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A Novel Phenanthrene Dioxygenase from *Nocardioides* sp. Strain KP7: Expression in *Escherichia coli*

ATSUSHI SAITO, TOKURO IWABUCHI,† AND SHIGEAKI HARAYAMA*

Marine Biotechnology Institute, Kamaishi Laboratories, Kamaishi, Iwate 026-0001, Japan

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***Nocardioides* sp. strain KP7 grows on phenanthrene but not on naphthalene. This organism degrades phenanthrene via 1-hydroxy-2-naphthoate, *o*-phthalate, and protocatechuate. The genes responsible for the degradation of phenanthrene to *o*-phthalate (*phd*) were found by Southern hybridization to reside on the chromosome. A 10.6-kb DNA fragment containing eight *phd* genes was cloned and sequenced. The *phdA*, *phdB*, *phdC*, and *phdD* genes, which encode the α and β subunits of the oxygenase component, a ferredoxin, and a ferredoxin reductase, respectively, of phenanthrene dioxygenase were identified. The gene cluster, *phdAB*, was located 8.3 kb downstream of the previously characterized *phdK* gene, which encodes 2-carboxybenzaldehyde dehydrogenase. The *phdCD* gene cluster was located 2.9 kb downstream of the *phdB* gene. PhdA and PhdB exhibited moderate (less than 60%) sequence identity to the α and β subunits of other ring-hydroxylating dioxygenases. The PhdC sequence showed features of a [3Fe-4S] or [4Fe-4S] type of ferredoxin, not of the [2Fe-2S] type of ferredoxin that has been found in most of the reported ring-hydroxylating dioxygenases. PhdD also showed moderate (less than 40%) sequence identity to known reductases. The *phdABCD* genes were expressed poorly in *Escherichia coli*, even when placed under the control of strong promoters. The introduction of a Shine-Dalgarno sequence upstream of each initiation codon of the *phdABCD* genes improved their expression in *E. coli*. *E. coli* cells carrying *phdBCD* or *phdACD* exhibited no phenanthrene-degrading activity, and those carrying *phdABD* or *phdABC* exhibited phenanthrene-degrading activity which was significantly less than that in cells carrying the *phdABCD* genes. It was thus concluded that all of the *phdABCD* genes are necessary for the efficient expression of phenanthrene-degrading activity. The genetic organization of the *phd* genes, the phylogenetically diverged positions of these genes, and an unusual type of ferredoxin component suggest phenanthrene dioxygenase in *Nocardioides* sp. strain KP7 to be a new class of aromatic ring-hydroxylating dioxygenases.**

The aerobic degradation of polycyclic aromatic hydrocarbons (PAHs), especially of low-molecular-weight ones such as naphthalene and phenanthrene, has been extensively studied (8, 19, 20). Bacteria degrade phenanthrene by one of two distinct routes. Bacteria capable of growing on both phenanthrene and naphthalene mineralize these substrates via salicylate and catechol (the salicylate pathway), while those capable of growing on phenanthrene but not naphthalene degrade phenanthrene via protocatechuate (the protocatechuate pathway) (29).

On the salicylate pathway, phenanthrene and naphthalene are converted to 1-hydroxy-2-naphthoate and salicylate, respectively, by the same set of enzymes (30, 54). The amino acid sequences of these enzymes (48, 54) were >90% identical to those of naphthalene-degrading enzymes encoded by the NAH7 plasmid in *Pseudomonas putida* G7 (11, 13, 42), by the pDTG1 plasmid in *P. putida* NCIB 9816-4 (31), and by the C18 plasmid in *Pseudomonas* sp. strain C18 (10). This observation has led to the expectation that the sequence diversity in the genes for the degradation of naphthalene and phenanthrene in bacteria would be limited. However, this view has recently been modified. *Pseudomonas* sp. strain U2 isolated by selective enrichment on naphthalene converts this substrate to salicylate, but salicylate is transformed differently from other naphthalene-degrading bacteria to gentisate. The organization of genes for naphthalene dioxygenase in strain U2 was different from that in other naphthalene-degrading bacteria, the genes for salicylate 5-hydroxylase being inserted in the gene cluster for naphthalene dioxygenase (18). Similarly, the genes for the degradation of naphthalene and phenanthrene in *Burkholderia* sp. strain RP007 have been shown to be significantly different in sequence and gene order from the above-mentioned naphthalene- or phenanthrene-degrading genes (32).

The catabolic genes from bacteria capable of growing on phenanthrene but not naphthalene have not previously been characterized. *Nocardioides* sp. strain KP7 was isolated from a Kuwait beach on the basis of its ability to degrade phenanthrene 2 years after the oil pollution occurred in 1991 (27). This organism transforms phenanthrene via the protocatechuate pathway. The genes and enzymes involved in the transformation of 1-hydroxy-2-naphthoate to *o*-phthalate in this organism have recently been characterized (24–26). In the present study, we characterize the genes for phenanthrene dioxygenase, the enzyme involved in the first step of phenanthrene catabolism, in this organism.

(A preliminary account of this work was presented at the May 1998 IUPAC International Conference. "Degradation Processes in the Environment" [39].)

MATERIALS AND METHODS

Bacterial strains and culture conditions. A gene library of *Nocardioides* sp. KP7 (27), a phenanthrene-degrading bacterium, was constructed in *Escherichia coli* S17-1 (43). *E. coli* HB101 (40), JM109 (55), and BL21(DE3) (45) were used as the hosts for plasmid construction or gene expression. *E. coli* strains were grown at 37°C on Luria-Bertani medium (40) or M9-glucose medium (40) supplemented with 100 μg of ampicillin per ml. *Nocardioides* sp. KP7 was grown at

* Corresponding author. Mailing address: Marine Biotechnology Institute, Kamaishi Laboratories, 3-75-1 Heita, Kamaishi, Iwate 026-0001, Japan. Phone: 81-193-26-6544. Fax: 81-193-26-6592. E-mail: shigeaki.harayama@kamaishi.mbio.co.jp.

† Present address: Shiseido Research Center, Kohoku-ku, Yokohama, Kanagawa 223-0057, Japan.

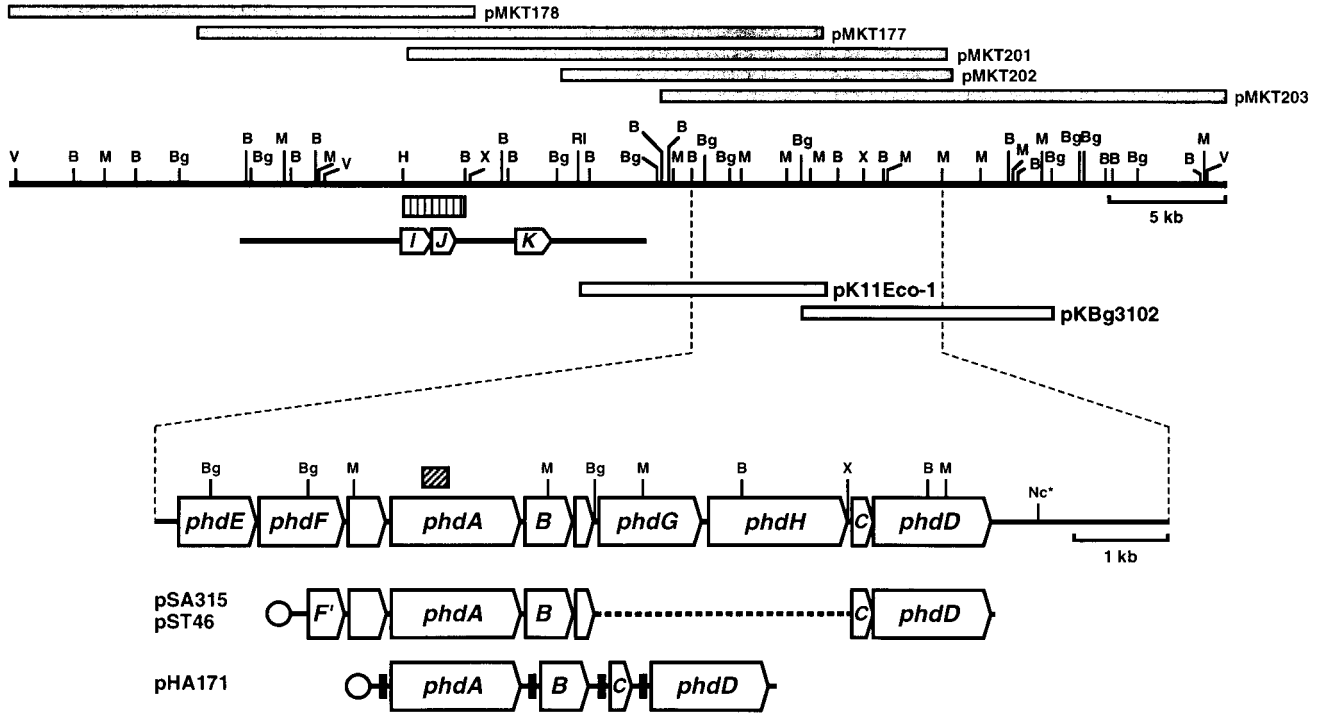

FIG. 1. Physical map and genetic organization of phenanthrene-degrading genes. Gray bars indicate the DNA inserts in the library clones. Open bars show inserts in the two clones used in this study. Arrows on the enlarged physical map show the locations and orientations of the phenanthrene-degrading genes: *phdA*, the α subunit of the oxygenase component; *B*, *phdB*, the β subunit of the oxygenase component; *C*, *phdC*, the ferredoxin component; *phdD*, the ferredoxin reductase component; *phdE*, dihydrodiol dehydrogenase; *phdF*, extradiol dioxygenase; *phdG*, hydratase-aldolase; *phdH*, aldehyde dehydrogenase. The box with oblique indicates the 302-bp PCR-amplified fragment that was used for probing the library clones. The typical expression plasmids are shown at the bottom. Circles indicate promoters. Transcription of DNA segments containing *phdABCD* genes cloned in pSA315 and pST46 is under the control of the *lac* and T7 promoters, respectively. The transcription in pHA171 of the *phdABCD* genes preceded by the SD sequence that is effective in *E. coli* (filled boxes) is under the control of the T7 promoter. The bar with three arrows indicates the previously reported sequence (AB000735 in DDBJ/GenBank/EMBL databases), arrows marked with *I*, *J*, and *K* showing the locations and orientations of the phenanthrene-degrading genes *phdI* (1-hydroxy-2-naphthoate dioxygenase), *phdJ* (*trans*-2′-carboxybenzalpyruvate hydratase-aldolase), and *phdK* (2-carboxybenzaldehyde dehydrogenase). The striped bar shows the 2.5-kb *Hin*dIII-*Bam*HI DNA fragment that was used as probe in the hybridization following PFGE (Fig. 2). Restriction sites: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII, M, *Mlu*I; Nc, *Nco*I; RI, *Eco*RI; V, *Eco*RV; X, *Xba*I. The asterisk indicates that the other *Nco*I restriction sites are not shown.

30°C in 5 ml of marine broth (Difco Laboratories) containing 10 mg of phenanthrene. The stationary-phase culture exhibited a tinge of pink.

PFGE and hybridization. The cells of *Nocardioides* sp. strain KP7 (2×10^7 to 5×10^7) were embedded in a gel plug made of 1.0% (wt/vol) CleanCut agarose (Bio-Rad Laboratories) and treated with restriction enzymes by using a CHEF genomic DNA plug kit (Bio-Rad). Plugged samples were placed in slots of a running gel made of 1.0% (wt/vol) pulsed-field certified agarose (Bio-Rad) and fixed by pouring molten 1.0% CleanCut agarose in the slots. Pulsed-field gel electrophoresis (PFGE) was performed with CHEF model DR II apparatus (Bio-Rad) under the following conditions: electrophoresis buffer, 0.5× Tris-borate-EDTA; voltage, 200 V (6 V/cm); reorientation angle, 120°; pulse time, linearly increased from 60 to 120 s; run times, 22 h; temperature, 15°C. Chromosome DNAs of *Saccharomyces cerevisiae* YNN295 and concatemers of lambda 50-kb DNA (Bio-Rad) were used as size standards. The DNA bands were visualized by ethidium bromide. DNAs in a pulsed-field gel were blotted onto a nylon membrane of Hybond-N+ (Amersham). The 2.5-kb *Hin*dIII-*Bam*HI DNA fragment (Fig. 1; nucleotides 6776 to 9312 of entry AB000735 in the DDBJ/GenBank/EMBL DNA databases), which contained almost the entire *phdI* gene (the structural gene for 1-hydroxy-2-naphthoate dioxygenase) (26) and the entire *phdJ* gene (the structural gene for *trans*-2′-carboxybenzalpyruvate hydratase-aldolase) (25), was used as a probe. Labeling of probe DNA, hybridization, and signal development were carried out by using the Amersham ECL (enhanced chemiluminescence) direct nucleic acid labeling and detection system.

Cloning and sequencing. The gene library of *Nocardioides* sp. strain KP7 based on pLAFR3 (44) was constructed as reported by Iwabuchi et al. (26). To produce a probe against the gene for the ring-hydroxylating dioxygenase of *Nocardioides* sp. strain KP7, the degenerate primers DO-1s [5′-TG(TC)AG(TC)T(AT)(TC)CA(TC)GG(GATC)TGG-3′] and DO-1a [5′-TC(GATC)(GA)C(GATC)GC(AG)AA(TC)TTCCA(AG)TT-3′] were designed from conserved amino acid sequences of the known α subunits of the ring-hydroxylating dioxygenase (10, 15, 23, 31, 34, 42, 47, 48, 58). A PCR with this primer pair and KP7 total DNA as a template amplified a 0.3-kb fragment. This fragment was cloned in the *Srf*I site of pCR-Script SK(+) (Stratagene Cloning Systems), giving pK11-300-5, and the 302-bp-long sequence was determined. The genomic library was then screened by colony hybridization, using the 302-bp fragment as a probe. Labeling of the probe DNA, hybridization, and signal development were carried out by using the Amersham ECL direct nucleic acid labeling and detection system. The four clones, pMKT177 (24, 26), pMKT201, pMKT202, and pMKT203, gave a positive signal.

The 10.5-kb *Eco*RI fragment of pMKT177 and the 10.5-kb *Bgl*II fragment of pMKT203 were cloned into the *Eco*RI site and *Bgl*II site, respectively, of pSL301 (7) to produce pK11Eco-1 and pKBg3102 (Fig. 1). Using pK11Eco-1, pKBg3102, and their subclone derivatives, the nucleotide sequence of the 10.6-kb segment encompassing the genes for phenanthrene dioxygenase (*phdABCD*) was determined using a *Taq* DyeDeoxy terminator cycle sequencing kit and a 373A DNA sequencer (Perkin-Elmer Applied Biosystems).

Homology search and phylogenetic analysis. The nucleotide sequence was processed by SeqEd version 1.0.3 and Genetyx version 8.0 software, and a homology search was performed with BLAST 2.0 (gapped BLAST) (1). Protein sequences were aligned by using CLUSTAL W version 1.7 (49), phylogenetic analysis was performed by the PHYLIP version 3.572c package (16), and a neighbor-joining tree was constructed by the NEIGHBOR program. The resulting unrooted tree was depicted by using the TreeView version 1.5 package (48).

Construction of expression plasmids. The 3-kb *Bgl*II fragment from pKEco-1 containing *phdE*, *phdA*, and *phdB* was cloned into the *Bgl*II site of pSL301 to give pKG1021. The 2-kb *Xba*I-*Nco*I fragment from pKBg3102 containing *phdC* and *phdD* was cloned between the *Xba*I and *Nco*I sites of pSL301 to give pKXN382. A *Hin*dIII site was introduced immediately after the termination codon of *phdD* by using a PCR primer, 5′-CCCCAAGCTTGGAATTCTTCCTCGGCTCATGCCGTCGGT-3′ (the underlined sequences are the *Hin*dIII and *Eco*RI sites), and the *Xba*I-*Hin*dIII fragment containing *phdC* and *phdD* was cloned between the *Xba*I and *Hin*dIII sites of pKG1021 to give pSA315 (Fig. 1). pT7-7(BglIIX) is a derivative of pT7-7 (3) in which the *Bgl*II site had been disrupted for the convenience of DNA cloning (39). The 4.6-kb *Eco*RI fragment from pSA315

including the *phdABCD* genes was cloned into the *Eco*RI site of pT7-7 (*Bgl*IIX), giving pST46 (Fig. 1). The *phd* genes in pSA315 and pST46 were arranged in the order *phdA-phdB-phdC-phdD* under the control of the *lac* and T7 promoters, respectively. The 1.36-kb fragment containing *phdA* was prepared by PCR using pKG1021 as the template and primers 5′-GGGAATTCCATATGTCGGTAGT CAGCGGGGAT-3′ (the underlined sequence is the *Nde*I site) and 5′-CCGG AATTCGGTCGCAACTCATAAGACAGC-3′ (the underlined sequence is the *Eco*RI site). The amplified product was cloned between the *Nde*I and *Eco*RI sites of pT7-7(BglIIX) to give pHA102. The entire *phdA* gene in pHA102 was located under the control of the T7 promoter and an efficient Shine-Dalgarno (SD) sequence derived from the pT7-7(BglIIX) vector. The 0.56-kb fragment containing *phdB* was prepared by PCR using pKG1021 as the template and primers 5′-CCGGAATTCAAGGAGATATACATATGCTGACTACTGTTGACGAG AATC-3′ (the underlined sequences are the *Eco*RI and *Nde*I sites) and 5′-CG CGGATCCAGATCTGCCTGCGGGCTAGAAG AAGAACGC-3′ (the underlined sequences are the *Bam*HI and *Bgl*II sites). The PCR product was cloned between the *Eco*RI and *Bam*HI sites of pT7-7(BglIIX) to give pHB103. The *phdB* gene in pHB103 was preceded by the efficient SD sequence, AAGGAG, derived from one of the primers used, and transcribed under the control of the T7 promoter. The 0.25-kb DNA fragments containing AAGGAG-*phdC* was PCR amplified by using pKXN382 as the template and the two primers 5′-CG CGGATCCAGATCTAAGGAGATATACATATGCGTGTGGATGTTGACC CACAGCGG-3′ (the underlined sequences are the *Bam*HI, *Bgl*II, and *Nde*I sites) and 5′-GCCCAAGCTTGGCCTCCCGTCACTGAGCGGG-3′ (the underlined sequence is the *Hin*dIII site) and then cloned between the *Bam*HI and *Hin*dIII sites of pT7-7(BglIIX) to give pHC102. The 1.26-kb fragment containing *phdD* was prepared by PCR using pKXN382 as the template and the two primers 5′-GGGAATTCCATATGACGGGAGGCCAGGTGGCGGCGC-3′ (the underlined sequence is the *Nde*I site) and 5′-CCCCAAGCTTGGAATTCTTCCT CGGCTCATGCCGTCGGT-3′ (the underlined sequence is the *Hin*dIII site), and the amplified fragment was cloned between the *Nde*I and *Hin*dIII sites of pT7-7(BglIIX) to give pHD115. To introduce the SD sequence upstream of *phdD*, two oligonucleotides, 5′-CTAGAAAGCTTAGATCTAAGGAGATATA CA-3′ (the underlined sequences are the *Hin*dIII and *Bgl*II sites) and 5′-TATG TATATCTCCTTAGATCTAAGCTTT-3′ (the underlined sequences are the *Bgl*II and *Hin*dIII sites), were annealed each other, and the double-stranded oligonucleotides were inserted between the *Xba*I-*Nde*I sites of pHD115 to give pHD101. The 0.56-kb *Eco*RI-*Bam*HI fragment from pHB103 containing AAG GAG-*phdB* was cloned between the *Eco*RI and *Bam*HI sites of pHA102 to give pHA111. *E. coli* BL21(DE3) carrying pHA111 overproduced three products: PhdA, PhdB, and a PhdB derivative most likely translated from the Met codon within the 3′ end of the *phdA* gene. To eliminate this by-product, pHA111 was digested with *Eco*RI, filled in with T4 DNA polymerase, and recircularized. Production of the undesired product was eliminated in the resulting plasmid, pHA111eV. pHB103 was digested with *Nde*I and recircularized to give pHB111, in which the *phdB* gene was located under the control of the T7 promoter and the efficient SD sequence derived from the pT7-7(BglIIX) vector. The 0.25-kb *Bam*HI-*Hin*dIII fragment from pHC102 containing AAGGAG-*phdC* was cloned between the *Bam*HI and *Hin*dIII sites of pHA102, pHA111eV, and pHB111, giving pHA121 (*phdA-phdC*), pHA141eV (*phdA-phdB-phdC*), and pHB121 (*phdB-phdC*), respectively. The 1.3-kb *Hin*dIII fragment from pHD101 containing AAGGAG-*phdD* was cloned into the *Hin*dIII sites of pHA121, pHA141eV, and pHB121, giving pHA162 (*phdA-phdC-phdD*), pHA171 (*phdA-phdB-phdC-phdD*), and pHB141 (*phdB-phdC-phdD*), respectively. pHA171 was digested with *Bgl*II (eliminating the *phdC* gene) and recircularized to give pHA151 (*phdA-phdB-phdD*).

Biotransformation of phenanthrene. *E. coli* BL21(DE3) or JM109 cells carrying one of the *phd*-expressing plasmids were grown overnight at 37°C in 100 ml of M9-glucose medium containing 100 μg of ampicillin per ml. The cells were collected by centrifugation, washed, and resuspended in a fresh M9-glucose medium, after which adjusting the cell densities were adjusted to an optical density at 600 nm of 1.0. An aliquot (10 ml) of the cell suspension was distributed into sealed vials (100 ml), and 40 μl of 50 mM phenanthrene dissolved in *N,N*-dimethylformamide was added to make the final concentration of phenanthrene 200 μM. When required isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 100 μM was added. The cells were incubated at 37°C for the time indicated. The volume of each culture was adjusted with methanol to 20 ml to completely dissolve the substrate and metabolites, mixed well, and centrifuged at 12,000 × *g* for 10 min. The resulting supernatant was loaded into a reverse-phase column (TSKgel ODS-80Ts; 4.6 by 250 mm; Tosoh) fitted to a high-pressure liquid chromatography (HPLC) system (Tosoh). Phenanthrene and its hydroxylation product were separated by using a methanol gradient (from 60% [vol/vol] to 90% [vol/vol] methanol in 5 min followed by 90% [vol/vol] methanol for 15 min) at a flow rate of 1 ml/min. Electron impact mass spectrometry (EI-MS) of the collected hydroxylation product was carried out with a JMS-SX102 mass spectrometer (JEOL, Tokyo, Japan).

Nucleotide sequence accession numbers. The nucleotide sequences reported in this paper have been submitted to the DDBJ/GenBank/EMBL DNA databases under accession no. AB017794, AB017795, and AB031319.

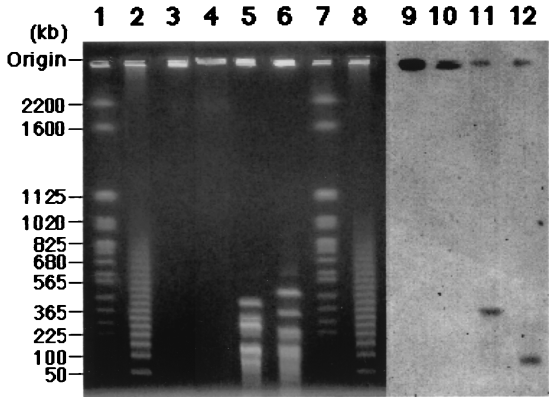

FIG. 2. Localization of phenanthrene-degrading genes on the chromosome DNA. Total DNA of *Nocardioides* sp. strain KP7 was subjected to PFGE (lanes 1 to 8) and hybridization with the 2.5-kb *Hin*dIII-*Bam*HI DNA fragment shown in Fig. 1 (lanes 9 to 12). Lanes 1 and 7, *S. cerevisiae* YNN295 chromosomal DNA as molecular mass markers; lanes 2 and 8, ladder of phage lambda DNA concatemers as molecular mass markers; lanes 3 and 9, undigested total DNA of KP7; lanes 4 and 10, *Dra*I-digested total DNA of KP7; lanes 5 and 11, *Xba*I-digested total DNA of KP7; lanes 6 and 12, *Hin*dIII-digested total DNA of KP7.

RESULTS

Localization of the *phd* gene cluster on the chromosome DNA. To examine whether the *phd* gene cluster lies on the chromosome or on a plasmid, the total cellular DNA of *Nocardioides* sp. strain KP7 was subjected to PFGE and then transferred to a membrane which was probed with the 2.5-kb *Hin*dIII-*Bam*HI DNA fragment containing the *phdI* and *phdJ* sequences (Fig. 1). Undigested KP7 DNA barely moved from the slot and hybridized with the probe (Fig. 2, lanes 3 and 9). No DNA band with a size between 50 and 2,200 kb was apparent. The *Dra*I digestion (lanes 4 and 10) gave a result similar to that with the nondigested DNA. In the cases of digestion with *Xba*I and *Hin*dIII, the probe hybridized to the 350- and 80-kb bands, respectively (lanes 11 and 12). These results indicate that the *phd* gene cluster resides on a very large DNA, most likely the chromosome DNA.

Cloning and sequencing of *phd* genes. The degenerate primers DO-1s and DO-1a, which were designed from the conserved amino acid sequences of the α subunits of ring-hydroxylating dioxygenases, were used to amplify a 302-bp fragment from the genomic DNA of *Nocardioides* sp. strain KP7 (Fig. 1). The amino acid sequence encoded by the fragment showed significant homology to the α subunits of known ring-hydroxylating dioxygenases. The gene library of strain KP7 was then screened by using the 302-bp fragment as a probe. Four clones gave a positive signal. One of them, pMKT177, has previously been characterized as a clone containing the *phdIJK* gene cluster, which encodes the enzymes responsible for the transformation of 1-hydroxy-2-naphthoate to *o*-phthalate (24–26). The other positive clones are designated pMKT201, pMKT202, and pMKT203. The physical map of 52-kb-long DNA encompassing pMKT178, pMKT177, and pMKT203 was constructed, and pK11Eco-1 and pKBg3102 were used for DNA sequencing of the 10.6-kb-long *Bam*HI-*Mlu*I fragment (Fig. 1). The gene cluster *phdEFABGHCD* plus two small open reading frames (ORFs) was identified downstream of the previously characterized *phdIJK* genes. In this study, the *phdA*, *phdB*, *phdC*, and *phdD* genes were further characterized.

PhdA showed significant but moderate sequence homology to the α subunits of known ring-hydroxylating dioxygenases. The amino acid sequences of the α subunits of PAH dioxyge-

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PhdA.KP7          91  CRHRGTLLCRTEAGNTSHFRCPYH  114----205  NWKLGADNFVGDAYHT-LMTH  224
NdoB.NCIB9816     81  CRHRGKTLVSVEAGNAKGFVCSYH  104----194  NWKAPAENFVGDAYHV-GWTH  213
NidA.I24          88  CRHRGMQVCRAEMGNASHFRCPYH  111----202  NWKLGADNFVGDAYHT-MMTH  221
CarAa.CB3         75  CRHRGGALCRGESGNTKNFICTYH   98----189  NWKLAAEQFTTDDFHF-LTSH  208
DxnA1.RW1         85  CRHRGNRLCLADRGNAKSFRCSYH  108----195  NWKWQAEQHATDHLHV-AVSH  214
DitA1.BKME-9      91  CPHRGMRISTADCGNTQIHKCIYH  114----205  NWKTAGEQSAADGFHT-LTLH  224
BnzA.BE-81        96  CRHRGMRICRADAGNAKAFTCSYH  119----208  NWKFAAEQFCSDMYHAGTTSH  228
BphA.LB400       100  CRHRGMRICRSDAGNAKAFTCSYH  123----219  NWKFAAEQFCSDMYHAGTTTH  239
TdnA1.UCC22       94  CSHRGASVCREHRGNAAGFTCPYH  117----208  NWKLVWDNAG-DGYHV-PFSH  226
CmtAb.F1          84  CPHRGATVCRERSGNSKNFQCFYH  107----198  NWKLLVENSI-DGYHA-VSTH  216
XylX.mt2          92  CSHRGATLCRFRSGNKATHTCSFH  115----208  NWKVQVENGA-DGYHV-STVH  226
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FIG. 3. Alignment of well-conserved sequence regions in the N-terminal and central part of the α subunits of ring-hydroxylating dioxygenases. The three-dimensional structure of the NdoBC oxygenase component from *Pseudomonas* sp. strain NCIB 9816-4 has recently been determined (28). Highlighted characters represent the residues involved in binding to the Rieske-type [2Fe-2S] cluster and to the mononuclear iron atom. Shadowed characters indicate the Asp residues hydrogen bonded to active-site ligand His on the same molecule and to [2Fe-2S] Rieske center ligand His on the neighboring α subunit. The Rieske-type [2Fe-2S] cluster-binding sequence, $CXHX_{17}CX_2H$, in PhdA was located at Cys91 to His114. The residues involved in coordinating the catalytic iron were also conserved as His219 and His224 (and Asp376). Asterisks indicate residues conserved in all proteins. Definition of abbreviations are given in the legend to Fig. 5.

nases from *Rhodococcus* sp. strain NCIMB 12038 (NarAa, AF082663) and strain I24 (NidA) (50) showed 55 to 56% identity with the PhdA sequence, whereas the other known α subunits exhibited a sequence identity lower than 40%. The amino acid sequence alignment of PhdA to the known α subunits revealed a limited number of conserved amino acid residues, the highly conserved ones corresponding to those involved in the coordination to the [2Fe-2S] Rieske-type cluster and to the catalytic nonheme iron (Fig. 3) (28).

The *phdB* gene was found downstream of the *phdA* gene, and PhdB exhibited significant sequence homology to the β subunits of the ring-hydroxylating dioxygenases. The β subunits of PAH dioxygenases from *Rhodococcus* sp. strain I24 (NidB) (50) and strain NCIMB 12038 (NarAb, AF082663) manifested relatively high sequence identity to PhdB (55 and 52%, respectively). The other β subunits exhibited sequence identity lower than 40%.

The *phdC* gene was located 2.9 kb downstream of the *phdB* gene. PhdC, which is predicted to be 69 amino acids long, did not show significant homology to any of the ferredoxin components of known ring-hydroxylating dioxygenases, except for DitA3 (33), but showed significant homology to the [3Fe-4S] and [4Fe-4S] ferredoxins.

The *phdD* gene was found just downstream of the *phdC* gene, and the encoded gene product showed significant sequence similarity to the proteins of the reductase family. PhdD contained amino acid sequences typical of ADP-binding βαβ folds (53) which may constitute putative flavin adenine dinucleotide (Val6 to Ala37)- and NAD (Ser148 to Glu176)-binding sites (35) with the completely conserved consensus motif $GXGX_2GX_3A$.

Involvement of the *phdABCD* genes in enzyme activity. To determine whether the *phdABCD* genes actually encode the components of the ring-hydroxylating dioxygenase, the genes were introduced into *E. coli*. Plasmids pSA315, containing the *phdABCD* genes under the control of the *lac* promoter, and pST46, containing the *phdABCD* genes under the control of the T7 promoter, were introduced into *E. coli* JM109 and BL21(DE3), respectively. The phenanthrene transformation activity in these cells was then examined in the presence or absence of IPTG. Both *E. coli* cultures showed very low phenanthrene transformation activity. One possible reason for this was inefficient translation from the *phdABCD* messages in the *E. coli* cells. Another possibility was inefficient transcription prevented by extra DNA sequences (e.g., the 3′-half region of *phdF* and two small ORFs) (Fig. 1).

To improve the expression of the *phdABCD* genes, an SD sequence whose effectiveness in *E. coli* had previously been demonstrated was introduced upstream of each of the *phdABCD* genes as described in Materials and Methods. The constructed plasmids were subsequently introduced into BL21(DE3), and the phenanthrene-transforming activity in the absence of IPTG was then examined in BL21(DE3) cells carrying one of these plasmids. The cells carrying pHA171 (*phdABCD*) showed the highest activity to transform phenanthrene to a more hydrophilic product (Fig. 4). The EI-MS spectrum of the product showed the molecular ion at *m/z* 212 (M^+), indicating the formation of phenanthrene dihydrodiol. Phenanthrene decreased linearly to 26% of the input value up to 12 h, while the concentration of dihydrodiol increased linearly during this period. After extension of the incubation to 24 h, phenanthrene had completely disappeared (data not shown).

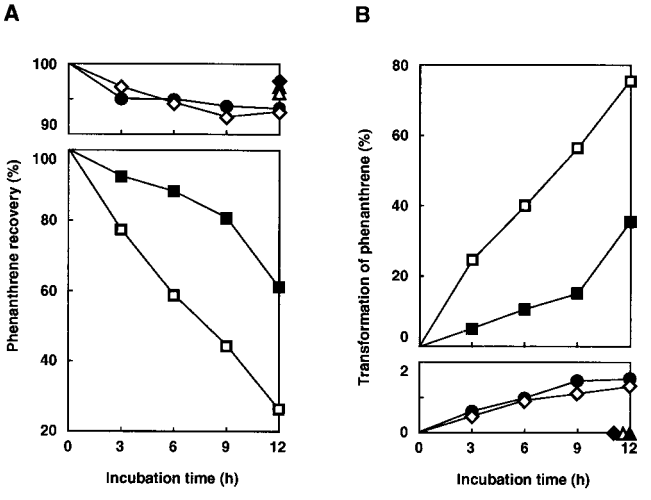

FIG. 4. Requirements of the *phdABCD* genes for phenanthrene transformation activity. The removal of phenanthrene (A) and formation of the dihydrodiol (B) were monitored by HPLC at 254 nm in a suspension of BL21(DE3) cells carrying expression plasmids pHA171 (*phdABCD*) (□), pHA141eV (*phdABC*) (■), pHA151 (*phdABD*) (●), pHA111eV (*phdAB*) (◇), pHB141 (*phdBCD*) (◆), pHA162 (*phdACD*) (△), and pT7-7 (BglIIX) (▲). Prolonged incubation (24 h) of the cells carrying pHA171 resulted in the complete removal of phenanthrene. Therefore, the rate of dihydrodiol formation was calculated by taking the amount of dihydrodiol at 24 h as 100%. Values are averages from two independent experiments.

Cells carrying pHA141eV (*phdABC*) lacking the reductase gene, *phdD*, showed one-third of the transforming activity of the pHA171 (*phdABCD*). Cells carrying pHA151 (*phdABD*) lacking the ferredoxin gene, *phdC*, and those carrying pHA111eV (*phdAB*) showed much less activity than cells carrying pHA171 (*phdABCD*). These results indicated that both electron transport proteins, PhdC and PhdD, were involved in the enzyme activity. Cells carrying pHA162 (*phdACD*), pHB141 (*phdBCD*), and pT7-7(BglIIX) showed no transformation activity, demonstrating the indispensability of the α and β subunits for the enzyme activity.

PhdA and PhdB expressed in *E. coli* cells from pHA171 (*phdABCD*) and pHA141eV (*phdABC*) were detected as major bands by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) even without the addition of IPTG, and its addition at a final concentration of 0.1 mM increased the level of their expression. However, the IPTG addition markedly reduced the activity of the phenanthrene transformation in these cells (data not shown), suggesting that the high-level expression of the *phd* genes caused the formation of inclusion bodies. In contrast to the PhdA and PhdB expression, PhdC and PhdD could not be identified in the *E. coli* cells by SDS-PAGE in the presence or absence of IPTG (data not shown).

DISCUSSION

We report in this study the cloning, sequencing, and functional expression in *E. coli* of a novel PAH ring-hydroxylating dioxygenase which is composed of the PhdABCD proteins. The gene clusters responsible for PAH degradation have been localized on both plasmids (*nah*, *P. putida* strain [31]; *ndo*, *P. putida* strain [42]; *dox*, *Pseudomonas* sp. strain C18 [10]; *nag*, *Pseudomonas* sp. strain U2 [18]; *phn*, *Burkholderia* sp. strain RP007 [32]) and chromosomes (*pah*, *P. putida* OUS82 [48]; *nah*, *P. stutzeri* AN10 [6]). The results of this study show that the *phd* gene cluster was localized on the chromosome (Fig. 2) and that the order of the *phd* genes (Fig. 1) was quite different from that of analogous gene sets so far reported. Each of the subunits of the PhdABCD dioxygenase showed a modest (<60%) sequence identity to all of the known dioxygenase subunits, although the basic sequence features of each protein family were conserved.

By phylogenetic analysis, PhdA did not form a cluster with most of known α-subunit sequences but formed a deep branch with two newly described α subunits from *Rhodococcus* sp., NarAa (AF082663) and NidA (50) (Fig. 5). Phylogenetic analysis of the β subunits gave an unrooted tree similar to that for the α subunits, indicating that PhdB formed a distinct cluster with the two β subunits, NarAb (AF082663) and NidB (50) (data not shown). In both unrooted trees, the branches of PhdA and PhdB were supported by low bootstrap values which might be due to the distant relationships of PhdA and PhdB to other counterparts.

The three-dimensional structure of the oxygenase component (α_3 [NdoB], β_3 [NdoC]) of naphthalene dioxygenase from *Pseudomonas* sp. NCIB 9816-4 was determined (28). A long narrow gorge which may provide access for substrates to catalytic iron was found in NdoB. The five residues constituting the narrowest part of the channel near the catalytic iron in NdoB were completely conserved in PhdA as Asn212, Phe213, His219, His 224, and Phe366. However, the residues lining the substrate-binding pocket below the catalytic iron and those covering the upper part of the catalytic iron were diverged. This sequence diversity may contribute to the substrate specificity difference between NdoB and PhdA.

The Lys97, Gly98, Val100, Gln115, Ser116, Pro118, and Trp211 residues in NdoB and the Ser75, Arg77, Arg78, Pro105, and Trp108 residues in NdoC form a possible interaction domain with the [2Fe-2S] ferredoxin. As discussed below, the ferredoxin component, PhdC, of the PhdABCD dioxygenase is a [3Fe-4S] or [4Fe-4S] ferredoxin, different from the [2Fe-2S] ferredoxins adopted in other ring-hydroxylating dioxygenases. Therefore, the binding site for PhdC on PhdA and PhdB is thought to be distinct from the binding site on NdoB and NdoC. As expected, the residues forming the putative ferredoxin-binding site in NdoB and NdoC were not conserved in PhdA and PhdB, except for Pro128 in PhdA (corresponding to Pro118 in NdoB) and Trp88 in PhdB (corresponding to Trp108 in NdoC).

Twenty-four residues at the N terminus of NdoC are involved in formation of the NdoC trimer (28). Alignment of the PhdB sequence with the NdoC sequence indicates that PhdB was 19 residues shorter than NdoC in the N-terminal region, and the 10 N-terminal residues of PhdB did not show any homology with NdoC. The long loop formed by residues 68 to 85 in NdoC is also involved in trimer formation and in the interaction with the Rieske domain of NdoB. The corresponding sequence in PhdB exhibited only 11% (2/18) amino acid identity and 55% (10/18) similarity. It is thus expected that the mode of interaction between the PhdB subunits would be quite different from that of NdoC.

The ferredoxin component of the PhdABCD dioxygenase, PhdC, was the first example of the [3Fe-4S] or [4Fe-4S] type of ferredoxin (39), the other ferredoxin components being the [2Fe-2S] type (4). More recently, another example of the [3Fe-4S] or [4Fe-4S] type of ferredoxin, DitA3, has been reported as a component of the diterpenoid dioxygenase of *Pseudomonas abietaniphila* BKME-9 (33). A homology search showed the wide and diverse distribution of the PhdC-like bacterial ferredoxin family among the high-G+C (9, 37) and low-G+C (14) gram-positive bacteria and members of the taxa *Proteobacteria* (17, 33), *Thermotogales* (36), and *Archaea* (22, 52). The ferredoxins closely related to PhdC are the electron transport proteins in the multicomponent P-450 systems in *Streptomyces griseolus* (37, 51). The ferredoxins from the hyperthermophilic archaea *Pyrococcus furiosus* and *Thermococcus litoralis* have been shown to bind a [4Fe-4S] cluster which can readily be converted to a stable [3Fe-4S] form (56). The three Cys residues which serve as ligands for the iron-sulfur cluster are conserved in all [3Fe-4S] and [4Fe-4S] types of ferredoxin, while substitution of the Cys residue second from the N termini has been observed in some proteins in this family. The Asp residue at this position can act as a ligands for the [4Fe-4S] cluster, as Cys does (57), while the Ala residue in ferredoxin-2 (SubB) from *S. griseolus* coordinates a [3Fe-4S] cluster in place of the second Cys (37). SubB was most similar to PhdC (51% sequence identity), whereas Tyr was found at the second Cys position of PhdC as in the case of DitA3, which shows less sequence identity (36%) to PhdC. It is not known whether the Tyr residues can act as ligands to the [4Fe-4S] cluster. The two Pro residues (Pro21 and Pro55 in PhdC) are also conserved in many ferredoxins, suggesting that these residues should be relevant to the function and/or protein conformation of the members in this ferredoxin family.

By a phylogenetic analysis of ferredoxin reductase components of the ring-hydroxylating dioxygenases, PhdD was shown to be distantly related to other reductases: PhdD constituted a cluster together with BphG (46), CarAd (41), CmtAa (12), and RedA2 (2) (data not shown), but all of these reductases branch near the root of this cluster and are equally distant from each other, showing a radial pattern of evolution. The ferredoxin

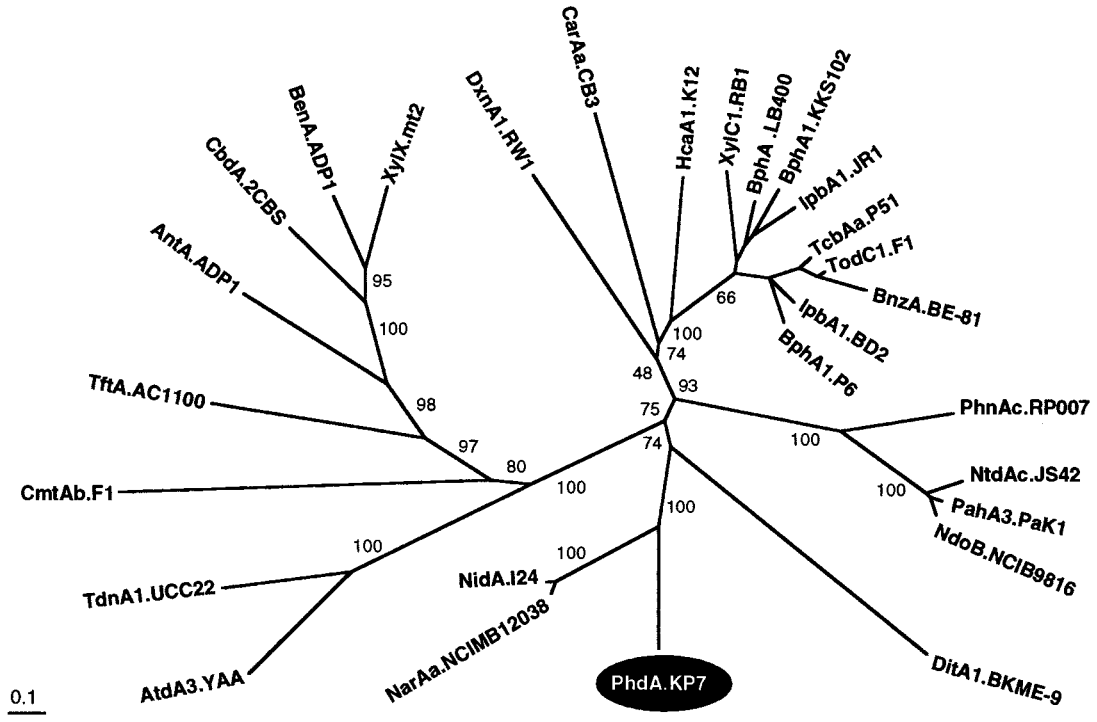

FIG. 5. Phylogenetic tree obtained from the alignment of PhdA with related proteins. The protein sequences of the 28 α subunits of ring-hydroxylating dioxygenases including PhdA are classified. The multiple-alignment analysis was performed with the PHYLIP software package, and the phylogenetic unrooted tree was drawn by using TreeView. The numbers on some branches refer to the percentage confidence estimated by a bootstrap analysis with 1,000 replications. The scale bar indicates the percentage divergence. The sequence abbreviations, enzyme substrates, species, and DDBJ/GenBank/EMBL references are as follows: NdoB.NCIB9816, naphthalene, *P. putida* NCIB 9816, M23914; PahA3.PaK1, naphthalene, *P. aeruginosa* PaK1, D84146; NtdAc.JS42, 2-nitrotoluene, *Pseudomonas* sp. strain JS42, U49504; PhnAc.RP007, phenanthrene, *Burkholderia* sp. strain RP007, AF061751; BnzA.BE-81, benzene, *P. putida* BE-81, M17904; TodC1.F1, toluene, *P. putida* F1, J04996; TcbAa.P51, chlorobenzene, *Pseudomonas* sp. strain P51, U15298; IbpA1.BD2, isopropylbenzene, *Rhodococcus erythropolis* BD2, U24277; BphA1.P6, biphenyl, *R. globerulus* P6, X80041; IbpA1.JR1, isopropylbenzene, *Pseudomonas* sp. strain JR1, U53507; BphA1.KKS102, biphenyl, *Pseudomonas* sp. strain KKS102, D17319; BphA.LB400, biphenyl, *Burkholderia cepacia* LB400, M86348; XylC1.RB1, substrate unknown, *Cycloclasticus oligotrophus* RB1, U51165; HcaA1.K12, phenylpropionate, *E. coli* K-12, AE000340; DxnA1.RW1, dibenzo-*p*-dioxin, *Sphingomonas* sp. strain RW1, X72850; CarAa.CB3, carbazole, *Sphingomonas* sp. strain CB3, AF060489; XylX.mt2, toluate, *P. putida* mt2, M64747; BenA.ADP1, benzoate, *Acinetobacter calcoaceticus* ADP1, AF009224; CbdA.2CBS, 2-halobenzoate, *B. cepacia* 2CBS, X79076; AntA.ADP1, anthranilate, *A. calcoaceticus* ADP1, AF071556; TftA.AC1100, 2,4,5-trichlorophenoxyacetic acid, *B. cepacia* AC1100, U11420; CmtAb.F1, *p*-cumate, *P. putida* F1, U24215; TdnA1.UCC22, aniline, *P. putida* UCC22, D85415; AtdA3.YAA, aniline, *Acinetobacter* sp. strain YAA, AB008831; NidA.I24, indene, *Rhodococcus* sp. strain I24, AF121905; NarAa.NCIMB12038, naphthalene, *Rhodococcus* sp. strain NCIMB 12038, AF082663; DitA1.BKME-9, diterpenoid, *P. abietaniphila* BKME-9, AF119621; PhdA.KP7, phenanthrene, *Nocardioides* sp. strain KP7, AB017794.

reductase component of DitA dioxygenase (33), of which ferredoxin is of the [3Fe-4S] or [4Fe-4S] type, has not yet been identified. Furthermore, the ferredoxin and ferredoxin reductase components of NarA (AF082663) and Nid (50) dioxygenases from *Rhodococcus* sp. also remain to be clarified. The relationships between these unknown electron transport proteins and the PhdCD components are of interest.

It has been shown that all four proteins are required for the full activity of the PhdABCD dioxygenase. PhdA and PhdB were essential for the enzyme activity. On the other hand, the ferredoxin component, PhdC, and the ferredoxin reductase component, PhdD, were dispensable in *E. coli* (Fig. 5). This may have been due to replacement of the function of the electron transport proteins of the ring-hydroxylating dioxygenase by that of *E. coli*. This result has been observed for other ring-hydroxylating dioxygenases (5, 31, 33), suggesting the relatively low specificity of the electron transport systems toward the oxygenase components. This tolerance between the oxygenase components and the electron transport systems has been advocated to play a role in the evolutionary process of ring-hydroxylating dioxygenases (21).

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REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Armengaud, J., B. Happe, and K. N. Timmis.** 1998. Genetic analysis of dioxin dioxygenase of *Sphingomonas* sp. strain RW1: catabolic genes dispersed on the genome. J. Bacteriol. **180:**3954–3966.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1990. Current protocols in molecular biology, Suppl. 11. unit 16.2, John Wiley & Sons, New York, N.Y.
4. **Batie, C. J., D. P. Ballou, and C. C. Correll.** 1992. Phthalate dioxygenase reductase and related flavin-iron-sulfur containing electron transferases, p. 543–556. *In* F. Muller (ed.), Chemistry and biochemistry of flavoenzymes, vol. 3. CRC Press, Boca Raton, Fla.
5. **Bergeron, J., D. Ahmad, D. Barriault, A. Larose, M. Sylvestre, and J. Powlowski.** 1994. Identification and mapping of the gene translation products involved in the first steps of the *Comamonas testosteroni* B-356 biphenyl/chlorobiphenyl biodegradation pathway. Can. J. Microbiol. **40:**743–753.

6. **Bosch, R., E. Garcia-Valdes, and E. R. B. Moore.** 1999. Genetic characterization and evolutionary implications of a chromosomally encoded naphthalene-degradation upper pathway from *Pseudomonas stutzeri* AN10. Gene **236:**149–157.
7. **Brosius, J.** 1989. Superpolylinkers in cloning and expression vectors. DNA **8:**759–777.
8. **Cerniglia, C. E., and M. A. Heitkamp.** 1989. Microbial degradation of polycyclic aromatic hydrocarbons (PAH) in the aquatic environment, p. 41–68. *In* U. Varanasi (ed.), Metabolism of polycyclic aromatic hydrocarbons in the aquatic environment. CRC Press, Inc., Boca Raton, Fla.
9. **Crespi, M., D. Vereecke, W. Temmerman, M. Van Montagu, and J. Desomer.** 1994. The *fas* operon of *Rhodococcus fascians* encodes new genes required for efficient fasciation of host plants. J. Bacteriol. **176:**2492–2501.
10. **Denome, S. A., D. C. Stanley, E. S. Olson, and K. D. Young.** 1993. Metabolism of dibenzothiophene and naphthalene in *Pseudomonas* strains: complete DNA sequence of an upper naphthalene catabolic pathway. J. Bacteriol. **175:**6890–6901.
11. **Eaton, R. W.** 1994. Organization and evolution of naphthalene catabolic pathways: sequence of the DNA encoding 2-hydroxychromene-2-carboxylate isomerase and *trans-o*-hydroxybenzylidenepyruvate hydratase-aldolase from the NAH7 plasmid. J. Bacteriol. **176:**7757–7762.
12. **Eaton, R. W.** 1996. *p*-Cumate catabolic pathway in *Pseudomonas putida* F1: cloning and characterization of DNA carrying the *cmt* operon. J. Bacteriol. **178:**1351–1362.
13. **Eaton, R. W., and P. J. Chapman.** 1992. Bacterial metabolism of naphthalene: construction and use of recombinant bacteria to study ring cleavage of 1,2-dihydroxynaphthalene and subsequent reactions. J. Bacteriol. **174:**7542–7554.
14. **Elliott, J. I., S. S. Yang, L. G. Ljungdahl, J. Travis, and C. F. Reilly.** 1982. Complete amino acid sequence of the 4Fe-4S, thermostable ferredoxin from *Clostridium thermoaceticum*. Biochemistry **21:**3294–3298.
15. **Erickson, B. D., and F. J. Mondello.** 1992. Nucleotide sequencing and transcriptional mapping of genes encoding biphenyl dioxygenase, a multicomponent PCB-degrading enzyme in *Pseudomonas* strain LB400. J. Bacteriol. **174:**2903–2912.
16. **Felsenstein, J.** 1993. Phylogeny Inference Package, version 3.5c. Department of Genetics, University of Washington, Seattle, Wash. (Distributed by the author.)
17. **Freiberg, C., R. Fellay, A. Bairoch, W. J. Broughton, A. Rosenthal, and X. Perret.** 1997. Molecular basis of symbiosis between *Rhizobium* and legumes. Nature **387:**394–401.
18. **Fuenmayor, S. L., M. Wild, A. L. Boyes, and P. A. Williams.** 1998. A gene cluster encoding steps in conversion of naphthalene to gentisate in *Pseudomonas* sp. strain U2. J. Bacteriol. **180:**2522–2530.
19. **Gibson, D. T., and V. Subramanian.** 1984. Microbial degradation of aromatic hydrocarbons, p. 181–252. *In* D. T. Gibson (ed.), Microbial degradation of organic compounds. Marcel Dekker, Inc., New York, N.Y.
20. **Harayama, S.** 1997. Polycyclic aromatic hydrocarbon bioremediation design. Curr. Opin. Biotechnol. **8:**268–273.
21. **Harayama, S., M. Kok, and E. L. Neidle.** 1992. Functional and evolutionary relationships among diverse oxygenases. Annu. Rev. Microbiol. **46:**565–601.
22. **Heltzel, A., E. T. Smith, Z. H. Zhou, J. M. Blamey, and M. W. Adams.** 1994. Cloning, expression, and molecular characterization of the gene encoding an extremely thermostable [4Fe-4S] ferredoxin from the hyperthermophilic archaeon *Pyrococcus furiosus*. J. Bacteriol. **176:**4790–4793.
23. **Irie, S., S. Doi, T. Yorifuji, M. Takagi, and K. Yano.** 1987. Nucleotide sequencing and characterization of the genes encoding benzene oxidation enzymes of *Pseudomonas putida*. J. Bacteriol. **169:**5174–5179.
24. **Iwabuchi, T., and S. Harayama.** 1997. Biochemical and genetic characterization of 2-carboxybenzaldehyde dehydrogenase, an enzyme involved in phenanthrene degradation by *Nocardioides* sp. strain KP7. J. Bacteriol. **179:** 6488–6494.
25. **Iwabuchi, T., and S. Harayama.** 1998. Biochemical and genetic characterization of *trans*-2′-carboxybenzalpyruvate hydratase-aldolase from a phenanthrene-degrading *Nocardioides* strain. J. Bacteriol. **180:**945–949.
26. **Iwabuchi, T., and S. Harayama.** 1998. Biochemical and molecular characterization of 1-hydroxy-2-naphthoate dioxygenase from *Nocardioides* sp. KP7. J. Biol. Chem. **273:**8332–8336.
27. **Iwabuchi, T., Y. Inomata-Yamauchi, A. Katsuta, and S. Harayama.** 1998. Isolation and characterization of marine *Nocardioides* capable of growing and degrading phenanthrene at 42°C. J. Mar. Biotechnol. **6:**86–90.
28. **Kauppi, B., K. Lee, E. Carredano, R. E. Parales, D. T. Gibson, H. Eklund, S. Ramaswamy.** 1998. Structure of an aromatic-ring-hydroxylating dioxygenase-naphthalene 1,2-dioxygenase. Structure **6:**571–586.
29. **Kiyohara, H., K. Nagao, K. Kouno, and K. Yano.** 1982. Phenanthrene-degrading phenotype of *Alcaligenes faecalis* AFK2. Appl. Environ. Microbiol. **43:**458–461.
30. **Kiyohara, H., S. Torigoe, N. Kaida, T. Asaki, T. Iida, H. Hayashi, and N. Takizawa.** 1994. Cloning and characterization of a chromosomal gene cluster, *pah*, that encodes the upper pathway for phenanthrene and naphthalene utilization by *Pseudomonas putida* OUS82. J. Bacteriol. **176:**2439–2443.
31. **Kurkela, S., H. Lehvaslaiho, E. T. Palva, and T. H. Teeri.** 1988. Cloning, nucleotide sequence and characterization of genes encoding naphthalene dioxygenase of *Pseudomonas putida* strain NCIB9816. Gene **73:**355–362.
32. **Laurie, A. D., and G. Lloyd-Jones.** 1999. The *phn* genes of *Burkholderia* sp. strain RP007 constitute a divergent gene cluster for polycyclic aromatic hydrocarbon catabolism. J. Bacteriol. **181:**531–540.
33. **Martin, V. J. J., and W. W. Mohn.** 1999. A novel aromatic-ring-hydroxylating dioxygenase from the diterpenoid-degrading bacterium *Pseudomonas abietaniphila* BKME-9. J. Bacteriol. **181:**2675–2682.
34. **Masai, E., A. Yamada, J. M. Healy, T. Hatta, K. Kimbara, M. Fukuda, and K. Yano.** 1995. Characterization of biphenyl catabolic genes of gram-positive polychlorinated biphenyl degrader *Rhodococcus* sp. strain RHA1. Appl. Environ. Microbiol. **61:**2079–2085.
35. **Mason, J. R., and R. Cammack.** 1992. The electron-transport proteins of hydroxylating bacterial dioxygenases. Annu. Rev. Microbiol. **46:**277–305.
36. **Nelson, K. E., R. A. Clayton, S. R. Gill, M. L. Gwinn, R. J. Dodson, D. H. Haft, E. K. Hickey, J. D. Peterson, W. C. Nelson, K. A. Ketchum, L. McDonald, T. R. Utterback, J. A. Malek, K. D. Linher, M. M. Garrett, A. M. Stewart, M. D. Cotton, M. S. Pratt, C. A. Phillips, D. Richardson, J. Heidelberg, G. G. Sutton, R. D. Fleischmann, O. White, S. L. Salzberg, H. O. Smith, J. C. Venter, and C. M. Fraser.** 1999. Evidence for lateral gene transfer between *Archaea* and bacteria from genome sequence of *Thermotoga maritima*. Nature **399:**323–329.
37. **O'Keefe, D. P., K. J. Gibson, M. H. Emptage, R. Lenstra, J. A. Romesser, P. J. Litle, and C. A. Omer.** 1991. Ferredoxins from two sulfonylurea herbicide monooxygenase systems in *Streptomyces griseolus*. Biochemistry **30:**447–455.
38. **Page, R. D.** 1996. TreeView: an application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. **12:**357–358.
39. **Saito, A., T. Iwabuchi, and S. Harayama.** 1999. Characterization of genes for enzymes involved in the phenanthrene degradation in *Nocardioides* sp. KP7. Chemosphere **38:**1331–1337.
40. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
41. **Shepherd, J. M., and G. Lloyd-Jones.** 1998. Novel carbazole degradation genes of Sphingomonas CB3: sequence analysis, transcription, and molecular ecology. Biochem. Biophys. Res. Commun. **247:**129–135.
42. **Simon, M. J., T. D. Osslund, R. Saunders, B. D. Ensley, S. Suggs, A. Harcourt, W. C. Suen, D. L. Cruden, D. T. Gibson, and G. J. Zylstra.** 1993. Sequences of genes encoding naphthalene dioxygenase in *Pseudomonas putida* strains G7 and NCIB 9816-4. Gene **127:**31–37.
43. **Simons, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system of *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**784–790.
44. **Staskawicz, B., D. Dahlbeck, N. Keen, and C. Napoli.** 1987. Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. **169:**5789–5794.
45. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorff.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185:**60–89.
46. **Sylvestre, M., M. Sirois, Y. Hurtubise, J. Bergeron, D. Ahmad, F. Shareck, D. Barriault, I. Guillemette, and J. M. Juteau.** 1996. Sequencing of *Comamonas testosteroni* strain B-356-biphenyl/chlorobiphenyl dioxygenase genes: evolutionary relationships among Gram-negative bacterial biphenyl dioxygenases. Gene **174:**195–202.
47. **Taira, K., J. Hirose, S. Hayashida, and K. Furukawa.** 1992. Analysis of *bph* operon from the polychlorinated biphenyl-degrading strain of *Pseudomonas pseudoalcaligenes* KF707. J. Biol. Chem. **267:**4844–4853.
48. **Takizawa, N., N. Kaida, S. Torigoe, T. Moritani, T. Sawada, S. Satoh, and H. Kiyohara.** 1994. Identification and characterization of genes encoding polycyclic aromatic hydrocarbon dioxygenase and polycyclic aromatic hydrocarbon dihydrodiol dehydrogenase in *Pseudomonas putida* OUS82. J. Bacteriol. **176:**2444–2449.
49. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **11:**4673–4680.
50. **Treadway, S. L., K. S. Yanagimachi, E. Lankenau, P. A. Lessard, G. Stephanopoulos, and A. J. Sinskey.** 1999. Isolation and characterization of indene bioconversion genes from *Rhodococcus* strain I24. Appl. Microbiol. Biotechnol. **51:**786–793.
51. **Trower, M. K., R. Lenstra, C. Omer, S. E. Buchholz, and F. S. Sariaslani.** 1992. Cloning, nucleotide sequence determination and expression of the genes encoding cytochrome P-450$_{soy}$ (*soyC*) and ferredoxin$_{soy}$ (*soyB*) from *Streptomyces griseus*. Mol. Microbiol. **6:**2125–2134.
52. **Wang, P. L., A. Donaire, Z. H. Zhou, M. W. Adams, and G. N. La Mar.** 1996. Molecular model of the solution structure for the paramagnetic four-iron ferredoxin from the hyperthermophilic archaeon *Thermococcus litoralis*. Biochemistry **35:**11319–11328.
53. **Wierenga, R. K., P. Terpstra, and W. G. J. Hol.** 1986. Prediction of the occurrence of the ADP-binding bab-fold in proteins, using an amino acid sequence fingerprint. J. Mol. Biol. **187:**101–107.

54. **Yang, Y., R. F. Chen, and M. P. Shiaris.** 1994. Metabolism of naphthalene, fluorene, and phenanthrene: preliminary characterization of a cloned gene cluster from *Pseudomonas putida* NCIB 9816. J. Bacteriol. **176:**2158–2164.
55. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
56. **Zhou, Z. H., and M. W. W. Adams.** 1992. Proton NMR investigation of the oxidized three-iron clusters in the ferredoxins from the hyperthermophilic archae *Pyrococcus furiosus* and *Thermococcus litoralis*. Biochemistry **31:**11952–11962.
57. **Zhou, Z. H., and M. W. W. Adams.** 1997. Site-directed mutations of the 4Fe-ferredoxin from the hyperthermophilic archaeon *Pyrococcus furiosus*: role of the cluster-coordinating aspartate in physiological electron transfer reactions. Biochemistry **36:**10892–10900.
58. **Zylstra, G. J., and D. T. Gibson.** 1989. Toluene degradation by *Pseudomonas putida* F1. Nucleotide sequence of the *todC1C2BADE* genes and their expression in *Escherichia coli*. J. Biol. Chem. **264:**14940–14946.