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Identification and Characterization of Genes Encoding Polycyclic Aromatic Hydrocarbon Dioxygenase and Polycyclic Aromatic Hydrocarbon Dihydrodiol Dehydrogenase in *Pseudomonas putida* OUS82

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Naphthalene and phenanthrene are transformed by enzymes encoded by the *pah* gene cluster of *Pseudomonas putida* OUS82. The *pahA* and *pahB* genes, which encode the first and second enzymes, dioxygenase and *cis*-dihydrodiol dehydrogenase, respectively, were identified and sequenced. The DNA sequences showed that *pahA* and *pahB* were clustered and that *pahA* consisted of four cistrons, $pahA_a$, $pahA_b$, $pahA_c$, and $pahA_d$, which encode ferredoxin reductase, ferredoxin, and two subunits of the iron-sulfur protein, respectively.

Pseudomonas putida OUS82 can assimilate naphthalene and phenanthrene as its sole carbon sources. The strain converts naphthalene and phenanthrene to salicylate and 1-hydroxy-2-naphthoate, respectively, by a shared catabolic pathway (the upper pathway; Fig. 1). Salicylate and 1-hydroxy-2-naphthoate are further degraded by other catabolic enzymes. The enzymes in the upper pathway have broad substrate specificities, and various polycyclic aromatic hydrocarbons other than naphthalene and phenanthrene are oxidized by a high-density suspension of OUS82 cells (9).

Previously, we cloned the gene cluster encoding the enzymes of the upper pathway and named it *pah* (polycyclic aromatic hydrocarbon; 9). The *pah* region strongly hybridized to a corresponding region of plasmid NAH7 of *P. putida* G7, which degrades naphthalene (4). All recombinant plasmids carrying *pahA* have 6.5- and 3.0-kb *Sal*I fragments. The two fragments were seen to be necessary for the dioxygenase phenotype (PahA). A restriction endonuclease map of a region in the fragments resembles that of the *nahA* region of NAH7 and pDTG1 in *P. putida* G7 and NCIB 9816-4, which degrade naphthalene (2, 4, 23). The *pahA* gene was expected to be in that region.

Here, we describe the identification and characterization of the *pahA* and *pahB* genes, which encode dioxygenase PahA, which is the first enzyme of the pathway and converts polycyclic aromatic hydrocarbon (PAH) to the corresponding *cis*-dihydrodiol, and dehydrogenase PahB, the second enzyme of the pathway, which converts the product of PahA to the corresponding diol.

P. putida OUS8211 (*trp-82 Δpah-821*), a derivative of strain OUS82 that is defective in naphthalene and phenanthrene utilization, and plasmid pDI1, which carries the *pahAB* gene cluster, were described previously (9). Plasmid NAH7 was described elsewhere (4, 5). *Escherichia coli* JM109 and plasmid pUC119 were described by Yanisch-Perron et al. (21) and Vieira and Messing (20), respectively. Broad-host-range vector pTS1210 (Ap^r Km^r mob^+), a derivative of pSa, was a gift from A. Nakazawa, (Yamaguchi University) (11). Helper plasmid pRK2013 (Km^r tra^+) was a gift from M. Fukuda (Nagaoka University of Technology) (7). *tac* promoter expression vector pKK223-3 (1) was purchased from Pharmacia LKB Biotechnology, Uppsala, Sweden. The rich medium (LB) and the minimal medium used in this study were described previously (9). SMT medium was the minimal medium plus 0.3% disodium succinate and 30 μg of tryptophan per ml. Restriction enzymes and T4 DNA ligase were obtained from Toyobo Co., Ltd., Osaka, Japan, and Nippon Gene Co., Ltd., Toyama, Japan. An in vitro packaging kit was obtained from Amersham International plc, Buckinghamshire, United Kingdom. DNA and amino acid sequence similarities were analyzed with DNASIS-Mac software (version 2.0; Hitachi Software Engineering Co., Ltd., Yokohama, Japan).

Nucleotide sequencing and characterization of *pahA* and *pahB*. Previously, the *pahA* gene was suggested to be in a 4.1-kb *Eco*RI-*Hpa*I fragment in pDI1 (Fig. 2). The *pahB* gene was thought to flank the *pahA* gene because genes that encode catabolic enzymes are often clustered (13, 19, 22). We sequenced the nucleotides of a 6-kb region between the *Eco*RV and *Sac*I sites (Fig. 2). DNA was sequenced by the dideoxy-chain termination procedure (15) with alkali-denatured plasmid DNA and biotinylated oligonucleotides (New England BioLabs, Beverly, Mass.) as the primer and a Sequenase DNA sequencing kit (United States Biochemical Corp., Cleveland, Ohio) or a *Bca*-Best DNA sequencing kit (Takara Shuzo Co., Ltd., Kyoto, Japan). Electrophoresis was done with 0.5× TBE buffer (1 M Tris base, 83 mM boric acid, 1 mM disodium EDTA) in the upper chamber (anode) and a mixture of 1× TBE and 0.5 volume of 3 M sodium acetate in the lower chamber (cathode) (17). The result of electrophoresis was electroblotted onto a Biodyne A (Pall Biosupport Div., East Hills, N.Y.) nylon membrane with an electroblotting apparatus (NB-1600; Nihon Eido Co., Ltd., Tokyo, Japan) at 100 mA (constant current) for 15 min with 0.2× TBE buffer and detected with a Uniplex chemiluminescence detection subkit (Millipore Corp., Bedford, Mass.). Five complete open reading frames (ORF1 to ORF5) and the 5′-terminal sequence of ORF6, each preceded by an *E. coli* consensus ribosome-

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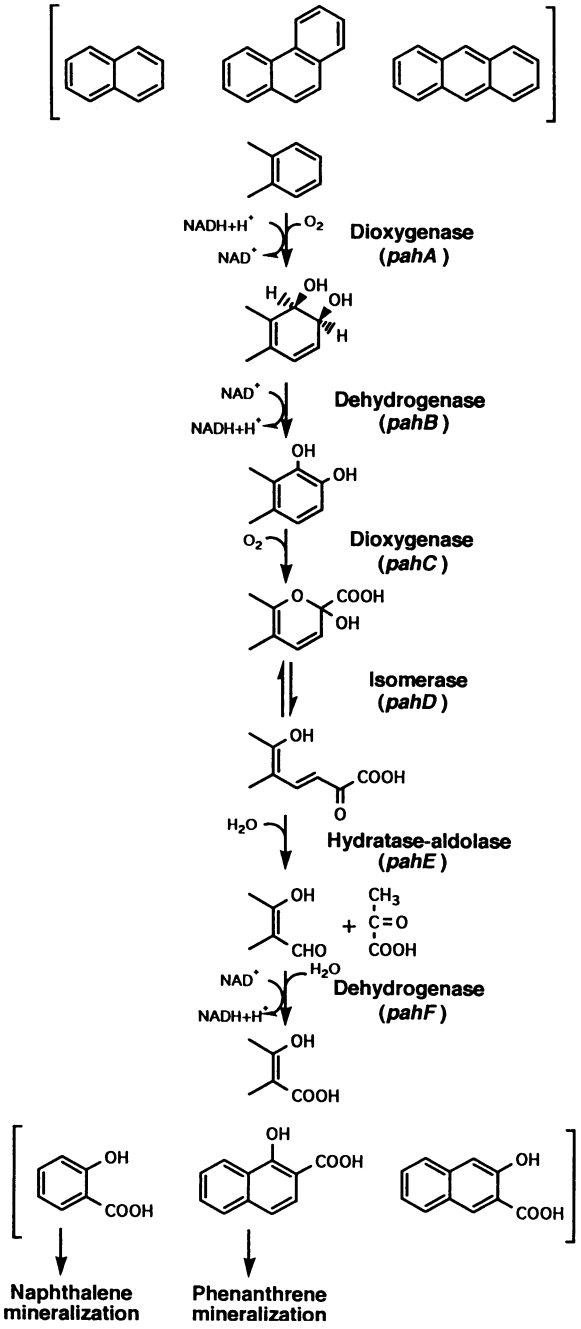


FIG. 1. Possible upper pathway for degradation of PAHs in *P. putida* OUS82.

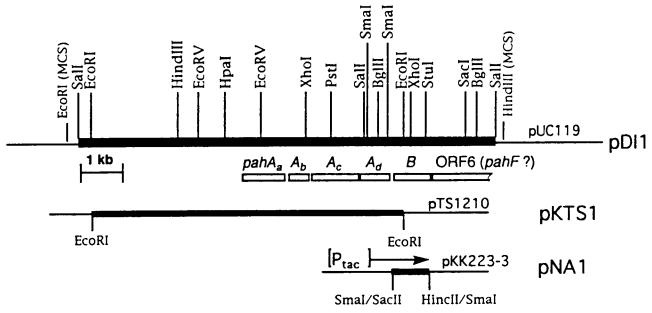


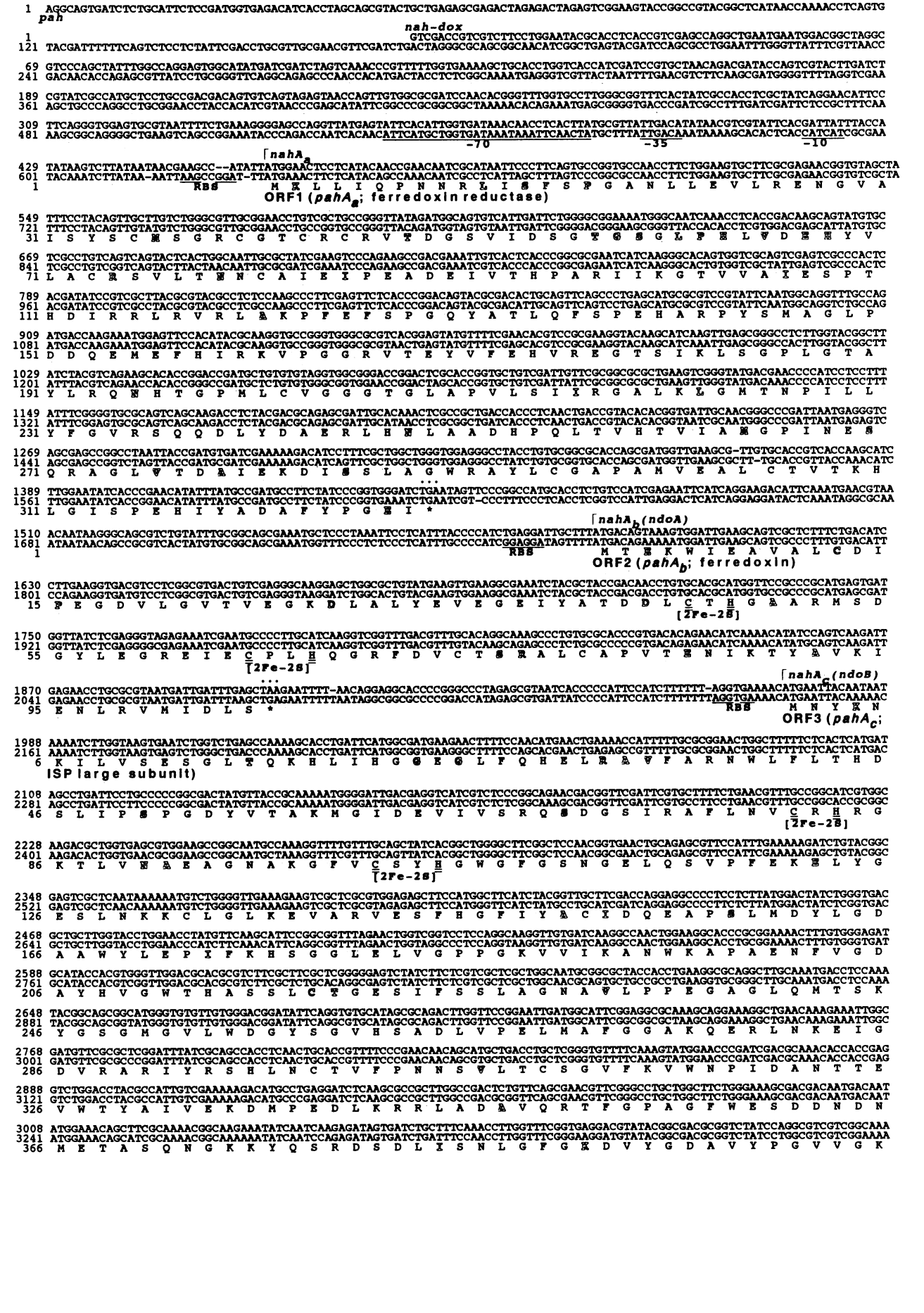
FIG. 2. Cloned DNA regions (thick lines) of *P. putida* OUS8211 in pDI1, pKTS1, and pNA1. The thin lines represent vector plasmids. The open boxes indicate the gene locations determined by nucleotide sequencing. P_{tac} and the arrow indicate the *tac* promoter and the direction of transcription, respectively. MCS, multiple cloning site in pUC119.

binding site, were found in that region (Fig. 3). ORF1 to ORF5 spanned 987, 315, 1,350, 585, and 780 nucleotides, respectively, and encoded polypeptides with deduced molecular masses of 35.6, 11.5, 49.3, 22.9, and 27.5 kDa, respectively. In this region, there was a pair of putative −10 and −35 promoter sequences similar to the *E. coli* consensus sequence upstream from the initiation codons of ORF1. The nucleotides and deduced amino acid residues of ORF1 to ORF4 were very similar to those of the naphthalene dioxygenase (NDO) genes of *P. putida* G7, NCIB 9816-4 (18), and NCIB 9816 (Table 1) (10). Rieske-type iron-sulfur centers (that is, [2Fe-2S]-binding sites) were found in the deduced amino acid sequences of ORF2 (Cys-45 to His-47 and Cys-64 to His-67) and ORF3 (Cys-81 to His-83 and Cys-101 to His-104). A sequence similar to an NahR-binding sequence (−70 sequence) (16, 24) was found upstream from ORF1.

These results indicate that the *pahA* gene encodes an NDO-type enzyme with four components: ferredoxin reductase, ferredoxin, and the iron-sulfur protein large and small subunits (6). We defined ORF1 to ORF4 as $pahA_a$, $pahA_b$, $pahA_c$, and $pahA_d$, respectively.

The protein predicted by ORF5 (27.4 kDa) was similar in molecular mass to the naphthalene dihydrodiol (NDD) dehydrogenase NahB (25.5 kDa) and the toluene dihydrodiol dehydrogenase TodD (27.0 kDa) (12, 14), and its primary structure was similar to the structures of the toluene dihydrodiol, biphenyl dihydrodiol, and benzene dihydrodiol dehydrogenases (TodD, [40.0% similarity], BphB [39.0% similarity], and protein 5 [40.9% similarity], respectively) listed in the GenBank DNA data base. The 0.5-kb *Eco*RI-*Stu*I region in ORF5 was strongly hybridized to a 4.7-kb *Sal*I fragment of NAH7 containing the *nahB* gene (5; data not shown). These results suggest that ORF5 encodes dihydrodiol dehydrogenase, and we defined ORF5 as *pahB*.

Conversion of naphthalene by *P. putida* OUS8211 carrying *pahA*. A 7.4-kb *Eco*RI fragment of pDI1 harboring *pahA* was cut out and inserted into pTS1210 to obtain pKTS1 (Fig. 2). A high-density suspension of OUS8211 cells carrying pKTS1 was exposed to naphthalene, and metabolites were extracted from the supernatant and analyzed by high-pressure liquid chromatography (D-6100 three-dimensional chromatography system; Hitachi Ltd., Tokyo, Japan; column, Intersil ODS-2 [4.6 by 250 mm]; G-L Science, Tokyo, Japan; mobile phase, 60% acetonitrile containing 0.05% trifluoroacetic acid; pressure, 150 kg/cm^2; flow rate, 0.5 ml/min). One main metabolite was purified and crystallized. The metabolite was converted to 1-naphthol and 2-naphthol by boiling under acidic conditions. Because NDD is converted to naphthol under acidic conditions (8), the metabolite was probably NDD. The metabolite was further analyzed by ^{13}C and ^{1}H nuclear magnetic resonance analyses. Peaks representing two hydroxylated carbons in positions 1 and 2 were detected in the ^{13}C nuclear magnetic resonance spectrum (δ 68.03 and 70.85), and the ^{1}H nuclear magnetic resonance spectra of the metabolite (δ 4.68 [d, J = 5.2 Hz, 1H, H-1], δ 4.38 [dd, J = 5.2 and 4.0 Hz, 1H, H-2], δ 6.05 [dd, J = 4.0 and 10 Hz, 1H, H-3], δ 6.53 [d, J = 10 Hz 1H, H-4], δ 2.04 and 2.38 [each s, 1H, OH], δ 7.10 to 7.55 [m]) agreed with those


pah
nah-dox
nahA_a
RBS
ORF1 (pahA_a; ferredoxin reductase)
nahA_b (ndoA)
RBS
ORF2 (pahA_b; ferredoxin)
[2Fe-2S]
[2Fe-2S]
nahA_c (ndoB)
RBS
ORF3 (pahA_c;
ISP large subunit)
[2Fe-2S]
[2Fe-2S]

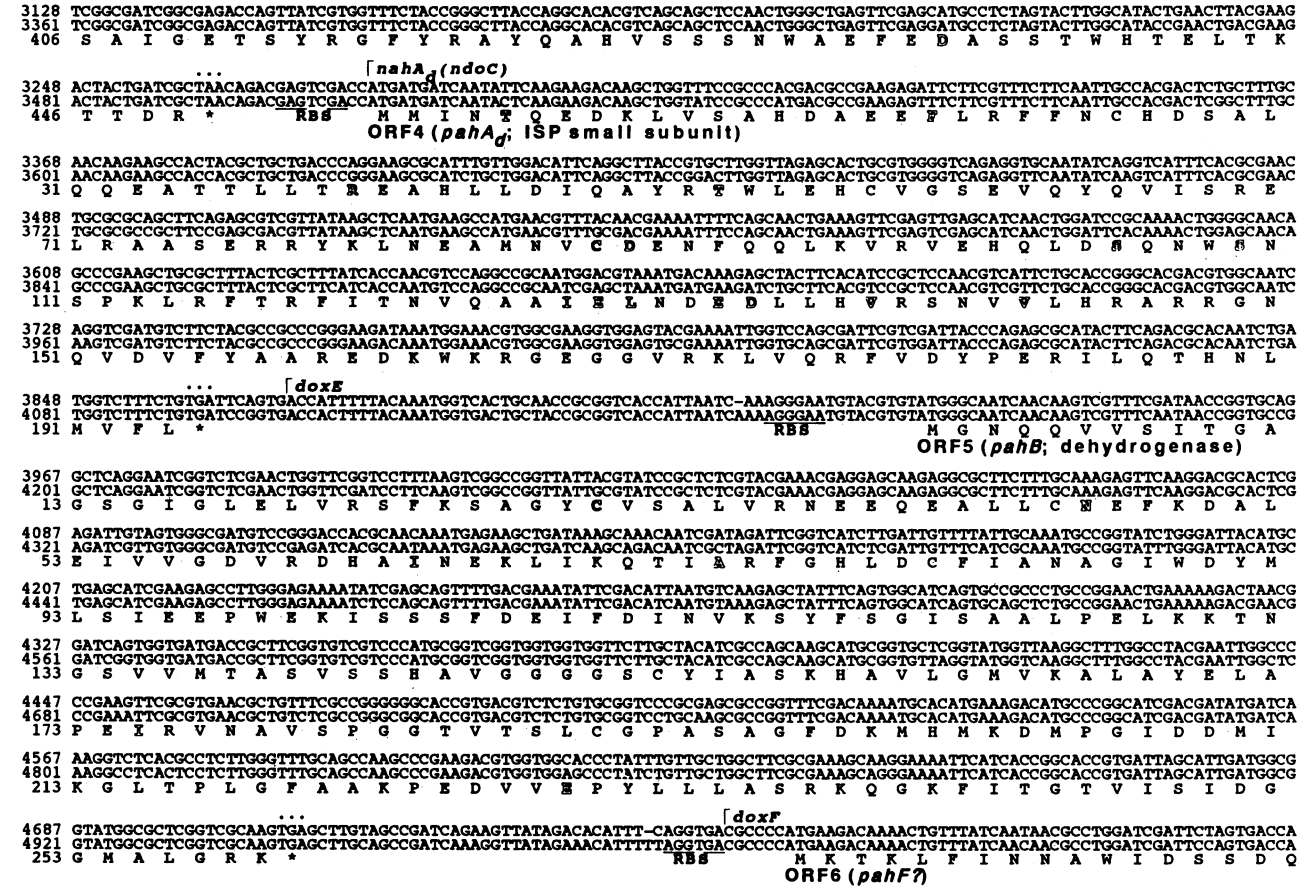


FIG. 3. Comparison of the nucleotide sequences of the *pahA* and *pahB* genes and the flanking regions identified in this study (*pah*; middle line) and the corresponding regions of *nah*, *ndo*, and *dox* (*nah-dox*; upper line), i.e., the *nahA* operon of *P. putida* NCIB 9816-4 and 9816 (positions 1 to 3911) and the *dox* operon of *Pseudomonas* sp. strain C18 (positions 1477 to 5605), respectively. The sequences of *nah* and *dox* between positions 1477 and 3911 are identical. The symbol ⌜ and three dots above *nah-dox* indicate initiation and stop codons of ORFs predicted by Simon et al. (18), Kurkela et al. (10) and Denome et al. (3). The deduced amino acid sequences for the *pah* genes (bottom line) are shown in the one-letter code. Amino acid residues not conserved between the *pah* products and the $nahA_a$ to $nahA_d$ and the *doxE* to *doxF* products are outlined. The asterisks indicate stop codons. A putative promoter (−35 and −10) and probable ribosome-binding sequences (RBS) are underlined. Estimated [2Fe-2S]-binding sites are doubly underlined. The sequence similar to the NahR binding sequence (−70) is also underlined.

of NDD reported by Jerina et al. (8). These results indicate that the *pahA* gene encodes dioxygenase and that naphthalene was converted to NDD by PahA.

Phenanthrene was also converted to *cis*-3,4-phenanthrene dihydrodiol by a high-density suspension of OUS8211 cells carrying pKTS1 (data not shown). The efficiency of conversion of phenanthrene was 1/10 of that of naphthalene.

Conversion of NDD by the *pahB* gene product. Because we could not find a typical product of naphthalene catabolism in the high-density suspension of OUS8211 cells carrying the *pahAB* cluster in a preliminary test, the *pahB* gene was joined to the *tac* promoter and expressed in *E. coli*. A 0.9-kb *Sac*II-*Hin*cII fragment containing the *pahB* gene was inserted into the *Sma*I site of *tac* promoter expression vector pKK223-3,

TABLE 1. Nucleotide and amino acid sequence similarities between PahA and other NDO-type enzymes[a]

NDO-type enzyme	*P. putida* OUS82		*P. putida* G7				*P. putida* NCIB 9816-4, NCIB 9816				*P. putida* F1				*P. pseudoalcaligenes* KF707			
	Gene (% G+C)	No. of amino acid residues	Gene (% G+C)	No. of amino acid residues	% Similarity[b] of:		Gene (% G+C)	No. of amino acid residues	% Similarity[b] of:		Gene (% G+C)	No. of amino acid residues	% Similarity[b] of:		Gene (% G+C)	No. of amino acid residues	% Similarity[b] of:	
					Nucleotides	Amino acids			Nucleotides	Amino acids			Nucleotides	Amino acids			Nucleotides	Amino acids
Ferredoxin reductase	$pahA_a$ (54.4)	328	$nahA_a$ (55.1)	328	97	96	$nahA_a$ (55.7)	328	90	90	*todA* (63.7)	410	53	28	*bphA4* (69.0)	408	52	27
Ferredoxin	$pahA_b$ (51.2)	104	$nahA_b$ (52.0)	107	94	93	$nahA_b$[c] (50.3)	104	91	90	*todB* (57.0)	107	58	38	*bphA3* (56.3)	109	58	39
ISP[d] large subunit	$pahA_c$ (53.7)	449	$nahA_c$ (53.6)	449	95	96	$nahA_b$[e] (52.6)	449	93	94	*todC1* (60.4)	450	56	39	*bphA1* (62.2)	458	56	37
ISP small subunit	$pahA_d$ (51.5)	194	$nahA_d$ (52.1)	194	95	91	$nahA_b$[f] (50.0)	194	93	92	*todC2* (58.7)	187	55	32	*bphA2* (58.2)	213	55	29

[a] The nucleotide and amino acid sequences of the dioxygenase are from the GenBank DNA data base.
[b] Compared with *P. putida* OUS82 gene and enzyme
[c] $nahA_b$, *ndoA*.
[d] ISP, iron-sulfur protein.
[e] $nahA_b$, *ndoB*.
[f] $nahA_b$, *ndoB*.

and pNA1 was obtained (Fig. 2). *E. coli* JM109 carrying pNA1 was cultured with induction by isopropyl-β-D-thiogalactopyranoside (IPTG), and an extract was prepared from the cells. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the extract gave an IPTG-induced protein band (data not shown). The molecular mass of the protein was estimated to be 27 kDa.

Conversion of NDD by the extract was analyzed by high-pressure liquid chromatography. NDD, which was prepared by conversion of naphthalene by *P. putida* OUS8211 carrying pKTS1; NAD^+; and the cell extract prepared from *E. coli* carrying pNA1 were mixed and incubated at 30°C. Naphthalene diol was transiently detected after 1 min of incubation, and accumulation of β-naphthoquinone, to which naphthalene diol was converted by oxidation in air, was detected after 15 min of incubation. The amino-terminal amino acid sequence of the 27-kDa protein was MGNQQVVSITG, which agreed with that of the deduced protein product of the *pahB* gene. These results indicate that the *pahB* gene encodes PAH dihydrodiol dehydrogenase.

In this study, we identified the PAH dioxygenase gene, *pahA*, and the PAH dihydrodiol dehydrogenase gene, *pahB*, and sequenced them. PahA is a multicomponent enzyme, like NDO (6). The nucleotide and amino acid sequences of PahA are similar to those of NDOs (more than 90% similarity), but the similarities between PahA and toluene dioxygenase or biphenyl dioxygenase are less at 52 to 58% for nucleotides and 27 to 39% for amino acids. The *pahB* gene is expected to be similar to the *nahB* gene, the nucleotide sequence of which has not been reported, because *pahB* and *nahB* hybridized strongly (data not shown). The *pah* and *nah* clusters were probably derived from the same ancestor.

A search of a DNA sequence data base showed that the region downstream from the *pahB* gene containing the 5′-terminal region of ORF6 is similar to the sequences that encode *E. coli* aldehyde dehydrogenase and *Alcaligenes eutrophus* acetaldehyde dehydrogenase. In plasmid NAH7, the *nahF* gene encoding aldehyde dehydrogenase is located between *nahB* and *nahC* (5). ORF6 is probably the *pahF* gene.

After we submitted this report for publication, the nucleotide sequence of the *dox* operon of *Pseudomonas* strain sp. C18 was reported by Denome et al. (3). The sequence between *doxA* and *doxF* is very similar to the sequence between $pahA_b$ and *pahB* reported here, but the *dox* sequence does not contain the region corresponding to the promoter and $pahA_a$ (Fig. 3). Some of the ORFs in the *dox* operon predicted by them do not initiate at an ATG or GTG codon and do seem strange. We have also determined the sequence of all regions of the *pah* cluster. We will report that nucleotide sequence, together with biochemical evidence, elsewhere.

Nucleotide sequence accession number. The nucleotide sequence in Fig. 3 will appear in the DDBJ, EMBL, and GenBank data bases under accession no. D16629.

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