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SHORT COMMUNICATION

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Cloning and sequence analyses of a 2,3-dihydroxybiphenyl 1,2-dioxygenase gene (*bphC*) from *Comamonas* sp. SMN4 for phylogenetic and structural analysis

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Abstract A genomic library of biphenyl-degrading *Comamonas* sp. SMN4 for isolating fragments containing the 2,3-dihydroxybiphenyl 1,2-dioxygenase (23DBDO) gene was constructed. The smallest subclone (pNPX9) encoding 23DBDO activity was sequenced and analyzed. The C-terminal domain of 23DBDO from *Comamonas* sp. SMN4 had five catalytically essential residues and was more highly conserved than the N-terminal domain. Phylogenetic and structural relationships of 23DBDO from *Comamonas* sp. SMN4 were analyzed.

Keywords Dihydroxybiphenyl dioxygenase · *bphC* · Extradiol dioxygenase · *Comamonas* · Biphenyl degradation

Introduction

Biphenyl/polychlorinated biphenyls are environmental pollutants that are widely distributed throughout the world. One means for their removal from the environment is microbial degradation. A number of microorganisms with this capability have been isolated and characterized, such as *Pseudomonas cepacia* LB400 [12], *Pseudomonas* sp. strain KKS102 [9], and *Rhodococcus* sp. strain RHA1 [11]. In a previous report, a microorganism having high degrading activity toward biphenyl was isolated and identified as *Comamonas* sp. SMN4 [14].

The major pathway for microbial degradation of biphenyl has been established: four specific enzymes—multi-component biphenyl dioxygenase, dihydrodiol dehydrogenase, 2,3-dihydroxybiphenyl 1,2-dioxygenase (23DBDO), and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid hydrolase—are sequentially involved in degradation of biphenyl into benzoic acid [9, 12].

Of these four enzymes, 23DBDO, encoded by the *bphC* gene, a class of extradiol dioxygenase, catalyzes *meta* cleavage of 2,3-dihydroxybiphenyl [9, 12]. These extradiol dioxygenases play a key role in the degradation pathway of biphenyl and other aromatic compounds [12, 14].

Most reports on extradiol dioxygenases [5, 12] are limited to the characterization of functional rather than structural properties, except for 23DBDO from *Pseudomonas* sp. KKS102 and *Pseudomonas* sp. LB400 [6, 16]. The three-dimensional structure of 23DBDO from *Comamonas* sp. SMN4 was elucidated by alignment of amino acid sequences of 23DBDO from SMN4 with those of KKS102 and LB400 [6, 16].

In this report, we cloned the *bph* gene cluster from *Comamonas* sp. SMN4, and subcloned and sequenced the *bphC* gene. The phylogenetic relationships among extradiol dioxygenases from various strains were also analyzed by comparing the amino acid sequences of enzymes. In addition, the structural relationships between N-terminal and C-terminal domains were analyzed.

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Materials and methods

Baterial strains, plasmids and materials

Comamonas sp. SMN4 was the strain capable of growth on biphenyl. *Escherichia coli* S17–1, used as the recipient strain in the cosmid cloning experiments, was kindly supplied by Timmis (GBF-National Center for Biotechnology, Braunschweig, Germany). *E. coli* NM522 (Promega; Madison, Wis.) and SG13009 (Qiagen,

Hilden, Germany) were used as the recipient strains for other cloning, and expression. The cosmid cloning vector pWE15 was obtained from Stratagene (La Jolla, Calif.), and the pGEM and pUC series of cloning vectors were the products of Promega and Novagen (Madison, Wis.). The pQE expression vector was also obtained from Qiagen. The plasmids constructed in the present study are shown in Fig. 1. Bacterial strains were grown in mineral salts basal medium [7] and Luria-Bertani broth. Biphenyl was provided as a carbon source. *Comamonas* sp. SMN4 was grown at 30°C, and *E. coli* strains at 37°C. Biphenyl, catechol, 3-methylcatechol and 4-methylcatechol were obtained from Sigma (St. Louis, Mo.) and 2,3-dihydroxybiphenyl was purchased from Wako Chemicals (Osaka, Japan).

DNA manipulations

Total genomic DNA from *Comamonas* sp. SMN4 was prepared by the method of Olsen et al. [13], and plasmid DNA was isolated by the alkaline-sodium dodecyl sulfate procedure [2]. Fragments of partially *Bgl*II-digested total DNA of *Comamonas* sp. SMN4 in the 30–40 kb size range were pooled and ligated to *Bam*HI-digested pWE15. The ligated DNA was transfected into *E. coli* S17–1, using Gigapack II Packaging Extract Kit (Stratagene). Subcloning was performed in pGEM or pUC vectors, and the resulting DNA was transformed into competent *E. coli* NM522.

DNA sequencing and sequence analysis

DNA nucleotide sequence was determined by an automated DNA sequencing system (Applied Biosystems, Foster City, Calif.) with fluorescent-labeled primers. Sequence analysis, database searches and sequence comparisons were performed using programs from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov).

All amino acid sequences searched were aligned using Clustal X with all parameters set at their default values [17]. The phylogenetic analyses were performed on these data sets using algorithm of PHYLIP (phylogenetic inference package, version 3.6 a2.1, University of Washington, Wash., http://evolution.genetics.washington.edu/phylip.html).

23DBDO activity assay

The 23DBDO activity in cell-free extracts was measured spectrophotometrically in 0.1 M glycine buffer (pH 9.0) containing 2,3-dihydroxybiphenyl or catechol derivatives as substrates [14]. One unit of 23DBDO activity is defined as the formation of 1 μmol *meta* cleavage compound/min at 25°C. The extinction coefficients for the products formed from the substrates were the same as those in the literature [4, 7]. The protein concentration was determined by the method of Lowry et al. [10] using bovine serum albumin as a standard.

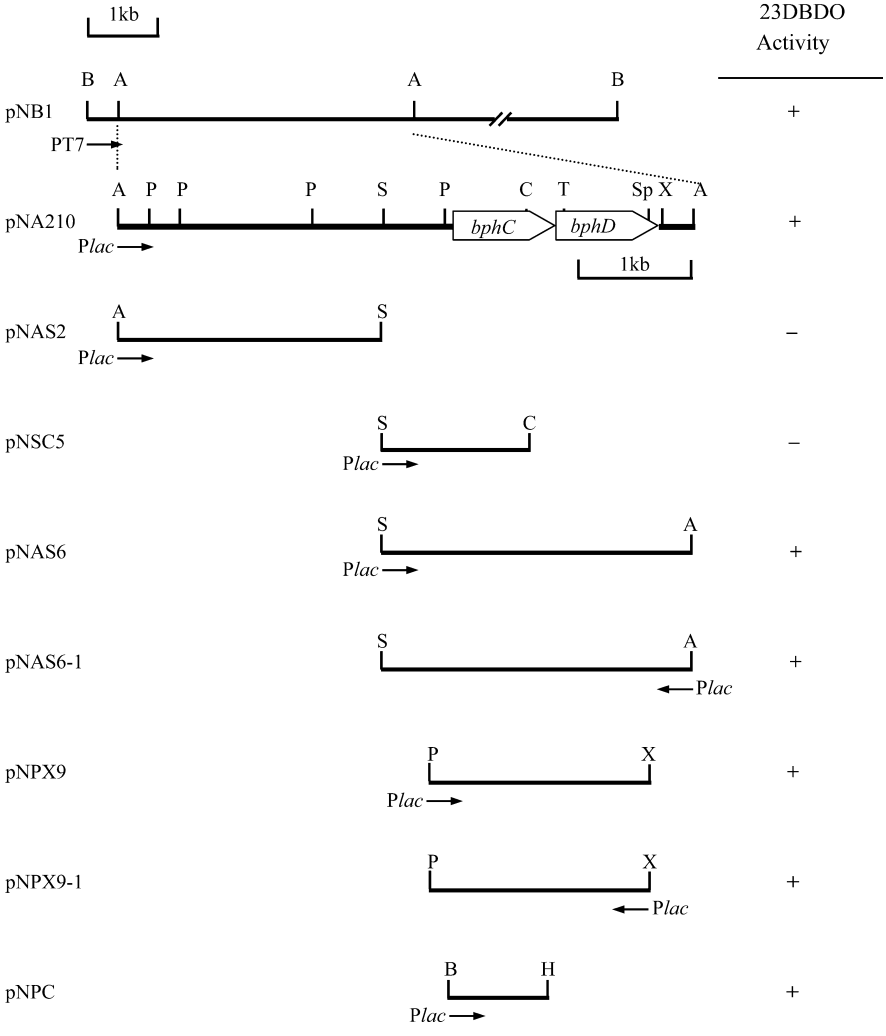

Fig. 1 Restriction map of the cloned region in pNA210 and localization of the 2,3-dihydroxybiphenyl 1,2-dioxygenase (23DBDO) gene. The arrowed boxes indicate the deduced positions of *bphC* and *bphD*. The activity column designates whether the clone having 23DBDO activity was expressed (+) or not (–). The direction of transcription is indicated by arrows. pNB1 is the recombinant plasmid cloned into the cosmid vector pWE15. The restriction enzyme sites of *Bam*HI (B) and *Hin*dIII (H) in pNPC are inserted sites for cloning into expression vector pQE30. A *Apa*I, C *Cla*I, P *Pst*I, S *Sal*I, Sp *Sph*I, T *Stu*I, X *Xba*I

Results and discussion

Cloning of *bph* genes from *Comamonas* sp. SMN4

From a cosmid library (1,000 clones) of *Comamonas* sp. SMN4 constructed with approximately 35 kb of the genome, two cosmid clones having 23DBDO activity screened by spraying an ethanol solution of 2,3-dihydroxybiphenyl were isolated and purified [7, 9, 12]. The recombinant cosmids conferring this activity were designated pNB1 and pNB2.

A variety of derivative plasmids were constructed from the 35 kb DNA inserted in pNB1. The *Apa*I fragments of pNB1 DNA were cloned into pGEM5Zf(+). The resultant clone, pNA210 (4.9 kb in size) demonstrated 23DBDO activity. Based on a restriction map of pNA210 (Fig. 1), subclones of pNA210 were constructed. The *bphC* gene is located between the *Pst*I and *Stu*I of pNA210. Subclones pNAS6 (2.6 kb in size) and pNPX9 (1.9 kb in size) showed 23DBDO activity, indicating that the *bphC* gene is expressed in *E. coli*. Interestingly, subclones pNAS6-1 and pNPX9-1, in which pNAS6 and pNPX9 were cloned in opposite orientation to the *lac* promoter of the vector (Fig. 1), also expressed the *bphC* gene. This suggests that the *bphC* gene may be expressed from its own promoter in *E. coli*. A subclone containing a 1.2 kb *Sal*I-to-*Cla*I fragment (pNSC5) did not show 23DBDO activity, indicating that the restriction enzyme *Cla*I cleaves the *bphC* gene.

23DBDO activities in 2,3-dihydroxybiphenyl and other dihydroxylated substrates (Table 1) indicated that subclones harboring the *bphC* gene showed the highest cleavage activity for 2,3-dihydroxybiphenyl and could cleave 3-methylcatechol as efficiently as *Comamonas* sp. SMN4. These subclones also have substantial cleavage activity for catechol and 4-methylcatechol.

The 23DBDO activities of pNAS6-1 and pNPX9-1 were much lower than those of pNAS6 and pNPX9 (Table 1). These results support the suggestion that the structural gene of 23DBDO in pNAS6-1 and pNPX9-1 was expressed from its own promoter as mentioned above (Fig. 1). Similar observations have been made with clone pUPX5014 and pGJZ1513, which carry the 23DBDO genes of *Pseudomonas* sp. DJ77 and *Beijerinckia* sp. B1, respectively [7, 8]. The 23DBDO activity of pNCP strain, harboring only the *bphC* gene, was higher than that of pNAS6 and pNPX9 (both have *bphC* and *bphD* genes). In strains harboring pNAS6 and pNPX9, the BphD enzyme may further process the product formed by reaction of BphC. This might explain the higher 23DBDO activity in subclone pNCP.

Genetic structure of *bphC*

In order to characterize the genetic structure of *bphC* in detail, the nucleotide sequence of subclone pNPX9 was determined: sequence analysis revealed two complete ORFs (open reading frames) corresponding to *bphC* and *bphD*. The accession number for the nucleotide sequences of *bphC* and *bphD* from *Comamonas* sp. SMN4 in the GenBank database is AY028943. The *bphC* and *bphD* ORFs are 879 bp and 831 bp in length, respectively, with the corresponding deduced polypeptide sequences consisting of 293 and 277 residues.

Upstream of the *bphC* sequence encoding 23DBDO, a sequence called "promoter-like" (-AGGCTCCGGG-GGCAA-) in *Pseudomonas* sp. KKS102 [9] was also found in *Comamonas* sp. SMN4. This sequence is similar to the consensus promoter sequences (A-AGGC-T and GCAATA) proposed in *Pseudomonas putida* [1], and may function as a promoter to express the *bphC* gene in subclones pNAS6-1 and pNPX9-1.

Table 1 2,3-Dihydroxybiphenyl 1,2-dioxygenase (23DBDO) activities in cell-free extracts of strains containing a *bphC* gene, with different dihydroxylated substrates

Strains	*bph* gene	23DBDO activity[a] (units/mg enzyme)			
		2,3-Dihydroxy biphenyl	Catechol	3-Methyl catechol	4-Methyl catechol
Comamonas sp. SMN4	*bphA1A2A3BCDA4*	49[b]	4.3	30	17
Escherichia coli S17-1 (pNB1)	*bphA1A2A3BCDA4*	123	26	94	18.5
E. coli S17-1		0	0	0	0
E. coli NM522 (pNA210)	*bphA2A3BCD*	189	33	98	22
(pNAS6)	*bphCD*	2,941	607	2,150	301
(pNAS6-1)	*bphCD*	89	18	55	20
(pNPX9)	*bphCD*	4,545	911	2,745	27
(pNPX9-1)	*bphCD*	125	27	71	453
E. coli NM522		0	0	0	0
E. coli SG13009 (pNCP)	*bphC*	5,750	955	2,898	980
E. coli SG13009		0	0	0	0

[a]23DBDO activity was measured spectrophotometrically in 0.1 M glycine buffer (pH 9.0). The reaction was initiated by the addition of substrate at a final concentration of 20 μM (2,3-dihydroxybiphenyl and 3-methylcatechol), 500 μM (catechol) or 20 mM (4-methylcatechol)

[b]One unit of enzyme activity is defined as the formation of 1.0 μmol 23DBDO/min at 25°C

Analysis and comparison of amino acid sequences

The amino acid sequence of 23DBDO (BphC) from *Comamonas* sp. SMN4 was aligned with those of 13 other extradiol dioxygenases. The amino acid homologies between BphC of SMN4 and those of other strains were 92% (BphC of Tn4371), 90% (BphC of KKS102), 91% (BphC of SY5), 64% (BphC of LB400), 28% (NahC of pPG7) and 15% (XylE of pWWO). The BphC from SMN4 belongs to the bicyclic family in the dendrogram shown in Fig. 2a [3]. Interestingly, within the monocyclic family, TodE, which is responsible for cleavage of 3-methylcatechol [4], was more related to the extradiol dioxygenases, exhibiting a preference for bicyclic substrates. TodE shared 53% homology with BphC of SMN4 (Fig. 2a).

The amino acid sequence alignments between BphC from *Comamonas* sp. SMN4 and BphCs from *Pseudomonas* sp. SY5 and *Pseudomonas* sp. KKS102, showed that BphC from SMN4 was divided into two domains—N-terminal (residues 1–135) and C-terminal (residues 136–297)—similar to BphC from KKS102 (PDB Id: 1DHY).

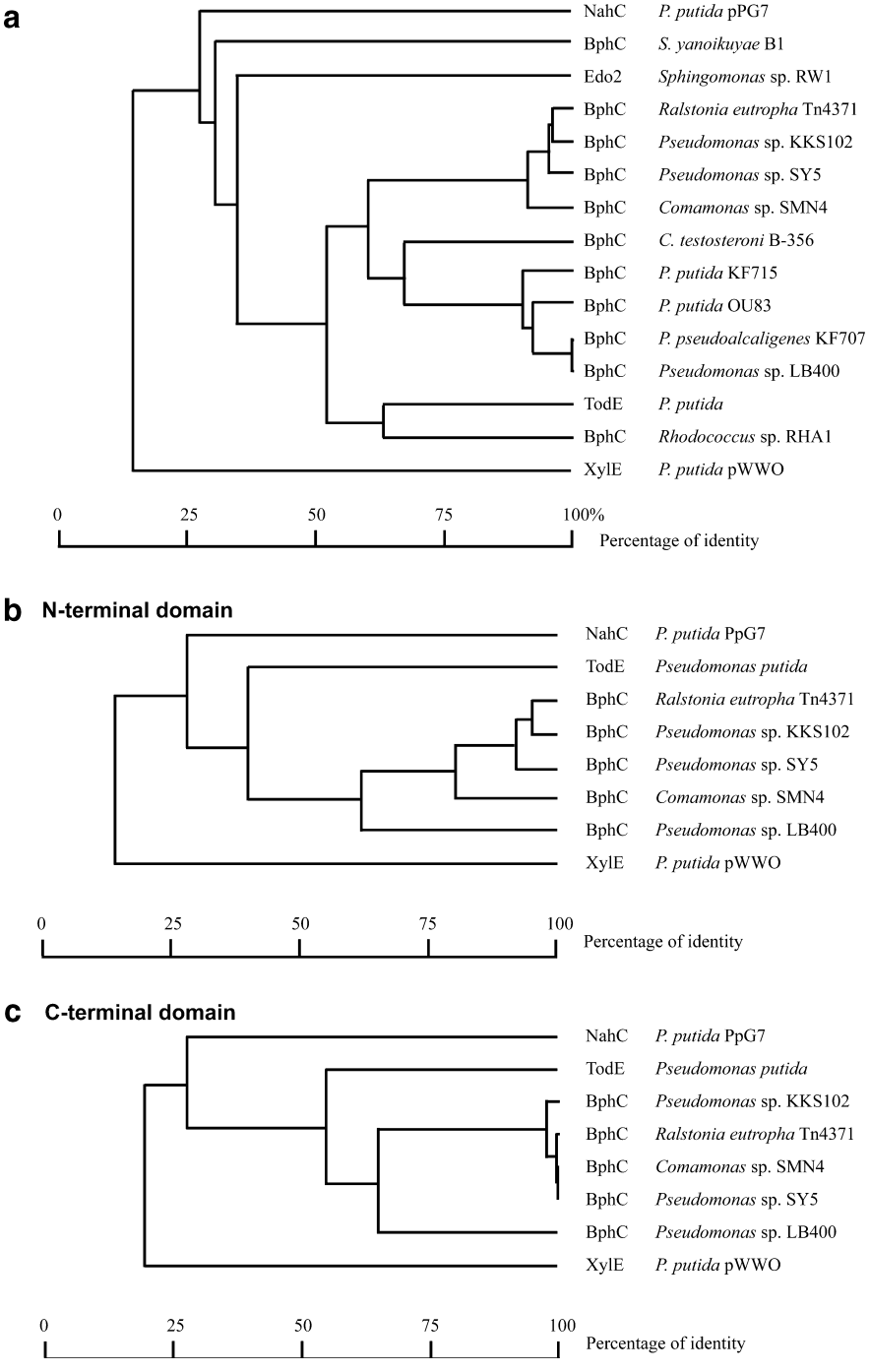

Fig. 2 Dendrogram showing the levels of homology among the amino acid sequences of extradiol dioxygenases of monocyclic and bicyclic aromatic compounds (**a**), and of the N- and C-terminal domains of extradiol dioxygenases (**b**). Sequences used (GenBank accession numbers in parenthesis): XylE, pWWO (M64747); NahC, PpG7 (J04994); BphC, B1 (U23374); Edo2, RW1 (AJ223219); BphC, Tn4371 (X97984); BphC, KKS102 (M264331); BphC, SY5 (AF190706); BphC, B-356 (U91936); BphC, KF715 (M33813); BphC, OU83 (X91876); BphC, KF707 (M83673); BphC, LB400 (X66122); TodE (Y18245); and BphC, RHA1 (D32142). XylE catechol 2,3-dioxygenase, NahC 1,2-dihydroxynaphthalene dioxygenase, BphC 2,3-dihydroxybiphenyl 1,2-dioxygenase, Edo2, 2,2′,3-trihydoxybiphenyl dioxygenase, TodE 3-methylcatechol 2,3-dioxygenase

Phylogenetic relationships between strains in terms of each domain

Figure 2b shows the phylogenetic relationships in each domain of BphC and related enzymes. TodE and XylE, classified as monocyclic enzymes in the toluene degradative pathway, and NahC, as a bicyclic enzyme degrading naphthalene [7], were included in the dendrograms for comparison (Fig. 2b). The BphC of LB400, having 64% identity with BphC from SMN4, is also included in the dendrogram due to its known tertiary structure (PDB Id: 1HAN). It is not clear whether extradiol dioxygenases other than BphC, i.e., TodE, XylE and NahC, are divided into N- and C-terminal domains. Therefore, the sequences of these enzymes were aligned into N- and C-terminal domains using the method of Eltis and Bolin [3] based on the two known crystal structures [6, 16]. The regions of the N- and C-terminal domains of BphC from *Comamonas* sp. SMN4 used for alignment were residues 6–125 and 144–270, respectively.

The amino acid sequence of the C-terminal domain of BphC from SMN4 exhibits 100% and 97% identity with those of *Pseudomonas* sp. SY5 and *Pseudomonas* sp. KKS102 [16], respectively, whereas the corresponding N-terminal domains show 78% and 82% identity (Fig. 2b). All other enzymes except NahC had higher homology in the C-terminal domains than N-terminal domains (Fig. 2b).

Han et al. [9] suggested that most of extradiol dioxygenase monomers consist of a five-coordinate square-pyramidal Fe^{2+} center in the C-terminal domain, with three His, one Tyr and one Glu residue. In BphC of SMN4, these amino acids residues coordinated at the Fe^{2+} center are conserved (data not shown). In addition, amino acids that could coordinate the Fe^{2+} responsible for various catalytic roles, are the same in LB400 [12] having 64% homology overall with BphC from SMN4 as well as with strains Tn4371, KKS102 and SY5 (Fig. 2b). Even in trihydroxybiphenyl dioxygenase (Edo 2, sharing 30% homology to BphC from SMN4) and catechol 2,3-dioxygenase (XylE; monocyclic enzyme showing 15% homology overall) (Fig. 2a), all five residues were completely conserved in the C-terminal domains.

Structural relationships between the N- and C-terminal domains

The similarity of two phylogenetic trees analyzed in the C-terminal domain and the N-terminal domain (Fig. 2b) suggests that each domain has co-evolved within the same family and is subject to similar selective pressures in the majority of cases. The crystal structures of LB400 and KKS102 show that the supersecondary structure of N- and C-terminal domains ($\beta\alpha\beta\beta\beta$) is similar, especially in KKS102 [16], even though the sequence homology between the two domains is less than 10% (data not shown).

The role of the C-terminal domain of BphC is substrate binding and catalysis [16]. However, the function of the N-terminal domain is not known despite its high structural homology to the C-terminal domain. The N-terminal domain could play some role other than catalysis because of the absence of an iron-binding site. The function of the N-terminal domain in BphC is regulating the enzyme activity of the C-terminal domain, like the subsidiary domain of aspartate transcarbamoylase (ATCase) [15]. Often, sequence homology in the subsidiary domain evolves more rapidly than in the catalytic domain. In fact, the sequence homology between ATCase of *E. coli* and that of *P. putida* showed that the catalytic domain was more conserved than the regulatory domain [15]. Similarly, this model explains the higher homology in C-terminal domains (catalytic domain) of BphC amino acid residues required for catalytic action of extradiol dioxygenases, with regulation being exerted by N-terminal domains.

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