 39, 40, 48), whose chemical structures are shown in Fig. 4. Phenanthrene, naphthalene, 1-methylnaphthalene, and 2-methylnaphthalene were converted to their *cis*-dihydrodiol forms, which are the typical products of aromatic ring dioxygenases, whereas the products obtained from dibenzofuran, dibenzothiophene, biphenyl, and diphenylmethane were detected not as *cis*-dihydrodiols but as monohydroxylated forms. The *cis*-dihydrodiols of the latter compounds generated enzymatically seemed to be converted to the monohydroxylated compounds shown in Fig. 4 nonenzymatically due to the structural instability of the *cis*-dihydrodiols.

Expression of the *phnC* gene in *E. coli*. An expression vector was constructed to analyze the activities of PhnC with different



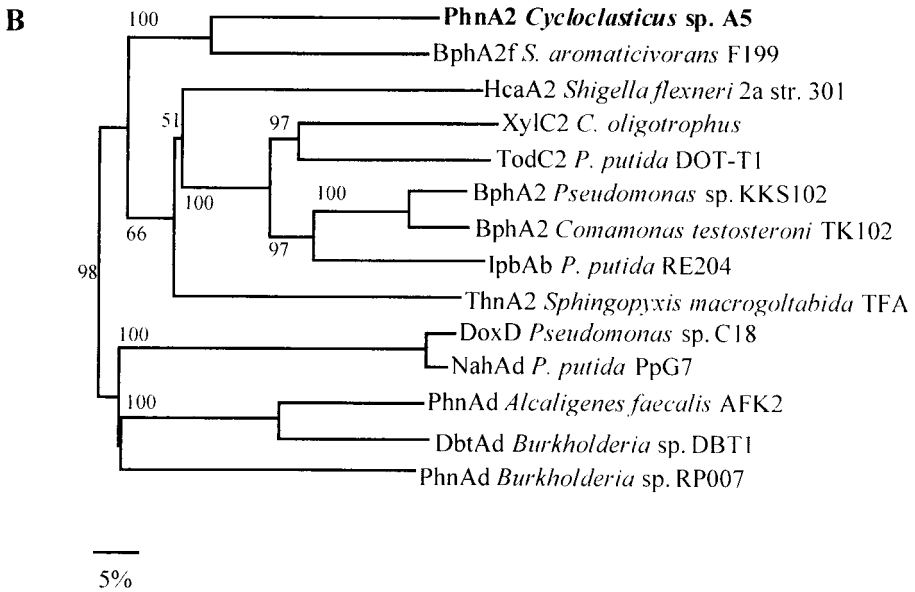


FIG. 2. Dendrograms based on the large subunits (A) and small subunits (B) of the aromatic ring-hydroxylating dioxygenases. The dendrograms were constructed by using the previously published amino acid sequences processed by Clustal W. Scale bar = 5% divergence. The numbers at nodes are bootstrap values expressed as percentages. The following sequences were used: *P. putida* NCIB 9816 *nah* genes (GenBank accession no. M83950), *Pseudomonas* sp. strain C18 *dox* genes (M6045), *S. aromaticivorans* strain F199 *bph* genes (AF079317), *Burkholderia* sp. strain DBT1 *dbt* genes (AF380367), *Burkholderia* sp. strain RP007 *phn* genes (AF061751), *A. faecalis* AFK2 *phn* genes (AB024945), *Sphingomonas* sp. strain P2 *ahd* genes (AB091692), *P. putida* PpG7 *nahAd* gene (M83949), *Pseudomonas* sp. strain JS42 *nahAc* gene (U49496), *P. stutzeri* AN10 *nahAc* gene (AF039533), *P. putida* NCIB9816 *ndoB* gene (M23914), *P. fluorescens* ATCC 17483 *ndoC2* gene (AF004283), *P. aeruginosa* Pak1 *pahA3* gene (D84146), *Agrobacterium sanguineum* IAM12620 *bphA* gene (AB062104), *P. aeruginosa* 142 *ohbB* gene (AF121970), *P. aeruginosa* JB2 *hybB* gene (AF087482), *Delfita* sp. strain T7 *terZa* gene (AB081091), *Burkholderia cepacia* DB01 *andAc* gene (AY223539), *Ralstonia* sp. strain U2 *nagG* gene (AF036940), *Cycloclasticus oligotrophus xylC2* gene (U51165), *Shigella flexneri* 2a str. 301 *hcaA2* gene (AAN44085), *Comamonas testosteroni* TK102 *bphA2* gene (AB086835), *S. macrogoltabida* TFA *thnA2* gene (AAN26444), *P. putida* RE204 *ipbAb* gene (AF006691), *Pseudomonas* sp. strain KKS102 *bphA2* gene (Q52439), and *P. putida* DOT-T1 *todC2* gene (Y1825).

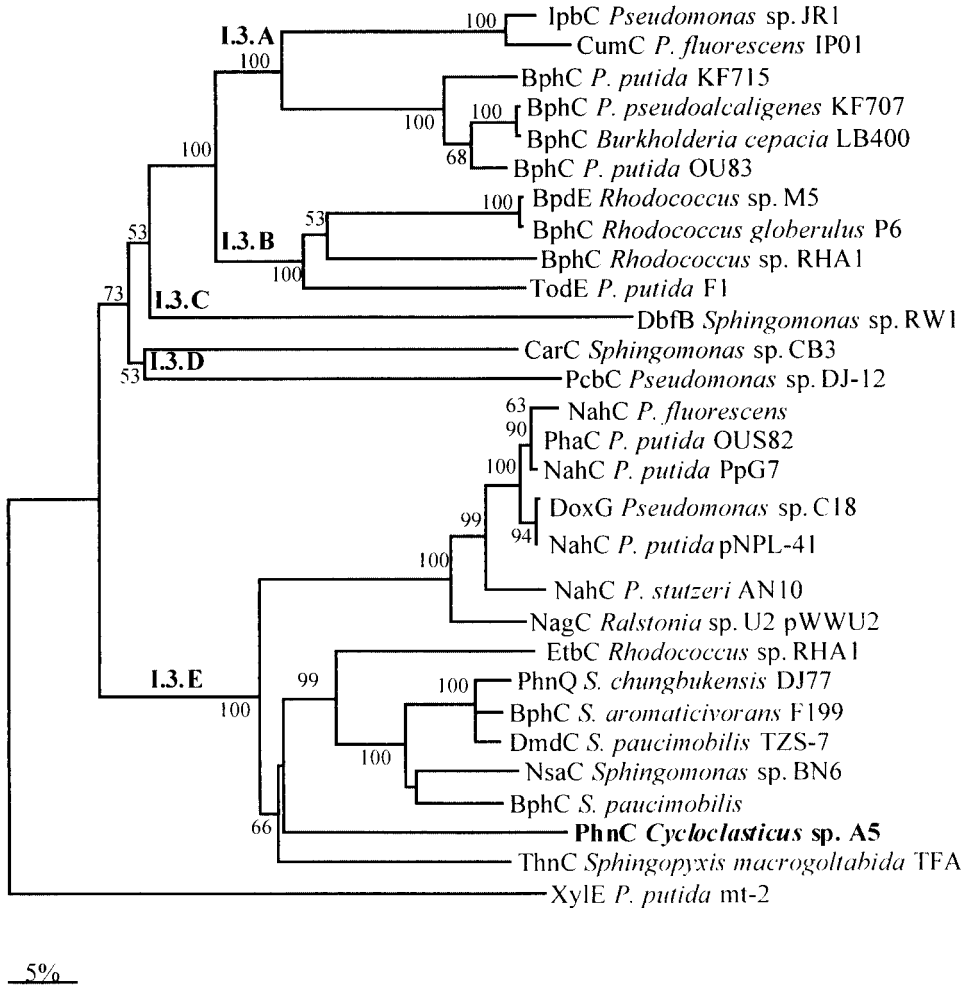


FIG. 3. Dendrogram based on the extradiol dioxygenases. The dendrogram was constructed by using the previously published amino acid sequences processed by Clustal W. Scale bar = 5% divergence. The numbers at nodes are bootstrap values expressed as percentages. The following sequences were used: *Pseudomonas* sp. strain JR1 *ipbC* gene (GenBank accession no. U53507), *Pseudomonas fluorescens* IP01 *cumC* gene (D37828), *P. putida* KF715 *bphC* gene (M33813), *Pseudomonas pseudoalcaligenes* KF707 *bphC* gene (M83673), *Burkholderia cepacia* LB400 *bphC* gene (X66122), *P. putida* OU83 *bphC* gene (X91876), *Rhodococcus* sp. strain M5 *bpdE* gene (U27591), *Rhodococcus globerulus* P6 *bphC* gene (X75663), *Rhodococcus* sp. strain RHA1 *bphC* gene (D32142), *P. putida* F1 *todE* gene (JO4996), *Sphingomonas* sp. strain RW1 *dbfB* gene (X72850), *Sphingomonas* sp. strain CB3 *carC* gene (AF060489), *Pseudomonas* sp. strain DJ-12 *pcbC* gene (D44550), *P. fluorescens nahC* gene (AY048760), *P. putida* OUS82 *pahC* gene (D16629), *P. putida* PpG7 *nahC* gene (J04994), *Pseudomonas* sp. strain C18 *doxG* gene (M60405), *P. putida* pNPL41 *nahC* gene (Y14173), *P. stutzeri* AN10 *nahC* gene (AF039533), *Ralstonia* sp. strain U2 *nagC* gene (AF036940), *Rhodococcus* sp. strain RHA1 *etbC* gene (D78322), *Sphingomonas chungbukensis* DJ77 *phnQ* gene (AF061802), *S. aromaticivorans* F199 *bphC* gene (AF079317), *S. paucimobilis* TZS-7 *dmdC* gene (AB035677), *Sphingomonas* sp. strain BN6 *nsaC* gene (U65001), *S. paucimobilis bphC* gene (P11122), *S. macrogoltabida* TFA *thnC* gene (AF157565), and *P. putida* mt-2 *xylE* gene (V01161).

substrates. The 2.3-kb *Hin*dIII-*Eco*RV DNA fragment was cloned into the same site of pBluescript II SK+ (Fig. 1C) and expressed in *E. coli* JM109. Overproduction of PhnC was confirmed by SDS-PAGE (data not shown). The size estimated by SDS-PAGE (32 kDa) agrees well with the predicted size for PhnC (34 kDa). The activities of PhnC with different monocyclic and bicyclic catechol derivatives were examined spectrophotometrically by using triplicate samples. The values obtained in three independent experiments were averaged (Table 4). The highest activity of PhnC was observed with 3-methylcatechol. Interestingly, the activities of PhnC with fused, unfused, and monocyclic catechols were almost the same.

DISCUSSION

We describe in this paper the cloning, sequencing, and functional expression in *E. coli* of genes coding for a novel PAH ring-hydroxylating dioxygenase from *Cycloclasticus* sp. strain A5. It has been reported that the gene clusters responsible for PAH degradation are localized on plasmids (*nah* in a *Pseudomonas putida* strain [30]; *ndo* in a *P. putida* strain [55]; *dox* in *Pseudomonas* sp. strain C18 [7]; *nag* in *Pseudomonas* sp. strain U2 [13]; *phn* in *Burkholderia* sp. strain RP007 [32]) and on chromosomes (*pah* in *P. putida* OUS82 [57]; *nah* in *P. stutzeri* AN10 [6]). *Cycloclasticus* sp. strain A5 harbored no plasmid,

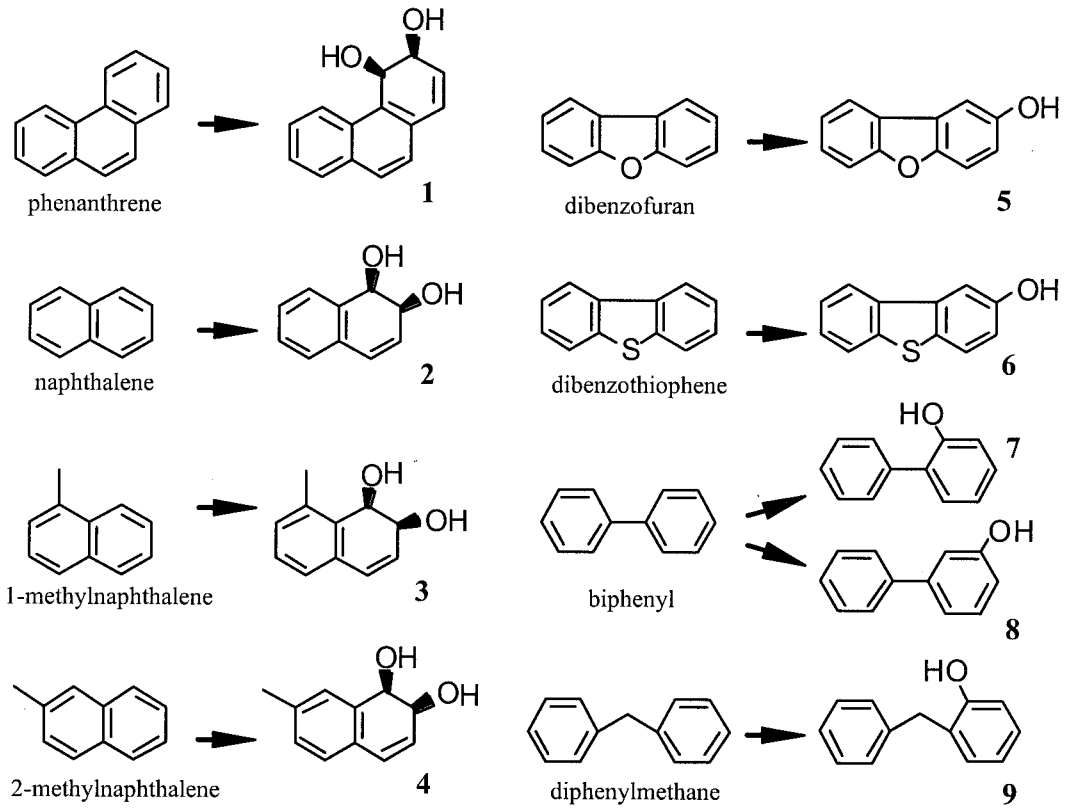


FIG. 4. Structures of products converted from aromatic substrates by *E. coli* cells harboring pPhnA.

and the dioxygenase genes described in this paper were localized on the chromosome (Kasai and Harayama, unpublished data). The order of the *phn* genes was found to be quite different from that of analogous genes reported previously (55, 61).

A phylogenetic analysis of the predicted amino acid sequence of the *phnA1* gene product revealed some divergence from the α-subunit sequences described previously in that it falls outside the major cluster, with which the level of amino acid sequence similarity is less than 44% (Fig. 2A), although the basic sequence features of the protein family are conserved. A phylogenetic analysis of the β subunits gave a tree similar to that constructed for the α subunits, indicating that PhnA2 formed a cluster with the β subunit BphA2f from *S. aromaticivorans* F199 (accession no. NP_049061), which fell outside the major cluster (Fig. 2B).

The *phnA3* and *phnA4* genes, which encode the ferredoxin and ferredoxin reductase components of the multicomponent PAH dioxygenase, were located 3.7-kb downstream of the β subunit (Fig. 1). Expression in *E. coli* JM109 of only the genes for the α and β subunits (*phnA1* and *phnA2*) did not result in the presence of active dioxygenase (data not shown), while dioxygenation activity was found when these genes were coexpressed with a cognate electron supply system consisting of the PhnA3 ferredoxin and the PhnA4 ferredoxin reductase from *Cycloclasticus* sp. strain A5. The genetic organization of most of the ring-hydroxylating dioxygenases that have been investigated involves cistrons encoding the α and β subunits which are contiguous with the genes of the specific electron carrier or at least are clustered in the same transcriptional unit, as is the case for the carbazole dioxygenase of *Pseudomonas* sp. strain CA10 (50) and the *p*-cumate dioxygenase of *P. putida* F1 (9). Moreover, the gene encoding the reductase associated with the electron carrier is also generally, although not always, present in such dioxygenase gene clusters (3). The genes encoding the PAH dioxygenase system of A5 were located on separate transcriptional units (data not shown). This suggests that the electron transport proteins of the PAH dioxygenase system may be shared with other redox systems, possibly to maximize the catabolic potential while limiting its genetic burden. Harayama et al. have proposed that this tolerance between redox and oxygenase partners may also function as an evolutionary process for multicomponent oxygenases (16). Although some potential catabolic and evolutionary benefit may result from multipurpose electron transport proteins, the data raise the

TABLE 4. Activity of PhnC extradiol dioxygenase

Substrate	Activity (μmol/min/mg)[a]	Relative activity (%)
1,2-DHN	186 (7)	53
2,3-Dihydroxybiphenyl	167 (27)	47
3-Methylcatechol	353 (75)	100
4-Methylcatechol	229 (6)	65
Catechol	154 (40)	44

[a] Average (standard error) for three determinations.

question of coordination of expression of the genes. However, the expression mechanism of the dioxygenase genes in *Cycloclasticus* remains unknown. It would be interesting to investigate whether these proteins are expressed constitutively or coordinately.

We demonstrated that the PhnA dioxygenase has a wide PAH substrate range, although this enzyme could not use anthracene as a substrate. The *E. coli* cells possessing *phnA* genes converted phenanthrene, naphthalene, 1-methylnaphthalene, and 2-methylnaphthalene, in addition to the tricyclic fused heterocycles dibenzofuran and dibenzothiophene, to their hydroxylated forms. Significantly, the *E. coli* cells expressing *phnA* were also able to transform biphenyl and diphenylmethane with low efficiency; these compounds are unfused linked aromatic compounds and ordinarily are substrates not of PAH dioxygenases but of biphenyl dioxygenases (37, 53). For example, phenanthrene dioxygenase that was isolated from the marine bacterium *Nocardioides* sp. strain KP7 transformed phenanthrene, anthracene, fluorene, naphthalene, dibenzofuran, and dibenzothiophene, which are polycyclic aromatic compounds, whereas it did not convert biphenyl and diphenylmethane to their hydroxyl products (53). Parales et al. have reported that several amino acids at the active site of the dioxygenase ISP α subunit were consistent with the enzyme's preference for an aromatic hydrocarbon substrate (42). The three-dimensional structure of the oxygenase component (α_3 [NdoB], β_3 [NdoC]) of the naphthalene dioxygenase of *Pseudomonas* sp. strain NCIB 9816-4 revealed a long narrow gorge which might provide access for substrates to catalytic iron in NdoB. The five residues constituting the narrowest part of the channel near the catalytic iron in NdoB were completely conserved in PhnA1 of *Cycloclasticus* sp. strain A5 as Asn-220, Phe-201, His-207, His-212, and Phe-350. However, the residues lining the substrate-binding pocket below the catalytic iron and the residues in the gorge above the catalytic iron were divergent. This sequence diversity may contribute to the wide range of PAHs that are degraded by *Cycloclasticus* sp. strain A5 (24).

A phylogenetic analysis of the predicted amino acid sequences of the *phnA1b* gene product revealed that PhnA1b clusters with α oxygenase subunits of salicylate 1-hydroxylase and salicylate 5-hydroxylase. These enzymes are monooxygenases that exhibit sequence similarity with dioxygenases such as naphthalene dioxygenase and require four gene products (i.e., the α and β subunits of a hydroxylase component, ferredoxin, and ferredoxin reductase) for full monooxygenation activity (43, 61). Because the other β-subunit gene of oxygenase was not found in the flanking region of *phnA1b*, it would be interesting to examine whether PhnA1b can combine with PhnA2 to form a functional ISP and utilize the same ferredoxin and ferredoxin reductase as the PhnA1PhnA2 oxygenase. Such a process was observed in S5H of *Ralstonia* sp. strain U2, which shared electron transport with naphthalene dioxygenase (61).

The *phnC* gene encodes the PAH extradiol dioxygenase. The results of the phylogenetic analysis showed that PhnC is one of the two-domain extradiol dioxygenases of the I.3 family which preferentially cleave bicyclic substrates (Fig. 3). The activities with fused, unfused, and single-ring compounds were not very different, and significant high PhnC activities were observed with substituted monocyclic catechol compounds (Table 4). Based on these results, there is a possibility that PhnC is involved in both the upper and lower pathways for degradation of naphthalene, phenanthrene, and biphenyl. Identification of the true substrates of PhnC and isolation of other ring cleavage enzymes, such as protocatechuate dioxygenases, would contribute to our understanding of the pathways for degradation of aromatic compounds in *Cycloclasticus*.

The presence of multiple dioxygenase genes in a single bacterium has recently been reported; for example, four to six dioxygenase α-subunit genes were found in various species of *Sphingomonas* that are able to efficiently degrade a wide range of aromatic hydrocarbons (4, 27, 44, 45). Geiselbrecht et al. have also reported that *Cycloclasticus* has at least two kinds of dioxygenase α-subunit genes (14). Since PhnA was not able to convert anthracene and monocyclic aromatic hydrocarbons, such as toluene and xylene (Table 3), other dioxygenase genes in *Cycloclasticus* should be examined to explain the efficient degradation of a wide range of aromatic hydrocarbons by this organism.

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