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The 2,3-Dihydroxybiphenyl 1,2-Dioxygenase Gene (*phnQ*) of *Pseudomonas* sp. DJ77: Nucleotide Sequence, Enzyme Assay, and Comparison with Isofunctional Dioxygenases

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2,3-Dihydroxybiphenyl 1,2-dioxygenase (2,3-DHBD), which catalyzes the ring *meta*-cleavage of 2,3-dihydroxybiphenyl, is encoded by the *phnQ* gene of biphenyl- and phenanthrene-degrading *Pseudomonas* sp. strain DJ77. We determined the nucleotide sequence of a DNA fragment of 1497 base pairs which included the *phnQ* gene. The fragment included an open reading frame of 903 base pairs to accommodate the enzyme. The predicted amino acid sequence of the enzyme subunit consisted of 300 residues. In front of the gene, a sequence resembling an *E. coli* promoter was identified, which led to constitutive expression of the cloned gene in *E. coli*. The deduced amino acid sequence of the PhnQ enzyme exhibited 85.6% identity with that of the corresponding enzyme in *Sphingomonas yanoikuyae* Q1 (formerly *S. paucimobilis* Q1) and 22.1% identity with that of catechol 1,2,3-dioxygenase from the same DJ77 strain. PhnQ showed broader substrate preference than previously-cloned PhnE, catechol 2,3-dioxygenase. Ten amino acid residues, considered to be important for the role of extradiol dioxygenases, were conserved.

Keywords: 2,3-Dihydroxybiphenyl 1,2-Dioxygenase, *phnQ*, Strain DJ77, Substrate preference.

Introduction

Extradiol type-dioxygenases are found in a variety of bacteria and are involved in aromatic ring fission at the meta position of dihydroxylated aromatics, intermediates of catabolic pathways of monocyclic and polycyclic compounds (Fortnagel *et al.*, 1990; Harayama *et al.*, 1992). This is one of the key reactions in the metabolism of various aromatic compounds such as toluene, naphthalene, biphenyl, and polyaromatic hydrocarbons (Dagley, 1975; Cerniglia, 1992; Harayama *et al.*, 1992; Furukawa, 1994). Catechol 2,3-dioxygenase, 2,3-dihydroxybiphenyl 1,2-dioxygenase, and 1,2-dihydroxynaphthalene dioxygenase are well known.

Generally, the extradiol dioxygenases can be divided into two families: those showing a preference for bicyclic substrates and those showing a preference for monocyclic substrates (Harayama *et al.*,1989). Eltis and Bolin aligned the sequences of over 30 extradiol dioxygenases with the assistance of two known crystal structures and analyzed their phylogenetic relationships (Eltis *et al.*, 1996). They revealed a number of strictly-conserved residues including the metal-ion ligands and several catalytically-active site residues, as well as a number of structurally-important residues that are remote from the active site.

A set of enzymes specified by *phn* genes enables a biphenyl- and phenanthrene-degrading *Pseudomonas* sp. strain DJ77 to degrade catechol and substituted catechols to TCA cycle intermediates (Kim *et al.*, 1992a; 1992b; Kim *et al.*, 1997a; 1997b; Shin *et al.*, 1997a). The *phn* gene cluster designated as *phnDEGH*, encoding 2-hydroxymuconic semialdehyde hydrolase, catechol 2,3-dioxygenase, 2-hydroxymuconic semialdehyde dehydrogenase, and 2-hydroxypent-2,4-dienoate hydratase, respectively, was cloned from the total chromosomal DNA of DJ77. We have recently cloned an additional extradiol dioxygenase gene (*phnQ*) that is not contained in the operon, including the catechol 2,3-dioxygenase gene (*phnE*) from DJ77 (Shin *et al.*, 1997b). The *phnQ* gene encodes 2,3-dihydroxybiphenyl 1,2-dioxygenase (2,3-DHBD), which catalyzes the oxidative ring *meta*-cleavage of 2,3-dihydroxybiphenyl to 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate. The *phnQ* and *phnE* genes are

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located approximately 6.5 kb away from each other and transcribed in opposite directions as determined by restriction mapping and partial sequencing. In the present study, we determined the complete nucleotide sequence of *phnQ*. In addition, we identified and then determined the functional relationships of the two extradiol dioxygenases present in strain DJ77 by comparing the substrate preference patterns and phylogenetic analysis.

Materials and Methods

Bacterial strains, plasmids, and culture conditions The bacterial strains and plasmids used and prepared in this study are listed in Table 1. *Pseudomonas* sp. strain DJ77 was grown in LB medium or minimal salt medium containing phenanthrene or biphenyl as the sole carbon source. The plasmid vector pBluescript SK (+) was used for subcloning.

DNA manipulations Total DNA of *Pseudomonas* sp. strain DJ77 was isolated by Murray's method (Sambrook *et al.*, 1989) and plasmid by the alkali lysis method (Murray *et al.*, 1980). Agarose gel electrophoresis, DNA digestion with restriction endonucleases, and ligation of DNA fragments were performed according to the standard conditions recommended by the supplier. The resulting plasmids were transformed into $CaCl_2$-prepared competent *E. coli* XL1-Blue cells (Murray *et al.*, 1980) and plated onto LB agar plates containing 100 μM ampicillin and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

DNA sequencing and sequence analysis The DNA sequence was determined by dideoxy chain termination method (Sanger *et al.*, 1977) using double-stranded DNA in an automated DNA-sequencing system (Applied Biosystems Inc.) with fluorescentely-labeled primers. Database searches were performed on-line using BLASTN, BLASTP, and TBLASTN programs (Altschul *et al.*, 1990) on the BLAST electronic mail server from the National Center for Biotechnology Information, Bethesda, USA. A putative promoter-like sequence was identified using the Web interface of the Promoter Prediction by the Neural Network program (Reese, 1994; Reese and Eeckmann, 1995; Reese *et al.*, 1996).

Sequence alignment All of the extradiol dioxygenase gene and protein sequences available from the GenBank library were searched and compared with the DJ77 *phnQ* gene and gene product sequences. Basically, the sequences were aligned using CLUSTAL_X with all parameters set at their default values (Thompson *et al.*, 1997). Then, these automatic sequence alignments, which introduced different gaps and perturbed the alignment of otherwise-conserved sequence patterns like those from Eltis and Bolin (1996), were adjusted to restore conservation of patterns manually (Fig. 3).

Detection of extradiol dioxygenase activity Strains harboring recombinant plasmids were grown as described above, washed with 100 mM sodium phosphate buffer (pH7.5), and resuspended in the same buffer. The resultant cell suspensions were sonicated and centrifuged at 15,000 rpm and 4°C for 30 min, and their supernatant (cell extract) was used immediately. Activity was spectrophotometrically measured at 25°C in 50 mM phosphate buffer (pH7.5) containing 500 μM 2,3-dihydroxybiphenyl or catechol derivatives as the substrate. One unit of enzyme activity was defined as the amount of enzyme that can convert 100 μmol of substrate per minute. The relative ring-cleavage activities were determined by the extinction coefficients of the respective ring-fission product formed from the following substrates: catechol, λ_{max} = 375 nm and ε = 36,000 cm^{-1} M^{-1}; 3-methylcatechol, λ_{max} = 388 nm and ε = 32,000 cm^{-1} M^{-1}; 4-methylcatechol, λ_{max} = 382 nm and ε = 17,000 cm^{-1} M^{-1} (Asturias *et al.*, 1993); 4-chlorocatechol, λ_{max} = 379 nm and (= 40,000 cm^{-1} M^{-1} (Sala-Trepat *et al.*, 1971); 2,3-dihydroxybiphenyl, λ_{max} = 434 nm and ε = 13,200 cm^{-1} M^{-1} (Eltis *et al.*, 1993). Specific activity was defined as the number of enzyme units per milligram of protein. Protein concentration was determined by the Lowry method (Lowry *et al.*, 1951).

Results and Discussion

Subcloning of the DNA fragment encoding 2,3-DHBD A cloned 5 kb *Xho*I fragment showing 2,3-DHBD activity was previously obtained (Fig. 1) (Shin *et al.*, 1997b). In a previous publication (Shin *et al.*, 1997b), a subclone containing a 1.5-kb *Xho*I-*Eco*RV insert which

Table 1. Bacterial strains and plasmids in this study.

Strain or plasmid	Description	Source or reference
Strains		
E. coli XL1-Blue	*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1 *lac* [*F' proAB*$^+$ *lacI*^{q}ZΔM15*Tn*10(*tet*^{r})]	Stratagene Co.
Pseudomonas sp. DJ77	Isolated from soil, Phn$^+$ and Bph$^+$	11
Plasmids		
pBluescript SK (+)	Cloning vector, Ap^r	Stratagene Co.
pPE17	1.7-kb *Pst*I-*Eco*RI fragment of pHENX7 in SK(+)	11
pUPX5	5-kb *Xho*I fragment of phage DNA	14
pUPX5013	1.5-kb *Xho*I-*Eco*RV fragment of pUPX5 in SK(+)	This work
pUPX5014	1.5-kb *Xho*I-*Eco*RV fragment of pUPX5013 in KS(+)	This work
pUPX5021	3-kb *Psp*1406I fragment of pUPX5 in SK(+)	This work
pUPX5022	1.3-kb *Psp*1406I-*Eco*RV fragment of pUPX5021 in SK(+)	This work

harbored the gene encoding 2,3-DHBD (PhnQ) was analyzed (on pUPX5013). Interestingly, clones pUPX5013 and pUPX5014 (in the latter, the *lac* promoter pBluescript SK (+) vector was reversely-oriented with respect to the same insert) constitutively expressed 2,3-DHBD. This means that the structural gene of 2,3-DHBD was expressed by its own promoter in *E. coli* and that no regulatory gene was present in the cloned fragments. Further subcloning and expression analyses allowed the localization of a gene for 2,3-DHBD activity to a 1.3-kb *Psp*1406I-*Eco*RV fragment (pUPX5022). The 1.5-kb DNA fragment containing the *phnQ* gene was subsequently sequenced.

Nucleotide sequence of the 2,3-DHBD gene *phnQ* A restriction map of the cloned region with the location of the *phnQ* gene is shown in Fig. 1. The determined nucleotide sequence and the deduced amino acid sequence of 2,3-DHBD are shown in Fig. 2. A total of 1497 bp, corresponding to the region between the *Xho*I and *Eco*RV restriction sites, were sequenced. Both strands of the DNA were sequenced in their entirety, and all of the restriction sites used for cloning were verified by sequencing overlapping clones. A 903 bp open reading frame (ORF) initiating at ATG (bp 382) and terminating at TGA (bp 1284) was identified within this sequence. The ORF predicts a 300 amino acid residue protein with a M_r of 33,270. This value is consistent with that of 34,000 per subunit determined by SDS-PAGE (data not shown). Ten base pairs upstream from the initiation codon is a putative Shine-Dalgarno site, the ribosome-binding sequence 5′-GGGAG-3′ (Shine *et al.*, 1975). Further upstream, a putative promoter-like sequence indicated at −35 and −10 was identified. We presume that this sequence functions as a promoter in *E. coli*. The G+C content of the *phnQ* gene was 60.8% which is different from that of the catechol 2,3-dioxygenase gene (57.4%) of the same strain (Kim *et al.*, 1992b).

Sequence comparisons with other extradiol dioxygenases The deduced amino acid sequence of PhnQ was closely related to the corresponding sequences of the members of the main superfamily of extradiol dioxygenases, which is represented by enzymes like 2,3-DHBD from *Pseudomonas* sp. KKS102 (Kimbara *et al.*, 1989), *S. yanoikuyae* Q1 (previously *paucimobilis* Q1) (Taira *et al.*, 1988), or *Rhodococcus* sp. RHA1 (Hauschild *et al.*, 1996), and was not significantly different from catechol 2,3-dioxygenase from *Sphingomonas* sp. HV3 (Yrjälä *et al.*, 1994), *S. yanoikuyae* sp. B1 (Kim and Zylstra, 1995), or *P. putida* mt-2 (Nakai *et al.*, 1983) and *P. putida* G7 (Ghosal *et al.*, 1987). The overall amino acid identities between the DJ77 *phnQ* gene product and those of others were 85.6% (BphC of Q1), 71.9% (EtbC of RHA1), 59.1% (NahC of *P. putida* G7), 39.9% (BphC of *P. cepacia* LB400) (Hofer *et al.*, 1993), 40.1% (BphC of KKS102), 20.5% (XylE of mt-2), 20.5% (NahH of G7), 22.1% (CmpE of HV3), and 22.1% (PhnE) (Kim *et al.*, 1992b).

The superfamily of extradiol dioxygenases can be divided into two subfamilies. Subfamily I consists of bicyclic-ring substrate *meta*-cleavage dioxygenase genes like *bphC* and *nahC*. Subfamily II consists of monocyclic-ring substrate *meta*-cleavage dioxygenase genes like *xylE* and *nahH* (Harayama and Rekik, 1989; Harayama *et al.*, 1992). The results of sequence homology studies show that PhnQ is more similar to the subfamily of extradiol dioxygenases that act on bicyclic-ring substrates than to those that act on single-ring substrates. In contrast, PhnE shows a reversed sequence identity pattern to PhnQ: CmpE of HV3 (100%), XylE of B1 (89.6%), NahH of NAH7

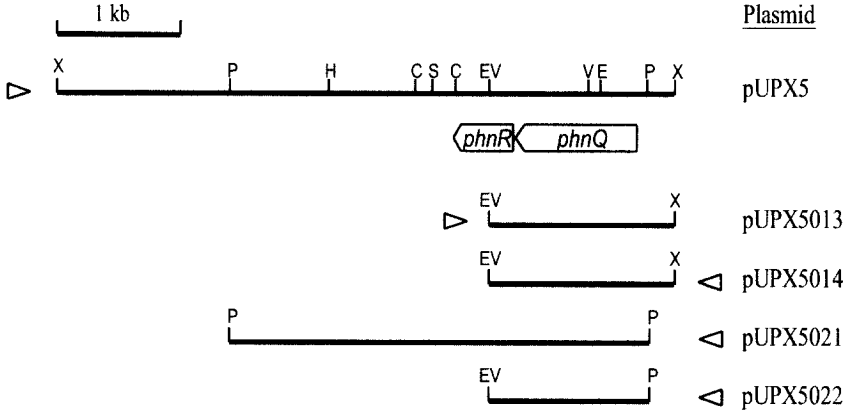

Fig. 1. Restriction map of the cloned region in pUPX5 and localization of the *meta*-cleavage dioxygenase gene. The arrowed boxes indicate the deduced positions of the genes for 2,3-DHBD and Rieske-type ferredoxin component of ring-hydroxylating dioxygenase. The direction of transcription of the *lac* promoter is indicated with arrowheads. Abbreviations: P, *Psp*1406I; Ev, *Eco*RV; X, *Xho*I.

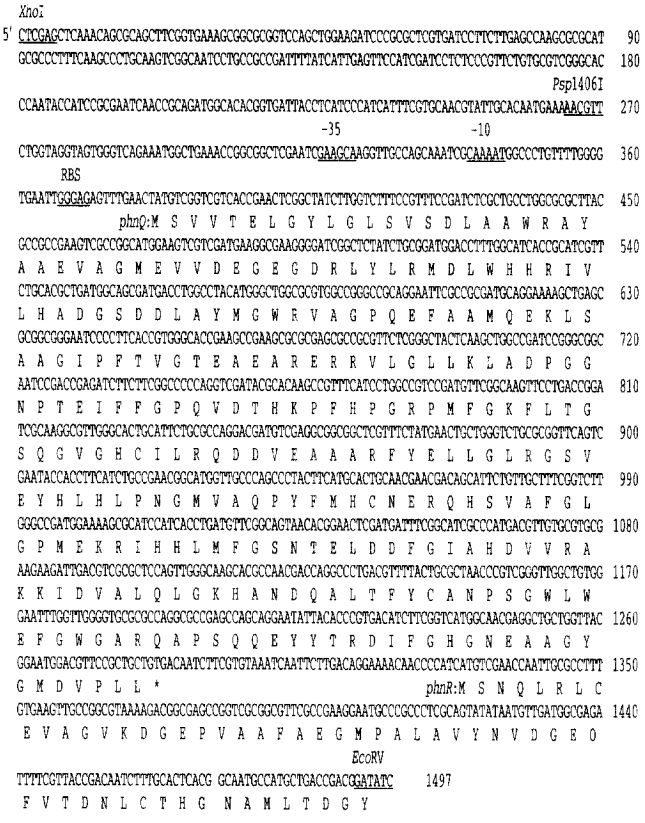

Fig. 2. Nucleotide sequence of the 1.5-kb *Xho*I-*Eco*RV fragment containing *phnQ* and its corresponding amino acid sequence (GenBank nucleotide database accession number AF061802). An asterisk indicates a stop codon. Putative ribosome binding site (RBS) and a putative promoter region (−10 and −35) are marked.

(50.2%), XylE of mt-2 (48.8%), DmpB of *P. putida* CF600 (52.1%) (Bartilson and Shingler, 1989), BphC of KKS102 (17.8%), BphC of LB400 (19.9%) (Hofer *et al.*, 1993), BphC of Q1 (22.1%), EtbC of RHA1 (23.5%), and NahC of G7 (19%). This sequence identity places PhnE within the monocyclic-ring substrate subfamily of extradiol dioxygenases.

Figure 3 shows the amino acid homology between the DJ77 *phnQ* product and five extradiol dioxygenases including BphCs of *Pseudomonas cepacia* LB400 and *Pseudomonas* sp. KKS102, whose crystal structures have been determined (Han *et al.*, 1995; Senda *et al.*, 1996), BphC of Q1, and XylE of mt-2, together with PhnE of the same DJ77 strain. Amongst the six enzymes shown in the alignment, 21 amino acids were found to be completely conserved. Among these conserved amino acids, ten amino acids which are considered to be important for the role of extradiol dioxygenase were all conserved. Studies showed that three of these are metal ligands, His-146, His-210, and Glu-260, and three are additional active site residues, His-

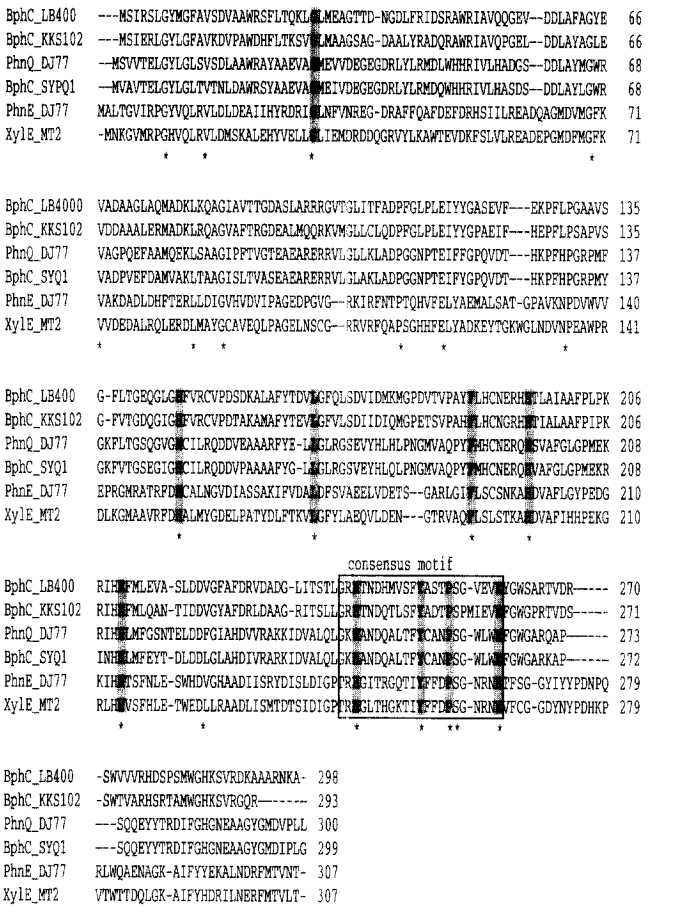

Fig. 3. Alignment of six extradiol dioxygenases as obtained by Clustal X and modified according to the crystal structures of BphC_LB400 and BphC-KKS102 as described by Eltis and Bolin (1996). Asterisks indicate the amino acids identical in all proteins. The iron ligands (His-146, His-210, and Glu-260) and the three conserved active site residues (His-195, His-241, and Tyr-250) that probably play a direct catalytic role are shaded light grey in bold letters. Conserved residues (Gly-28, Leu-165, and Pro254) that play a structural role and Phe-187, which interacts with the hydroxylated ring of the substrate in the enzyme-substrate complex (Senda *et al.*, 1996), are also shaded grey in bold letters. The numbering system refers to the sequence of BphC from *P. cepacia* LB400. The residues constituting the consensus motif are boxed. The sequences are as follows (references and GenBank accession numbers, respectively, are provided in parentheses): BphC_LB400, *P. cepacia* LB400, 2,3-dihydroxybiphenyl dioxygenase (Hofer *et al.*, 1993) (X66122); BphC_KKS102, *Pseudomonas* sp. strain KKS102, 2,3-dihydroxybiphenyl dioxygenase (Kimbara *et al.*, 1989) (M26433); PhnQ_DJ77, *Pseudomonas* sp. strain DJ77, 2,3-dihydroxybiphenyl dioxygenase (AF061802); BphC_SYQ1, *S. yanoikuyae* Q1 (formerly *S. paucimobilis* Q1), 2,3-dihydroxybiphenyl dioxygenase (Taira *et al.*, 1992) (M20640); PhnE_DJ77, *Pseudomonas* sp. strain DJ77, catechol 2,3-dioxygenase (Kim *et al.*, 1992b) (U83882); XylE_MT2, *P. putida* mt-2, catechol 2,3-dioxygenase (Nakai *et al.*, 1983) (V01161).

195, His-241, and Tyr-250 responsible for various catalytic roles. The other strictly-conserved residues, Gly-28, Leu-165, and Pro-254 are likely to have structural or folding roles (Han *et al.*, 1995; Eltis and Bolin, 1996). Phe-187, which interacts with the hydroxylated ring of the substrate in the enzyme-substrate complex, is also considered to be important (Senda *et al.*, 1996). The numbering system refers to the sequence of BphC from *P. cepacia* LB400. The extradiol dioxygenase fingerprint region (defining the organic substrate binding pocket) which spans residues 239 to 260 in the BphC_LB400 sequence were also conserved (Boldt *et al.*, 1997).

Enzyme activities in cell extracts from a clone carrying the recombinant plasmid Noticeably different migration distances were seen for the two *meta*-cleavage dioxygenases when samples were run on a nondenaturing polyacrylamide gel (data not shown). In addition, the catalytic functions of the two enzymes, PhnE and PhnQ, are significantly different from each other. The extradiol dioxygenase from pPE17 (PhnE) exhibited high ring-fission activity with catechol and 3-methylcatechol, but little activity with 2,3-dihydroxybiphenyl. The ability of the *meta*-cleavage dioxygenase encoded by pPE17 to cleave 3-methylcatechol and 4-chlorocatechol is between that for catechol and 2,3-dihydroxybiphenyl. In contrast, extradiol dioxygenase from pUPX5022 (PhnQ) shows broader substrate preference than PhnE. 2,3-DHBD encoded by pUPX5022 has maximal activity against 2,3-dihydroxybiphenyl, with significant activity against catechol, 3-methylcatechol, and 4-chlorocatechol (75.4, 86.7, and 71.6% of the activity against 2,3-dihydroxybiphenyl, respectively). These data suggest that pUPX5022 contains the *phnQ* gene, coding for an enzyme similar to 2,3-dihydroxybiphenyl 1,2-dioxygenase, while pPE17 contains the gene coding for an enzyme similar to catechol 2,3-dioxygenase (Table 2).

Genetic organization It is noteworthy to comment on the organization of *phnQ/phnE* compared to other biphenyl pathway genes. In the *nah* operon, the genes *nahC* and *nahH* encoding 2,3-dihydroxybiphenyl 1,2-dioxygenase and catechol 2,3-dioxygenase, respectively, are found in different operons with the same direction. However, the gene for 2,3-DHBD (*phnQ*) is located 6.5 kb upstream from *phnE* and these two *meta*-cleavage dioxygenases are transcribed in opposite directions (Shin *et al.*, 1997b). This is also the case for two *Sphingomonas* strains as revealed by Romine *et al.* (1999) (GenBank accession number AF079317) and Kim (1996).

Table 2. Relative activities for the oxidation of various aromatic diols by cell extracts of *E. coli* XL1-Blue (pUPX5022) encoding 2,3-DHBD (PhnQ) from strain DJ77 in comparison to those of PhnE (pPE17) from the same strain.

Substrates	λ_{max} of product (nm)	Enzyme Relative activity	
		PhnQ	PhnE
Catechol	375	75.4	100.0
3-Methylcatechol	385	86.7	99.7
4-Methylcatechol	382	13.9	24.9
4-Chlorocatechol	379	71.6	67.5
2,3-Dihydroxybiphenyl	434	100.0	11.5

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