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Biotransformation of Phenanthrene and 1-Methoxynaphthalene with *Streptomyces lividans* Cells Expressing a Marine Bacterial Phenanthrene Dioxygenase Gene Cluster

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The *phdABCD* gene cluster in a marine bacterium *Nocardioides* sp. strain KP7 codes for the multicomponent enzyme phenanthrene dioxygenase. *phdA* encoding an iron-sulfur protein large subunit α, *phdB* encoding its small subunit β, *phdC* encoding ferredoxin, and *phdD* encoding ferredoxin reductase, were replaced in such a way that the termination codons of the preceding open reading frames were overlapped with the initiation codons of the following genes. This manipulated *phdABCD* gene cluster was positioned downstream of the thiostrepton-inducible promoter *PtipA* in a high-copy-number vector pIJ6021, and introduced into the gram-positive, soil-inhabiting, filamentous bacterium *Streptomyces lividans*. The recombinant *S. lividans* cells converted phenanthrene into a *cis*-diol form, which was determined to be *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene by its UV spectral data as well as HPLC property, using the authentic sample for comparison. This biotransformation proceeded very efficiently; 200 μM and 2 mM of phenanthrene were almost completely converted to its *cis*-diol form in 6 h and 32 h, respectively. In addition, the *S. lividans* cells carrying the *phdABCD* gene cluster were found to transform 1-methoxynaphthalene to two products, which were identified to be 8-methoxy-2-naphthol in addition to 8-methoxy-1,2-dihydro-1,2-naphthalenediol by their EI-MS, ^{1}H- and ^{13}C-NMR spectral data.

Key words: arene dioxygenase; phenanthrene; 1-methoxynaphthalene; 8-methoxy-2-naphthol; *Streptomyces lividans*

Polycyclic aromatic hydrocarbons (PAHs) such as phenanthrene and anthracene, which are mutagenic or carcinogenic,[1] are environmental pollutants derived from coal and petroleum. PAHs are supposed to be degraded by microbial consortia. Bacteria mediating aerobic degradation of PAHs have been isolated, and the metabolic pathways have been analyzed.[2–4] One of the rate-limiting steps in the degradation of these pollutants is considered to be the first oxygenation reaction that is catalyzed by PAH dioxygenase. This dioxygenase, frequently named naphthalene or phenanthrene dioxygenase, is a multicomponent enzyme, which consists of ferredoxin, ferredoxin reductase, and an iron-sulfur protein, and introduces both atoms of molecular oxygen into an aromatic nucleus.[5] Many genes coding for PAH dioxygenase have been isolated from various bacteria such as *Pseudomonas putida* strains G7 and NCIB 9816–4,[6] *Pseudomonas* sp. strain C18,[7] *Pseudomanas* sp. strain U2,[8] *Burkhorderia* sp. strain RP007,[9] *Comamonas testosteroni* GZ39,[10] and *Nocardioides* sp. strain KP7.[11] The functions of the encoded enzymes have been extensively studied.[12–13] Among them, the phenanthrene dioxygenase genes (*phdABCD*) of *Nocardioides* sp. strain KP7 that was isolated from a Kuwait beach oil-polluted area appear to be unique, because of the phylogenetically diverged position of the genes and an unusual type (3Fe-4S or 4Fe-4S) of the ferredoxin component.[11] The multicomponent enzyme encoded by *phdABCD* has been shown to convert phenanthrene, anthracene, and naphthalene to corresponding *cis*-diols, i.e., *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene, *cis*-1,2-dihydroxy-1,2-

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dihydroanthracene, and *cis*-1,2-dihydroxy-1,2-dihydronaphthalene, respectively (Fig. 1) (A. Saito and S. Harayama, in preparation).

PAHs, especially of the higher-molecular-weight types, are refractory to biodegradation and persist in the natural environment. Although biological methods have been successfully used to treat municipal and industrial waste water, their application in land remediation is still at a stage of infancy.[4] On the other hand, the genus *Streptomyces* that constitutes one major group among soil-living prokaryotes does not participate in PAH biodegradiation due to the lack of related catabolic genes. We consider that giving the ability to degrade various PAHs to the soil-living *Streptomyces* strains should be feasible due to their flexibility in genetic manipulation. As a first step toward this goal, we have introduced the phenanthrene dioxygenase genes derived form *Nocardioides* sp. strain KP7 into *Streptomyces lividans*, expressed these genes effectively, and hydroxylated phenanthrene efficiently by use of the recombinant *S. lividans* cells. This is the first example of biotransformation of PAHs by recombinant *Streptomyces* species. We have also newly found that the recombinant cells are capable of converting 1-methoxynaphthalene into 8-methoxy-1,2-dihydro-1,2-naphthalenediol and 8-methoxy-2-naphthol.

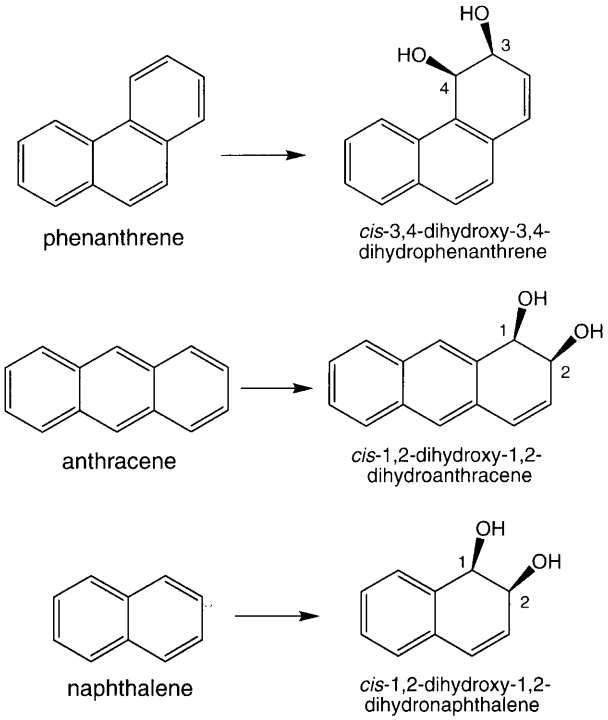

Fig. 1. Substrates Converted by *Escherichia coli* Harboring the Phenanthrene Dioxygenase Genes (*phdABCD*) of *Nocardioides* sp. Strain KP7 and the Structures of Converted Products.
These are unpublished data obtained from A. Saito and S. Harayama.

Materials and Methods

Plasmids, bacterial strains, and growth conditions. Plasmids pSA315 and pHA171 carrying the *phdABCD* gene cluster of *Nocardioides* sp. strain KP7 were described.[11] Vector pUC19 and *Escherichia coli* JM109[14] were purchased from Takara Shuzo Co. *E. coli* JM109 and BL21 (DE3)[15] were used as hosts for expression of *phdABCD*, and cultured in LB medium[14] at 30 or 37°C. Ampicillin (50 to 150 μg/ml) was added when needed.

The expression vector pIJ6021 for *Streptomyces* species, which carries the kanamycin resistance gene and the thiostrepton-inducible promoter *PtipA*,[16] was a gift from E. Takano and M. Bibb, John Innes Centre. *Streptomyces lividans* TK21[17] was used as a host for expression of *phdABCD*, and cultured in YEME medium or minimal medium[17] at 30°C. Minimal medium contained glucose (1%), L-asparagine (0.05%), K_2HPO_4 (0.05%), $MgSO_4 \cdot 7H_2O$ (0.02%), and $FeSO_4 \cdot 7H_2O$ (0.001%). Kanamycin was used at a final concentration of 5 μg/ml when necessary.

General recombinant DNA techniques. Restriction enzymes, T4 DNA ligase and *Taq* DNA polymerase were purchased from Takara Shuzo. DNA manipulation was done in *E. coli*[14] and in *S. lividans*,[17] as described. Nucleotide sequences were analyzed by the dideoxy chain termination method with the Thermo Sequenase fluorescence labeled primer cycle sequencing kit (Amersham) on an automated DNA sequencer.

Construction of expression plasmid. Five DNA fragments (Phd-1, Phd-2, Phd-3, Phd-4, and Phd-5) were amplified by PCR with appropriate primers and pSA315 as a template (Table 1, see also Fig. 2). PCR was done at 98°C for 20 sec, 60°C for 30 sec, and 72°C for 60 sec in a total of 25 cycles. These amplified DNA fragments were separated by agarose gel electrophoresis and purified using the Gene clean kit II (Bio101). The adjacent two fragments (Phd-1 and Phd-2, Phd-2 and Phd-3, Phd-3 and Phd-4, and Phd-4 and Phd-5) were ligated to each other and amplified by the annealing PCR method to produce connected DNA fragments (Phd-12, Phd-23, Phd-34, and Phd-45, respectively). Annealing PCR was done with the two DNA fragments at 98°C for 20 sec, 60°C for 30 sec, and 72°C for 2 min in a total of 10 cycles, followed by amplification by PCR of the ligated fragment using appropriate primers at 98°C for 20 sec, 60°C for 30 sec, and 72°C for 2 min in a total of 25 cycles. A 0.9-kb *Kpn*I-*Sal*I fragment of Phd-12 was cloned between the *Kpn*I and *Sal*I sites of pUC19, resulting in pPhd-12. pUC19-NcoI, a pUC19 derivative which had a unique *Nco*I site in the multicloning-sequence was constructed as follows. pUC19 was digested by *Sma*I followed by attachment

Table 1. Primers Used in This Study

primer[a]	sequence (5′ to 3′)[b]	bold letters	italic letters
1F	cccggtaccggaggtatacat**ATG**TCGGTAGTCAGCGGGGATAGG *Kpn*I *Nde*I	start codon of *phdA*	
1R	GTAGGACTCGAGCCGCGGgACCGGTGTCAG (T→g, elimination of a *Kpn*I cleavage site)		
2F	GGAACCTGACACCGGTcCCGCGGCTCGAG (A→c, elimination of a *Kpn*I cleavage site)		
2R	ctcgtcaacagtagtcag**ca***TCA*cagGACAGCGCTCTCGTCGTCGG (TAA→cag, alternation of a codon for Leu)	start codon of *phdB*	stop codon of *phdA*
3F	gtcctg*tga***TG**CTGACTACTGTTGACGAGAATCTG	start codon of *phdB*	stop codon of *phdA*
3R	caacatccacacg**ca***tcA*GAAGAAGAACGCAAGATTGTG	start codon of *phdC*	stop codon of *phdB*
4F	cttgcgttcttcttc*tg***ATG**CGTGTGGATGTTGACCCAC	start codon of *phdC*	stop codon of *phdB*
4R	GCACGCCGGTGACGAGCCGGATgCCTCCAGTGGC (C→g, elimination of a *Bam*HI cleavage site)		
5F	CTACGCCACTGGAGGcATCCGGCTCGTCACCGGC (G→c, elimination of a *Bam*HI cleavage site)		
5R	cccggatccgcgtcgcggaTCTTCCTCGGC*TCA*TGCCGTCGGTAC *Bam*HI		stop codon of *phdD*

[a] Refer to Fig. 1 for the position of each primer. [b]Capital and small letters indicate nucleotides matching to the template aequence and nucleotides designed for mutagenesis, respectively.

of an *Nco*I linker (gggccatggccc) to both ends. After digestion of the fragment by *Nco*I, the fragment was self-ligated, resulting in pUC19-NcoI. A 0.7-kb *Sal*I-*Nco*I fragment of Phd-23 was cloned between the *Sal*I and *Nco*I sites of pUC19-NcoI, resulting in pPhd-23. A 1.0-kb *Nco*I-*Sac*I fragment of Phd-34 was cloned between the *Nco*I and *Sac*I sites of pUC19-NcoI, resulting in pPhd-34. A 0.8-kb *Sac*I-*Bam*HI fragment of Phd-45 was cloned between the *Sac*I and *Bam*HI sites of pUC19-NcoI, resulting in pPhd-45. All fragments were sequenced to confirm there were no errors in the PCR. The 0.9-kb *Kpn*I-*Sal*I fragment of pPhd-12 and the 0.7-kb *Sal*I-*Nco*I fragment of Phd-23 were cloned together between the *Kpn*I and *Nco*I sites of pUC19-NcoI, resulting in pPhd-123. The 1.0-kb *Nco*I-*Sac*I fragment of pPhd-34 and the 0.8-kb *Sac*I-*Bam*HI fragment of pPhd-45 were cloned between the *Nco*I and *Bam*HI sites of pUC19-NcoI, resulting in pPhd-345. Finally, a 1.6-kb *Kpn*I-*Nco*I fragment of pPhd-123 and a 1.8 kb *Nco*I-*Bam*HI fragment of pPhd-345 were cloned together between the *Kpn*I and *Bam*HI sites of pUC19, resulting in pPhd-12345. A 3.4-kb *Nde*I-*Bam*HI fragment of pPhd-12345 was cloned between the *Nde*I and *Bam*HI sites of pIJ6021, resulting in pIJ6021-phdABCD.

Expression of phdABCD in S. lividans. *S. lividans* TK21 harboring pIJ6021-phdABCD was grown in 100 ml of YEME medium containing 5 μg/ml of kanamycin in a shaking (Sakaguchi) flask at 30°C with reciprocal shaking (120 rpm) for 2 days. One milliliter of this culture was inoculated into the same medium, and cultivated under the same conditions. After 24 h, thiostrepton was added to the culture at a final concentration of 5 μg/ml to induce transcription from *PtipA*. After an additional 24 h of incubation, about 3 g (wet wt) mycelium was collected by centrifugation. The cell extract of the mycelium prepared by sonication was analyzed by SDS-polyacrylamide gel electrophoresis according to the method of Laemmli.[18] The gel was stained with Coomassie Brilliant Blue R-250 (CBB).

Conversion of phenanthrene and 1-methoxynaphthalene. *S. lividans* cells harboring pIJ6021-phdABCD was grown as described above. The mycelium was collected by centrifugation and washed once with minimal medium. Then, 100 mg or 1 g (wet wt) of mycelium was resuspended in 100 ml of fresh minimal medium, and the substrate, phenanthrene (Kanto Chemical Co.) or 1-methoxynaphthalene (Tokyo Kasei Kogyo Co.), was added to the mycelium suspension at a final concentration of 200 μM or 2 mM, respectively. As stock solutions, 50 mM phenanthrene and 100 mM 1-methoxynaphthalene, dissolved in *N,N*-dimethylformamide, were used. The mycelium and substrates were incubated on a reciprocal shaker (120 rpm) at 30°C.

Extraction and HPLC analysis of products converted from phenanthrene and 1-methoxynaphthalene. To extract converted products as well as the substrate, methanol of the same volume was added to the reaction mixture and mixed for 30 min. After centrifugation to remove cells, the liquid phase was used for high-pressure liquid chromatography (HPLC) analysis or for further purification of the converted products.

The liquid phase (80 μl) was put through HPLC on

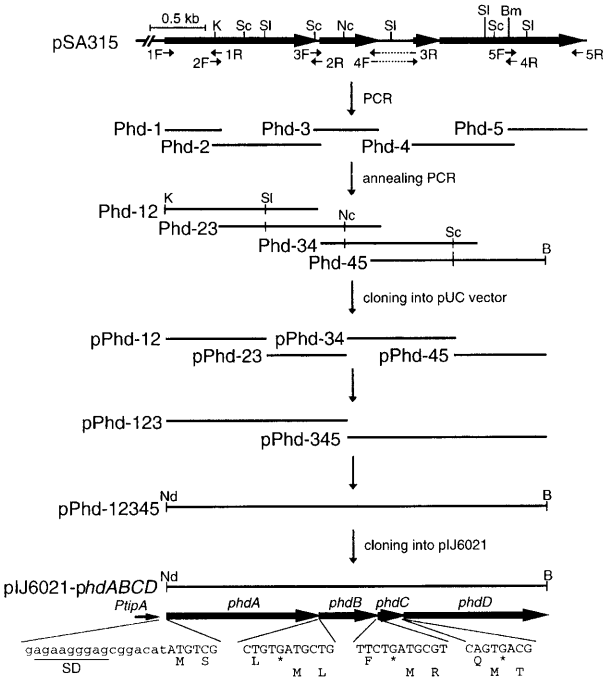

Fig. 2. Schematic Representation of the Strategy Used for Construction of pIJ6021-phdABCD.

The following abbreviations are used for restriction enzymes: Bm, *Bam*HI; K, *Kpn*I; Nc, *Nco*I; Nd, *Nde*I; Sc, *Sac*I; and Sl, *Sal*I. Below the arrows indicating the manipulated *phd* genes, the nucleotides sequence upstream of *phdA* and the junction regions of adjacent genes are shown. SD represents a Shine-Dalgarno sequence. Using pSA315 as a template, five DNA fragments (Phd-1, Phd-2, Phd-3, Phd-4, and Phd-5) were amplified by PCR with appropriate pairs of primers (1F and 1R, 2F and 2R, 3F and 3R, 4F and 4R, and 5F and 5R, respectively). The adjacent two fragments, for example, Phd-1 and Phd-2, were ligated to each other and amplified by the annealing PCR method, resulting in connected DNA fragments, Phd-12. A 0.9-kb *Kpn*I-*Sal*I fragment of Phd-12 was cloned into pUC19, resulting in pPhd-12. All the fragments (pPhd-12, pPhd-23, pPhd-34, and pPhd-45) thus obtained were sequenced to confirm there were no errors in the PCR. pPhd-123, for example, was constructed by combining the 0.9-kb *Kpn*I-*Sal*I fragment of pPhd-12 and the 0.7-kb *Sal*I-*Nco*I fragment of Phd-23. Finally, pPhd-12345 was constructed by combining the 1.6-kb *Kpn*I-*Nco*I fragment of pPhd-123 and the 1.8-kb *Nco*I-*Bam*HI fragment of pPhd-345. A 3.4-kb *Nde*I-*Bam*HI fragment of pPhd-12345 was cloned between the *Nde*I and *Bam*HI sites of pIJ6021, resulting in pIJ6021-phdABCD.

a Puresil C18 column (4.6×250 mm, Waters) and a photodiode array detector (model 996, Waters). It was developed at a flow rate of 1 ml/min with solvent A (H_2O-methanol, 1:1) for 5 min, followed by a 凸-shaped gradient (No.3; Waters) from solvent A to solvent B (methanol-2-propanol, 6:4) for 15 min, and with solvent B for 13 min.

Purification and identification of products converted from 1-methoxynaphthalene. The liquid phase (1,400 ml) obtained by the above-described procedure was concentrated *in vacuo*, and extracted by ethylacetate (500 ml×2). The organic layer was concentrated *in vacuo* and put on a silica gel [20 by 250 mm, Silica Gel 60 (Merck)] for chromatography, using a solvent system of hexane: ethylacetate = 1:1.

The structure of the converted products were identified by MS (EI-MS, JEOL DX-303) spectrum and NMR (500 MHz, JEOL α) analysis including some 2D spectra (DQF-COSY, C-H COSY, HMBC).

Results

Expression of phdABCD in S. lividans

The *phdABCD* genes of *Nocardioides* sp. strain KP7 encode a phenanthrene dioxygenase complex, which is a multicomponent enzyme composed of an iron-sulfur protein large (α) subunit (PhdA), a small (β) subunit (PhdB), ferredoxin (PhdC), and ferredoxin reductase (PhdD).[11] All these four proteins are required for the full dioxygenation activity.[11] To express the phenanthrene dioxygenase genes in *S. lividans*, we constructed plasmid pIJ6021-phdABCD, in which the coding region of *phdA* was placed just after an ideal Shine-Dalgarno sequence downstream of the thiostrepton-inducible promoter *PtipA*. The *phdB*, *phdC* and *phdD* genes were positioned in this order just downstream from *phdA*. The start codons of *phdB*, *phdC* and *phdD* were designed to overlap the stop codons of *phdA*, *phdB* and *phdC*, respectively (Fig. 2). These four genes were assumed to be co-transcribed and translationally coupled because of the absence of intervening sequences between the adjacent genes. In *Streptomyces*, translational coupling seems to be common, since the overlapping of a start codon and a stop codon between adjacent genes were often reported. For example, in two polycistrons (*crtEIBV* and *crtYTU*) that are the carotenoid biosynthesis gene clusters in *Streptomyces griseus*, the start codons of *crtI*, *B*, *V*, *T* and *U* overlap the stop codons of *crtE*, *I*, *B*, *Y* and *T*, respectively.[19] Therefore, the *phdABCD* genes on the pIJ6021-phdABCD were expected to be expressed efficiently in *S. lividans* cells.

We then checked the synthesis of the PhdABCD proteins in *S. lividans* harboring pIJ6021-phdABCD by SDS-polyacrylamide gel electrophoresis. The recombinant *S. lividans* strain was grown for 24 h in 100 ml of YEME medium containing 5 μg/ml kanamycin to maintain the plasmid, and thiostrepton was added to the culture at a final concentration of 5 μg/ml to induce transcription from *PtipA*. After an additional 24 h of incubation, a cell-free extract was prepared. Two proteins of about 48 kDa and 21 kDa, which represented PhdA (50 kDa) and PhdB (20 kDa), respectively, were detected as major bands on SDS-polyacrylamide gel electrophoresis (Fig. 3). These proteins were not detected in the cell-free extract prepared similarly from *S. lividans* harboring pIJ6021. On the other hand, no proteins correspond-

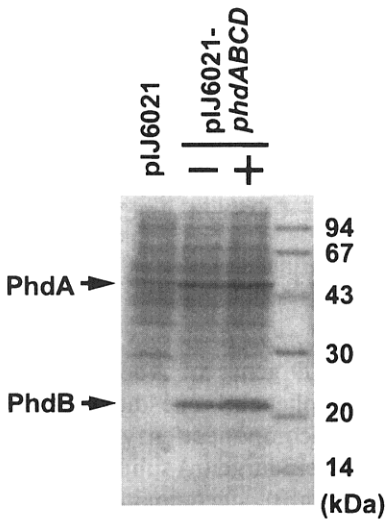

Fig. 3. Expression of *phdABCD* in *S. lividans*.

The cell extract of *S. lividans* cells harboring pIJ6021-phdABCD and pIJ6021, as a control, was analyzed by SDS-polyacrylamide gel electrophoresis. *S. lividans* cells harboring pIJ6021-phdABCD were grown with (+) or without (−) induction of *PtipA* by thiostrepton. Proteins were stained with CBB. The protein bands representing PhdA and PhdB are indicated by arrows.

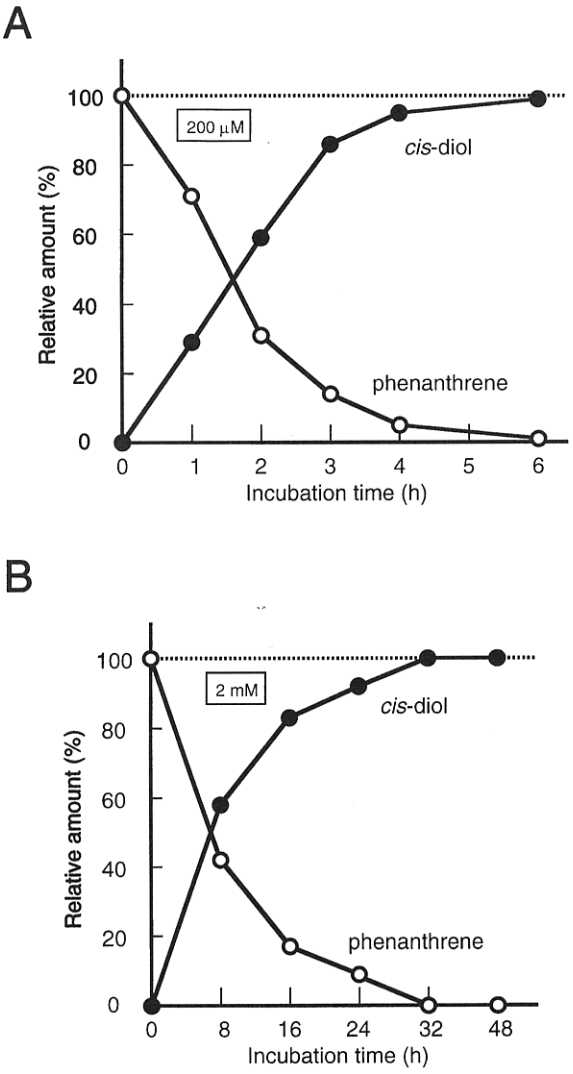

Fig. 4. Transformation of Phenanthrene with the *S. lividans* Mycelium Harboring pIJ6021-phdABCD.

The conversion experiments were performed using 100 mg of the induced mycelium in 100 ml of mimimal medium containing 200 μM (A) and 2 mM (B) of phenanthrene. See Materials and Methods for details. One milliliter of the reaction mixture was removed at the indicated times. The substrate and a biotransformed product were extracted and analysed by HPLC. The values are the means obtained from two independent experiments.

ing to PhdC (7 kDa) and PhdD (44 kDa) were detected in the recombinant *S. lividans*. Saito *et al.*[11] reported that, on expression of the *phdABCD* genes in *E. coli*, PhdC and PhdD were not detectable, although PhdA and PhdB were detected as major bands on SDS-polyacrylamide gel electrophoresis. It is therefore probable that PhdC and PhdD are not efficiently expressed or are unstable at least in these heterologous hosts.

Biotransformation of phenanthrene with S. lividans cells expressing phdABCD

We carried out biotransformation of phenanthrene using *S. lividans* horboring pIJ6021-phdABCD, as shown in Fig. 4. These conversion experiments were done using 100 mg of the mycelium in a shaking flask containing 100 ml of mimimal medium. Phenanthrene as the substrate at a final concentration of 200 μM (Fig. 4A) and 2 mM (Fig. 4B) was used. A biotransformed product was identified as a *cis*-diol, as described below. Almost all phenanthrene was converted to the *cis*-diol in 6 h (Fig. 4A). Figure 4A shows the HPLC profile of the reaction mixture at this time when almost all the substrate was converted. When 2 mM phenanthrene was used (Fig. 4B), all the substrate was converted to the *cis*-diol in 32 h.

Biotransformation of 1-methoxynaphthalene with S. lividans cells expressing phdABCD

Our preliminary experiments on the basis of the rather generous substrate specificity of PhdABCD showed that the dioxygenase enzyme complex used 1-methoxynaphthalene as the substrate and converted it to certain compounds. We therefore used 1-methoxynaphthalene as the substrate and the recombinant *S. lividans* cells as an enzyme source. The reaction mixture contained 1 g of the induced mycelium and 200 μM 1-methoxynaphthalene in a shaking flask containing 100 ml of mimimal medium. After 12 h of incubation at 30°C, 65% and 8% of 1-methoxynaphthalene were converted to compounds 3 and 4, respectively (Fig. 5B). Although we used a ten times larger amount of mycelium than that used in the case of phenanthrene, the conversion efficiency was low, compared with that for phenanthrene.

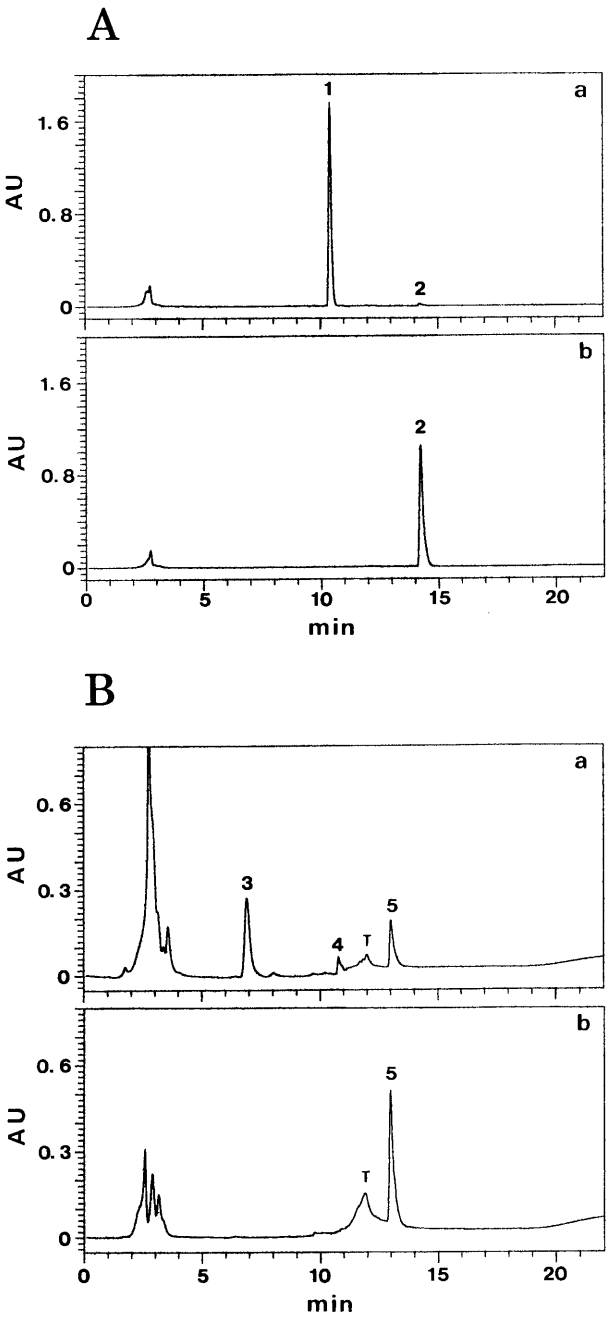

Fig. 5. HPLC Analysis of Aromatic Compounds Extracted from the Reaction Mixture Containing the *S. lividans* Cells Harboring pIJ6021-phdABCD (a) and the Vector pIJ6021 (b).

Aromatic compounds were extracted from the reaction mixture containing the cells and phenanthrene after 6 h of incubation (A) and from the reaction mixture containing the cells and 1-methoxynaphthalene after 12 h of incubation (B). Figure 4A and Fig. 4B were monitored at 252 nm and 220 nm, respectively. The peaks with retention times of 10.4 min (1), 14.2 min (2), 6.9 min (3), 10.8 min (4), and 13.0 min (5) were found to represent *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene, phenanthrene, 8-methoxy-1,2-dihyro-1,2-naphthalenediol, 8-methoxy-2-naphthol, and 1-methoxynaphthalene, respectively. The peaks with retention times of 11.9 min (T) represented thiostrepton.

Structual analysis of the products converted by S. lividans expressing phdABCD

Aromatic compounds were extracted from the medium after 6 h of co-cultivation with the cells of *S. lividans* harboring pIJ6021-phdABCD and phenanthrene as the substrate, and analyzed by HPLC (Fig. 5A). One major peak, 1, was observed at 10.4 min of retention time. This compound was identified to be *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene by its UV spectral data (λmax 219, 252, 262, and 309 nm) and its HPLC properties, when these data were compared with those of the authentic sample. This authentic sample was extracted from the co-cultivated medium with the cells of *E. coli* BL21 (DE3) harboring *phdABCD* (plasmid pHA171[11]) and with the substrate phenanthrene, as described by Saito *et al.*[11] Peak 2 at 14.2 min of retention (Fig. 5A) was phenanthrene.

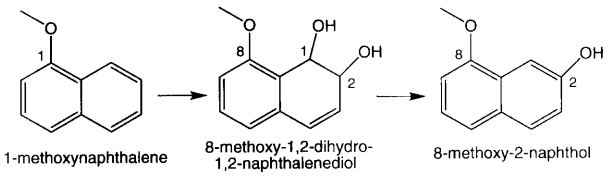

Fig. 6. Structures of 8-Methoxy-1,2-dihyro-1,2-naphthalenediol and 8-Methoxy-2-naphthol, and a Probable Pathway of Transformation of 1-Methoxynaphthalene to 8-Methoxy-2-naphthol *via* 8-Methoxy-1,2-dihyro-1,2-naphthalenediol in the Culture of *S. lividans* Harboring pIJ6021-phdABCD.

Aromatic compounds were extracted from the medium after 12 h of co-cultivation with the cells of *S. lividans* harboring pIJ6021-phdABCD and 1-methoxynaphthalene as the substrate, and analyzed by HPLC (Fig. 5B). Two peaks 3 and 4 were observed at 6.9 min and 10.8 min of retention time in addition to peak 5 (retention time, 13.0 min) of the substrate 1-methoxynaphthalene. The purification scheme described in Materials and Methods yielded 1.6 mg of compound 3 and 3.5 mg of compound 4. The molecular formula of compound 4 was determined to be $C_{11}H_{10}O_2$ by EI-MS [m/z 174 (M^+)], ^1H NMR and ^{13}C NMR spectra. Analysis of DQF-COSY, C-H COSY, and HMBC spectra (Table 2) showed that compound 4 was 8-methoxy-2-naphthol, the structure of which is shown in Fig. 6. Compound 3 was identified as 8-methoxy-1,2-dihydro-1,2-naphthalenediol (Fig. 6) by its spectral data. Physicochemical properties of these two hydroxylated products are shown in Table 2. 8-Methoxy-1,2-dihydro-1,2-naphthalenediol (compound 3) was gradually converted to 8-methoxy-2-naphthol (compound 4) by dehydration during the purification. It was therefore considered that compound 3 was converted to compound 4 non-enzymatically, due to the structural unstability of 3.

Discussion

In this study, we have for the first time demonstrated the functional expression of the aromatic com-

Table 2. Physico-chemical Properties and Spectral Data of 8-Methoxy-1,2-dihydro-1,2-naphthalenediol (3) and 8-Methoxy-2-naphthol (4)

	3	4
Appearance	Colorless oil	Colorless oil
Molecular formula	$C_{11}H_{12}O_3$	$C_{11}H_{10}O_2$
EI-MS	182 (M^+)	164 (M^+)
UV λmax nm (ε)	218.8 (17,300), 258.4 (4,300)	220.4 (40,600), 237.2 (30,100)
(MeOH)	300.8 (2,600), 337.2 (1,700)	282.8 (5,300), 294.4 (4,800) 322.8 (2,300), 336.0 (2,500)
IR ν(neat)cm $^{-1}$	3415, 2923, 1639, 1267, 1059, 746	3446, 2940, 1633, 1261, 1219, 1088, 995, 918
^{1}H-NMR	H1, 5.05; H2, 4.50; H3, 5.90;	H1, 7.52; H3, 7.10; H4, 7.70
(DMSO-d_6)	H4, 6.40; H5, 6.73; H6, 7.24; H7, 6.80; 8-CH_3, 3.87	H5, 7.34; H6, 7.22; H7, 6.77 8-CH_3, 3.97
^{13}C-NMR	C1, 63.6; C2, 69.5; C3, 131.6;	C1, 104.2; C2, 153.2; C3, 117.9;
(DMSO-d_6)	C4, 126.9; C4a, 133.1; C5, 119.8; C6, 130.1; C7, 110.6; C8, 154.3; 8-CH_3, 55.7; C8a, 122.5	C4, 129.5; C4a, 129.8; C5, 120.1; C6, 123.4; C7, 104.4; C8, 154.3; 8-CH_3, 55.7; C8a, 126.6

pound dioxygenase genes in *Streptomyces* species to hydroxylate phenanthrene and 1-methoxynaphthalene. The cells of *S. lividans* harboring the *Nocardioides phdABCD* genes on plasmid pIJ6021-phdABCD converted almost all of the phenanthrene (200 μM) to the *cis*-diol in 6 h of incubation (Fig. 4A). Saito *et al.*[11] tried biotransformation of phenanthrene using *E. coli* transformants under conditions similar to those in the case of *S. lividans*. In this experiment using the *E. coli* cells carrying *phdABCD* on plasmid pHA171, in which the corresponding genes were highly expressed, 74% of phenanthrene (200 μM) was converted to the *cis*-diol in 12 h of incubation. It is thus very likely that the recombinant *S. lividans* cells converted phenanthrene to the *cis*-diol more efficiently than *E. coli* cells.

Phenanthrene dioxygenase of *Nocardioides* sp. strain KP7 had the ability to convert four fused aromatic hydrocarbons such as pyrene in addition to three fused aromatic hydrocarbons such as phenanthrene and anthracene as well as the two fused aromatic hydrocarbon naphthalene (A. Saito and S. Harayama, unpublished observation). According to our studies using recombinant *E. coli* BL21 (DE3) harboring plasmid pHA171, this dioxygenation enzyme was incapable of transforming phenyl compounds such as toluene, ethylbenzene, *n*-butylbenzene, *sec*-butylbenzene, and *t*-butyl benzene (Shindo *et al.*, in preparation). Thus, PhdABCD seems to have an affinity for two or more fused aromatic hydrocarbons. Generally, polycyclic aromatic hydrocarbons (PAHs) with higher molecular weights are more refractory to biodegradation. Therefore, recombinant *Streptomyces* strains that express the PhdABCD-type dioxygenation genes may function as a trigger for biodegradation by microbial consortia in the soil environment. Since the members in the gram-positive bacteria *Streptomyces* species are soil-inhabiting microbes, which are flexible toward genetic manipulation, our study here would extend such a possibility in bioremediation for the soil environment polluted with PAHs. So far, among soil-inhabiting bacteria that can degrade environmental pollutants, the gram-negative bacteria *Pseudomonas* species have been the only strains, of which a host-vector system is well-established. Suyama *et al.* have already shown that the recombinant *Psudomonas* strains degrade a wider range of aromatic hydrocarbons with low-molecular weight, compared with the parent strains.[20]

We have also shown that the recombinant *S. lividans* expressing the *phdABCD* genes can convert 1-methoxynaphthalene to its diol, 8-methoxy-1,2-dihydro-1,2-naphthalenediol, which appears to be converted to the more stable monohydroxylated product, 8-methoxy-2-naphthol, non-enzymatically (Fig. 6). This is the first example in microbial hydroxylation of 1-methoxynaphthalene to our knowledge. Actinomycetes such as *Streptomyces* have so far been used for biotransformation of various chemical compounds.[21] One of the reasons may be that actinomycetes have an advantage in permeability of substrates through the cell membrane. Thus, recombinant *Streptomyces* strains may be promising also as a host for the production of *cis*-diols, which are shown to be very useful as building blocks for asymmetric synthesis.[12]

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