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Cloning and Characterization of a Chromosomal Gene Cluster, *pah*, That Encodes the Upper Pathway for Phenanthrene and Naphthalene Utilization by *Pseudomonas putida* OUS82

HOHZOH KIYOHARA,* SHIN TORIGOE, NAOFUMI KAIDA, TAKUYA ASAKI, TOSHIYA IIDA, HIROYUKI HAYASHI, AND NOBORU TAKIZAWA

Biotechnology Laboratory, Department of Applied Chemistry, Faculty of Engineering, Okayama University of Science, Okayama 700, Japan

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A 25-kb DNA *SalI* fragment cloned from the chromosomal DNA of *Pseudomonas putida* OUS82, which utilizes phenanthrene (Phn^+) and naphthalene (Nah^+), carried all of the genes necessary for upper naphthalene catabolism. Cosmid recombinant pIP7 complemented both the Nah^- and Phn^- defects of OUS8211 (Trp^- Nah^- Phn^- Sal^+ [salicylate utilizing] Hna^+ [1-hydroxy-2-naphthoate utilizing]) and only the Phn^- defect of OUS8212 (Trp^- Nah^- Phn^- Sal^- Hna^+). The results indicate that strain OUS82 uses different pathways after *o*-hydroxycarboxylic aromatics in the catabolism of naphthalene and phenanthrene.

Many bacteria that utilize a polycyclic aromatic hydrocarbon (PAH) as the source of carbon and energy have been isolated. A single strain has been reported to utilize or co-oxidize more than one PAH, i.e., naphthalene, phenanthrene, biphenyl, anthracene, pyrene, chrysene, fluorene, fluoranthene, and dibenzothiophene (1, 9, 12–14, 18, 20, 21, 24, 29, 30). Less is known about the relationship between the ways different PAHs are catabolized by a single strain.

Here, we show genetically that Phn^+ Nah^+ *Pseudomonas putida* OUS28 uses one system of enzymes that has broad substrate specificity for the upper catabolic pathways from naphthalene to salicylic acid and from phenanthrene 1-hydroxy-2-naphthoic acid and that this Phn^+ phenotype depends on the occurrence of the ability to metabolize 1-hydroxy-2-naphthoic acid. We designated the genes for the upper pathway *pah*.

All of the bacterial strains and plasmids used in this study are listed in Table 1. *P. putida* PpG1064 (*met*) carrying NAH7 was the gift of I. C. Gunsalus, University of Illinois, Urbana. A Phn^+ Nah^+ bacterium (strain OUS82) was selected from our collection of bacteria that were isolated from soil with various PAHs (18) and identified as *P. putida* at The National Collections of Industrial and Marine Bacteria Limited, Aberdeen, Scotland. The strain harbored two plasmids, 90 and 43 kb long.

The composition of the mineral salts solution (MM2), without any growth substrate, used as the minimal basal medium was the same as before (16). MM2S contained 10 mM disodium succinate in MM2, and MM2SY contained 0.005% yeast extract in MM2S. MM2Y contained only 0.005% yeast extract in MM2. The concentration of the PAH (naphthalene or phenanthrene) added as a growth substrate was 0.3% (wt/vol). For growth of mutants that require an amino acid, the appropriate amino acid was added to a final concentration of 30 μg/ml to MM2S or MM2 plus PAH. The complete medium used (LB) contained 1% polypeptone, 0.5% yeast extract, and 0.5% NaCl. When necessary, 100 μg of kanamycin per ml was added to the media. For agar plates, 1.5% agar was added. *Pseudomonas* bacteria were cultured at 30°C; *Escherichia coli* was cultured at 37°C.

P. putida OUS82, which had been isolated as a Phn^+ bacterium, was able to utilize naphthalene as the sole source of carbon and energy after an induction period of about 18 h (Fig. 1A). Other aromatic hydrocarbons, including anthracene and biphenyl, were not assimilated. 1-Hydroxy-2-naphthoic acid and salicylic acid, the final products of the upper pathways of the degradation of naphthalene and phenanthrene, respectively, were utilized at concentrations below 0.05% (wt/vol).

To test the abilities to assimilate naphthalene and phenanthrene, various mutants deficient in naphthalene catabolism were constructed from Nah^+ Phn^+ OUS821 (Trp^-) by treatment with 1-chloronaphthalene (25). All OUS821 Nah^- mutants also did not use phenanthrene but did use salicylic acid and 1-hydroxy-2-naphthoic acid. Figure 1B shows the utilization of naphthalene, phenanthrene, salicylic acid, and 1-hydroxy-2-naphthoic acid by one of the Nah^- Phn^- mutants, OUS8211. Sal^- mutant OUS8212, which was derived from OUS8211, retained the ability to utilize 1-hydroxy-2-naphthoic acid (Fig. 1C). These results suggest that OUS82 uses different pathways for the degradation of salicylic acid and 1-hydroxy-2-naphthoic acid and that the naphthoic acid derived from phenanthrene may be not catabolized through salicylic acid.

DNA was manipulated as described by Sambrook et al. (22). For cloning in *E. coli* or *P. putida* OUS82 of genes encoding enzymes that catalyze the upper pathway of naphthalene degradation, partial *SalI* digests of the total DNA from OUS82 were ligated at the *SalI* site in the tetracycline resistance gene of pSTK10, which was constructed by insertion of a 1.5-kb *HincII* digest carrying the kanamycin resistance gene from pUC4K into the *EcoRI* site of cosmid vector pLAFR1, packaged in vitro, and transfected into *E. coli* HB101. Of 200 tetracycline-sensitive and kanamycin-resistant HB101 clones, 17 formed blue indigo-forming colonies on LB plates. These cloned DNA fragments probably carry naphthalene dioxygenase-encoding genes (7).

For selection of DNA clones carrying all of the genes for the upper pathway, the 17 recombinants (designated pIP1 to pIP17) were transferred from *E. coli* HB101 to *P. putida* OUS8211 (*trp-82 Δpah-821* Sal^+), OUS8212 (*trp-82 Δpah-821 sal-8211*), or AC10 (*met*) with helper plasmid pRK2013. A

* Corresponding author. Mailing address: Biotechnology Laboratory, Department of Applied Chemistry, Faculty of Engineering, Okayama University of Science, 1-1 Ridai-cho, Okayama 700, Japan. Phone: +81-86-252-3161. Fax: +81-86-252-6891.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics[a]	Source or reference
P. putida		
OUS82	Wild type; Phn$^+$ Nah$^+$ Hna$^+$ Sal$^+$	This work
OUS821	*trp-82* Phn$^+$ Nah$^+$ Hna$^+$ Sal$^+$; tryptophan-requiring derivative of OUS82	This work
OUS8211	*trp-82 Δpah-821 Hna*$^+$ *Sal*$^+$; Nah$^-$ Phn$^-$ derivative of OUS821	This work
OUS8212	*trp-82 Δpah-821 sal-8211 Hna*$^+$; Sal$^-$ derivative of OUS8211	This work
AC10	*met*	4
PpG1064 (NAH7)	*trp* Nah$^+$	I. C. Gunsalus
A. faecalis AFK2	Wild type; Phn$^+$	17
E. coli HB101	*recA hsdR hsdM lacY endA str pro leu thi* F$^-$	3
Plasmids		
pLAFR1	Tc^r *cos*$^+$ *mob*$^+$	11
pUC4K	Km^r	28
pSTK10	Tc^r Km^r *cos*$^+$ *mob*$^+$; cosmid vector inserted with 1.5-kb *Hin*cII-fragment from pUK4K into *Eco*RI site of pLAFR1	This work
pUC119	Ap^r	31
pRK2013	Km^r *tra*$^+$	10
pIP7	Ind$^+$ Km^r *cos*$^+$ *mob*$^+$; recombinant of pSTK10 with 25-kb *Sal*I-*Sal*I DNA fragment carrying *pah* gene cluster from OUS82 chromosomal DNA	This work
pDI1	Ind$^+$ (*pahA*$^+$) Ap^r; recombinant of pUC119 with 9.5-kb *Sal*I-*Sal*I fragment subcloned from 25-kb fragment of pIP7	This work

[a] Abbreviations: Nah$^+$, Phn$^+$, Hna$^+$, and Sal$^+$, phenotypes for the ability to assimilate naphthalene, phenanthrene, 1-hydroxy-2-naphthoic acid, and salicylic acid, respectively; Tc^r, Km^r, and Ap^r, resistance to tetracycline, kanamycin, and ampicillin, respectively; Ind$^+$, formation of indigo on LB plates or MM2YS plates containing 1 mM indole; *pah*, gene cluster for the upper pathway of naphthalene and phenanthrene degradation; *pahA*, gene for PAH dioxygenase.

recombinant pIP7 complemented the Nah$^-$ phenotype of OUS8211 (Fig. 2A), suggesting that pIP7 carries all of the genes for the upper degradation pathway from naphthalene to salicylic acid. Transconjugant OUS8211 carrying pIP7 was able to use phenanthrene also. Introduction of pIP7 into Nah$^-$ Phn$^-$ Sal$^-$ Hna$^+$ OUS8212 enabled it to use phenanthrene but not naphthalene (Fig. 2B). This observation suggests that genes for the upper pathway of naphthalene degradation mediate the conversion of phenanthrene to 1-hydroxy-2-naphthoic acid, which may be metabolized through a pathway different from that proposed by Evans et al. (8).

A 9.5-kb *Sal*I-*Sal*I fragment (made up of 6.5- and 3.0-kb subunits) was cloned from pIP7 into the *Sal*I site of plasmid pUC119 in *E. coli* HB101. One of the clones (designated pDI1) had dioxygenase activity. The 9.5-kb *Sal*I fragment recovered from pDI1 strongly hybridized with *Sal*I digests of plasmid NAH7 (data not shown). A restriction map of the fragment

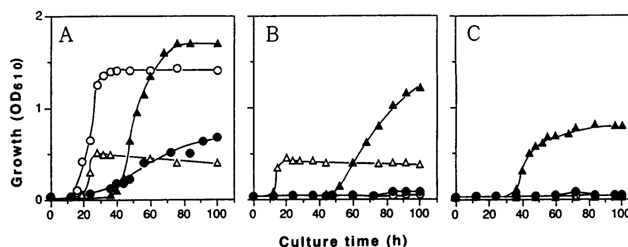

FIG. 1. Use of phenanthrene (●), naphthalene (○), salicylic acid (△), and 1-hydroxy-2-naphthoic acid (▲) by wild-type *P. putida* OUS82 (A), Nah$^-$ Sal$^+$ derivative OUS8211 (B), and Nah$^-$ Sal$^-$ derivative OUS8212 (C). OD_{610}, optical density at 610 nm.

cloned in pDI1 was then compared with the maps of the *nahA* genes ($nahA_{NAH7}$ and $nahA_{NCIB}$) of NAH7 from *P. putida* G7 and of pDTG1 from *P. putida* NCIB 1986 (26) (Fig. 3). Because the spaces between the *Eco*RV, *Xho*I, *Pst*I, and *Sma*I sites in the fragment were similar to those in $nahA_{NAH7}$ and $nahA_{NCIB}$, the region between *Hpa*I and *Eco*RI was tested for further restriction sites. *Sca*I, *Eco*RV, *Mul*I, and *Sph*I sites in the ferredoxin reductase genes ($nahA_a$), an *Xho*I site in the ferredoxin genes ($nahA_b$), *Pst*I, *Nco*I, *Kpn*I, *Mlu*I, and *Sca*I sites in genes ($nahA_c$) for the large subunits of the iron-sulfur protein, and *Bam*HI and *Sma*I sites in genes ($nahA_d$) for the small subunits of the iron-sulfur protein on NAH7 or pDTG1 previously reported were found in the *Hpa*I-*Eco*RI region on the cloned fragment. These observations suggest that the naphthalene-phenanthrene dioxygenase gene (*pahA*) of OUS82 is very similar to the *nahA* genes in *P. putida* plasmids.

The 9.5-kb *Sal*I fragment strongly hybridized with the total DNA from OUS82 but with neither the plasmid DNA nor the total DNA of OUS8211. This observation suggests that *pahA* is on the chromosome of OUS82 and is not present in Nah$^-$ derivative OUS8211.

Phenolic products are formed from these aromatic hydrocarbons through bacterial oxidation (2). Cells of Nah$^+$ or Phn$^+$ bacteria that had been cultured with naphthalene or phenanthrene were shaken for 48 h with various aromatic compounds (640 ppm for solid hydrocarbons; vapor for liquid hydrocarbons), and phenolic compounds produced in the supernatants of the cell suspensions were treated with Folin-Ciocalteau phenol reagent (2, 15). Their A_{760} was measured and used as an index of the concentration (millimolar) of sodium salicylate (Table 2). Wild-type OUS82 and recombinant strain OUS8211 carrying pIP7 yielded phenols from homocyclic PAHs naphthalene, phenanthrene, anthracene, fluorene, and acenaph-

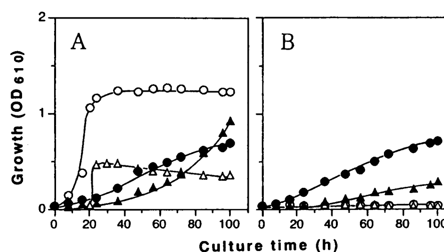

FIG. 2. Restoration of the ability to use naphthalene, phenanthrene, or both in deletion mutants OUS8211 (*trp-82* Nah$^-$ Phn$^-$ Sal$^+$ Hna$^+$) (A) and OUS8212 (*trp-82* Nah$^-$ Phn$^-$ Sal$^-$ Hna$^+$) (B) by introduction of cosmid recombinant pIP7. Symbols are the same as in Fig. 1. OD_{610}, optical density at 610 nm.

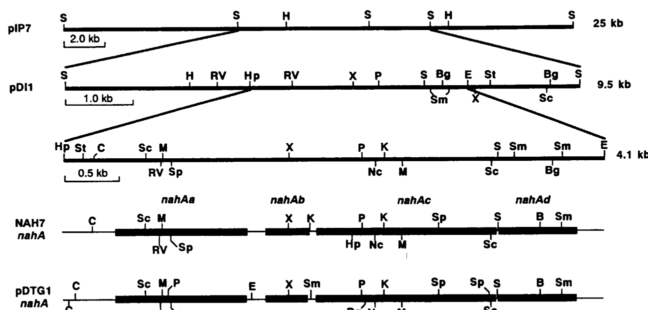

FIG. 3. Restriction map of the 9.5-kb *Sal*I-*Sal*I fragment carried by a recombinant that expresses dioxygenase, pDI1, subcloned from cosmid recombinant pIP7 into pUC19. Abbreviations for restriction endonucleases: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; K, *Kpn*I; M, *Mlu*I; Nc, *Nco*I; P, *Pst*I; RV, *Eco*RV; S, *Sal*I; Sc, *Sac*I; Sm, *Sma*I; Sp, *Sph*I; St, *Stu*I; X, *Xho*I.

thene and from heterocyclic PAHs dibenzothiophene, carbazole, dibenzofuran, xanthene, xanthone, and dibenzo-*p*-dioxin. Benzene derivatives, except biphenyl, were not oxidized. Nah$^-$ OUS8211 did not oxidize any of these aromatics. Nah$^+$ Phn$^-$ *P. putida* PpG1064 carrying NAH7 and Nah$^-$ Phn$^+$ *Alcaligenes faecalis* AFK2 also oxidized a range of PAHs, but their ability to degrade PAHs was not as great as that of OUS82 and OUS8211 carrying pIP7. Bacteria that use naphthalene or phenanthrene may degrade a range of two- and three-ring fused aromatics to phenols.

By high-pressure liquid chromatography (Hitachi L-6200 Intelligent Pump, L-3000 photodiode array detector, and D-6100 Data Station analyzer; Inertsil ODS-2 column; developing solvent, acetonitrile, and H_2O [60:40, vol/vol] containing 0.05% trifluoroacetic acid), *P. putida* AC10 carrying pIP7 was found to convert naphthalene into salicylic acid (retention time, 8.22 min) and phenanthrene into a small amount of 2-hydroxy-1-naphthoic acid (retention time, 11.36 min) and the major product 1-hydroxy-2-naphthoic acid (retention time, 13.97 min). An unassimilable PAH, anthracene, was also converted into 2-hydroxy-3-naphthoic acid (retention time, 11.97 min). Cloned DNA on pIP7 seemed to carry all of the genes for broad substrate specificity enzymes that convert these PAHs into the corresponding *o*-hydroxycarboxylic aromatics.

In *P. putida* OUS82, both of the pathways for catabolism of naphthalene to salicylate and of phenanthrene to naphthoic acids were mediated by a single enzyme system coded by a gene cluster on the chromosome. Because the substrate specificity of the enzyme system is broad, unassimilable dicyclic or tricyclic aromatics, including homocyclics and heterocyclics, might be converted into phenolic products. The observation that not only *P. putida* OUS82 but also Nah$^+$ Phn$^-$ *P. putida* PpG1064 carrying NAH7 and Nah$^-$ Phn$^+$ *A. faecalis* AFK2 converted various PAHs into phenols suggests that the broad substrate specificity of enzymes mediating the upper pathways for PAHs is common in bacteria that degrade PAHs. The finding that the restriction map of the 4.1-kb *Hpa*I-*Eco*RI region in the cloned DNA from OUS82 is similar to that of the *nahA* region in plasmid NAH7 suggests that all of the genes for the upper pathway in OUS82 have high sequence similarity to those in the *nah* operon. We report elsewhere on the cloning and sequencing of genes for the PAH dioxygenase and a gene for the PAH dihydrodiol dehydrogenase from OUS82 and on the similarity of *pahA* to $nahA_{\mathrm{NAH7}}$ and $nahA_{\mathrm{NCIB}}$ (27), which have similar nucleotide sequences (26). Zuniga et al. (32) also

TABLE 2. Conversion of 26 aromatic compounds into phenols by bacteria that utilize phenanthrene, naphthalene, or both

Aromatic	Amt of phenols produced (mmol)[a]			
	P. putida			*A. faecalis*
	OUS82 (wild type)	OUS8211 (pIP7)	PpG1064 (NAH7)	AFK2 (wild type)
Homocyclics				
Naphthalene	4.8	6.2	0.2	0.5
Phenanthrene	4.1	4.8	0.3	3.3
Anthracene	4.5	2.6	0.1	0.4
Pyrene	0.1	ND	ND	ND
Naphthacene	ND	ND	ND	ND
Benz[*a*]anthracene	ND	ND	ND	0.1
Fluorene	1.3	0.3	0.1	0.3
Acenaphthene	0.8	0.5	0.2	0.2
Fluoranthene	0.1	ND	ND	0.1
Heterocyclics				
Dibenzothiophene	0.7	1.1	0.6	1.3
Carbazole	0.4	0.1	0.1	0.2
Dibenzofuran	0.7	0.4	0.5	0.3
Xanthene	0.4	0.7	0.2	0.3
Xanthone	1.0	1.0	0.1	0.4
Dibenzo-*p*-dioxin	0.5	0.2	0.2	0.2
Monocyclics				
Biphenyl	0.2	0.1	0.1	0.2
Diphenyl ether	0.2	ND	ND	ND
Tetralin	0.1	ND	ND	ND
Cyclohexylbenzene	ND	ND	ND	ND
Diphenyl methane	0.1	ND	ND	ND
n-Decylbenzene	ND	ND	ND	ND
1-Phenylhexane	ND	ND	ND	ND
Diethylbenzene	ND	ND	ND	ND
o-Xylene	ND	ND	ND	ND
Toluene	ND	ND	ND	ND
Benzene	ND	ND	ND	ND

[a] Amounts of phenols converted from aromatics were determined by measuring salicylic acid concentrations. The conversion procedures are described in the text. ND, no phenol detected.

reported the chromosomal genes for the upper pathway of naphthalene degradation by *P. putida* PMD-1. Menn et al. (19) and Sanseverino et al. (23) have reported that bacteria carrying NAH7 and an NAH7-like plasmid can convert anthracene and phenanthrene into naphthoic acids. Recently, Denome et al. (5) have reported the complete DNA sequences of upper naphthalene-catabolic (*dox*) genes residing on a 75-kb plasmid harbored by dibenzothiophene-degrading *Pseudomonas* sp. strain C18, which can degrade but not assimilate naphthalene and phenanthrene. We also found here that plasmid NAH7 can mediate the oxidation of a variety of PAHs to phenols. Thus, PAH-catabolic (*pah*) genes similar to each other might have been conserved not only on plasmids but also on chromosomes in bacterial populations.

By introduction of a recombinant plasmid carrying the *pah* gene cluster, Phn$^-$ Nah$^-$ Sal$^+$ OUS8211 was transformed to the Phn$^+$ Nah$^+$ phenotype, but the Phn$^-$ Nah$^-$ Sal$^-$ Hna$^+$ mutant restored only the Phn$^+$ phenotype. These findings indicate that the ability of *P. putida* OUS82 to assimilate naphthalene and phenanthrene depends on the occurrence of both of the degradation pathways after salicylic and 1-hydroxy-2-naphthoic acids, which are formed through a common upper pathway. The pathway for catabolism of 1-hydroxy-2-naphthoic acid may be different from that for the catabolism of salicylic

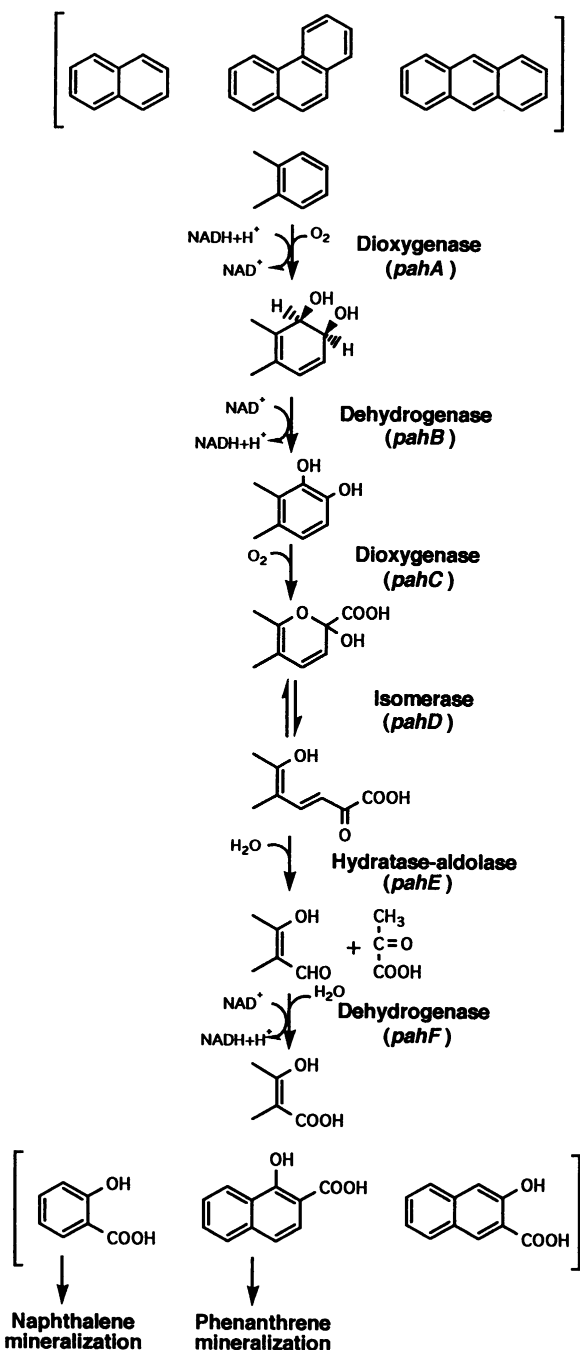

FIG. 4. Possible relationship between the upper pathway for PAH degradation in *P. putida* OUS82 and mineralization of those compounds.

acid. However, the mechanism for the further degradation of 1-hydroxy-2-naphthoic acid remains unclear. The ability of a single strain of bacteria to use various PAHs may depend on the presence of enzyme systems for further metabolism of metabolites produced through the upper pathway that is almost identical to that informed by plasmid NAH7 (6) (Fig. 4).

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