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Identification of the *bphC* Gene for *meta*-Cleavage of Aromatic Pollutants from a Metagenomic Library Derived from Lake Waters

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Abstract Useful genes can be screened from various environments by construction of metagenomic DNA libraries. In this study, water samples were collected from several lakes in mid Korea, and analyzed by T-RFLP to examine diversities of the microbial communities. The crude DNAs were extracted by the SDS-based freezing-thawing method, and then further purified using an UltraClean™ kit (MoBio, USA). The metagenomic libraries were constructed with the DNAs partially digested with *Eco*R I, *Bam*H I, and *Sac* II in *Escherichia coli* DH10B using the pBACe3.6 vector. About 44.0 Mb of metagenomic libraries were obtained with average inserts 13~15 kb in size. The *bphC* genes responsible for degradation of aromatic hydrocarbons via *meta*-cleavage were identified from the metagenomic libraries by colony hybridization using the *bphC* specific sequence as a probe. The 2,3-dihydroxybiphenyl (2, 3-DHBP) dioxygenase gene (*bphC*), capable of degradation of 2,3-DHBP, was cloned and its nucleotide sequences analyzed. The genes consisted of 966 and 897 base pairs with an ATG initiation codon and a TGA termination codon. The activity of the 2,3-DHBP dioxygenase was highly expressed to 2,3-DHBP and showed a broad substrate range to 2,3-DHBP, catechol, 3-methylcatechol and 4-methylcatechol. These results indicated that the *bphC* gene identified from the metagenomes derived from lake water might be useful in the development of a potent strain for degradation of aromatic pollutants.

Keywords: metagenomic libraries, *bphC*, 2,3-DHBP dioxygenase, aromatic pollutants

INTRODUCTION

The biosphere is dominated by a variety of microorganisms [1], yet most microbes in nature have not been studied. Traditional methods for culturing microorganisms limit analysis to those that grow under laboratory conditions [2]. The recent surge of research in molecular microbial ecology provides compelling evidence for the existence of many novel microorganisms in the environment in terms of numbers and varieties, dwarfing those of the comparatively few amenable to laboratory cultivation [3].

Metagenomic techniques can be used to address the challenge of studying prokaryotes in the environment that are as yet unculturable, representing more than 99% of the organisms in some environments, as shown in Table 1 [12]. Direct genomic cloning offers the opportunity to capture operons or genes encoding the pathways that may direct the synthesis of complex molecules, such as antibiotics and other useful substances [13,14].

Polychlorinated biphenyls (PCB) and nitrotoluene have been of public and scientific concern for several decades due to their persistence in the environment, ability to bioaccumulate and potential carcinogenicity. A promising approach for dealing with PCB and nitrotoluene contamination is bioremediation, as a number of biphenyl-degrading organisms are capable of transforming those pollutant chemicals [15,16]. The organisms that catabolize biphenyl to benzoate and 2-hydroxypenta-2,4-dienoate, via the so-called upper pathway, have been intensively studied [17]. The pathway consists of consecutive reactions catalyzed by four enzymes: biphenyl 2,3-dioxygenase (BphA), 2,3-dihydro-2,3-dihydroxybiphenyl-2,3-dehydrogenase (BphB), 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC) and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (BphD). The *bphABCD* gene cluster responsible for degradation of biphenyl to benzoic acid has been cloned from *Pseudomonas* sp. KKS102, *Pseudomonas* sp. DJ-12, *Pseudomonas aeruginosa* KF702 *Pseudomonas pseudoalcaligenes* KF707, *Burkholderia* sp. LB400 (*Burkholderia* sp. LB400 was originally identified as *Pseudomonas* sp. LB400) and *Rhodococcus globerulus* P6 [18-22]. Of these four enzymes, 2,3-dihydroxybiphenyl (2,3-DHBP) dioxygenase, which is encoded by the *bphC* gene, belongs to a class of extradiol dioxygenase catalyzes for the *meta*-cleavage of 2,3-DHBP [18,23]. These ex-

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Table 1. Culturability determined as a percentage of the culturable bacteria in comparison with the total cell counts

Habitat	Culturability (%)[a]	References
Seawater	0.001~0.1	[4,5,6]
Freshwater	0.25	[7]
Mesotrophic lake	0.1~1	[8]
Unpolluted estuarine waters	0.1~3	[4]
Activated sludge	1~15	[9,10]
Sediments	0.25	[7]
Soil	0.3	[11]

[a] Culturable bacteria are measured as CFU formation.

tradiol dioxygenases play key roles in the degradation pathways of biphenyl and other aromatic compounds [24].

Herein, the construction and initial screening of BAC libraries made with DNA isolated directly from lake waters in mid Korea are described. The *bphC* genes were identified from metagenomic DNA clones and examined for their potent activities. This means that the construction of a metagenomic library would be a useful method to detect a potent genome of valuable information, such as the *bphC* gene responsible for degradation of aromatic pollutants.

MATERIALS AND METHODS

DNA Extraction

The bacterial genomic DNAs were prepared from the waters of Chungju, Daecheong and Dunpo lakes in mid Korea, using the protocol described by Rhochelle *et al.* [25]. The samples were centrifuged at 14,000 × g and 4°C for 15 min, and the bacterial cell pellets obtained resuspended in 300 μL of lysozyme solution (0.15 M NaCl and 0.1 M EDTA, pH 8.0, lysozyme 15 mg·mL^{-1}) and incubated for 1 h at 37°C, while gently mixing the solution at 15 min intervals. After the addition of 300 μL SDS buffer (0.1 M NaCl and 0.5 M Tris-HCl, pH 8.0, SDS 4%), the samples were incubated for 10 min at -70°C and 10 min at 65°C. This freezing-thawing process was repeated three times. The bacterial genomic DNA was then extracted and purified from the cell lysates by two sequential phenol-chloroform extractions and isopropanol precipitation. The DNA pellets obtained were washed with 70% ethanol and dissolved in sterile TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). These genomic DNAs were then purified using an UltraCleanTM kit (Mo Bio, Solana Beach, California, USA), and subjected to electrophoresis in 0.8% agarose gels, and stored at -20°C.

16S rDNA T-RFLP

The 16S rDNA fragments were amplified by PCR using two eubacterial primers 27FB (*E. coli* numbering 8-27: 5'-AGAGTTTGATCMTGGCTCAG-3') and 785R (*E. coli* numbering 785-804: 5'-ACTACCRGGGTATCTAAT CC-3'). The 27FB was biotinylated at the 5'-end to separate terminal restriction fragments (T-RFs) from other digested fragments [26]. PCR was carried out in a 50 μL reaction mixture containing; 1× PCR buffer (100 mM Tris-HCl, 400 mM KCl, 1.5 mM $MgCl_2$ and 500 μg·mL^{-1} BSA at pH 8.3), 160 μM of each dNTP, 0.3 μM of each primer, 1.5 unit of *Taq* polymerase (Genenmed, Seoul, Korea) and 10~15 ng/μL of template DNA. An initial denaturation step (3 min at 95°C) was allowed by 30 cycles of denaturation (30 s at 95°C), annealing (30 s at 58°C) and extension (1 min at 72°C), with a final 10 min extension at 72°C. The PCR products were purified using an UltraCleanTM kit (MoBio) and subjected to electrophoresis in 0.8% agarose gels.

Purified PCR products were digested at 37°C for 5 h with 5 units of *Hae*III (TaKaRa, Otsu, Shiga, Japan). The biotinylated T-RFs were selectively isolated from the digested fragments using Streptavidin MagneSphere paramagnetic particles (SA-PMPs) and a magnetic separation stand (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions [23]. The samples were soaked in 0.2 M NaOH for 5 min at room temperature to denature the double stranded T-RFs. Biotinylated and single stranded T-RFs were mixed with 25% NH_4OH and incubated at 65°C for 10 min to separate the T-RFs from SA-PMPs. The NH_4OH was removed using a vacuum microcentrifuge, and the T-RF pellets obtained resuspended in distilled water. T-RFLP patterns were analyzed by electrophoresis on a 6% polyacrylamide gel (acrylamide:bisacrylamide = 19:1; 7.0 M urea; 1× TBE). After mixing the DNA samples with an equal volume of loading dye buffer (95% formamide, 10 mM NaOH, 20 mM EDTA, 0.02% bromophenol blue, 0.02% xylene cyanol FF), they were heated for 3 min at 95°C, and chilled on ice prior to electrophoresis at 1900 V and 50 W for 3 h. Silver staining was performed according to the manufacturer's instructions (Bioneer, Daejeon, Korea).

Library Construction

The plasmid, pBACe3.6, and *E. coli* DH10B were used as the vector and host strain, respectively. Total DNAs from the lake water samples and the pBACe3.6 vector digested with *Eco*RI, *Bam*HI, and *Sac* II, respectively, were ligated for 16 h at 16°C. The ligation mixtures were transformed using the calcium chloride procedure described by Sambrook *et al.* [27]. White colonies were picked onto Trypticase Soy Agar plates (TSA-Cm) supplemented with 12.5 μg/mL chloroamphenicol and 5% sucrose. The library was replicated into duplicate sets of TSA-Cm broth with glycerol and stored at -80°C.

Colonies were grown for 1 day at 37°C on LB agar plates supplemented with 12.5 μg/mL chloroamphenicol. *In situ* hybridization was performed using the ECL direct nucleic acid labeling and detection systems (Amersham, USA), according to the manufacturer's instructions. The

Table 2. Environmental conditions of the lake waters studied in this work

Parameter	Dunpo	Chungju	Daecheong
Location	Asan, Chungnam	Chungju, Chungbuk	Cheongju, Chungbuk
Surface area (km^2)	0.5	67.5	72.8
Depth (m)	3.0	97.5	72.0
Temperature (°C)	24.5	25.7	25.1
pH	7.34	7.48	7.48
Chlorophyll-a (mg/L)	68.88	1.14	0.97
Total nitrogen (mg/L)	5.635	1.469	1.342
Total phosphate (mg/L)	3.864	0.034	0.030
BOD (mg/L)	8.5	1.4	1.4

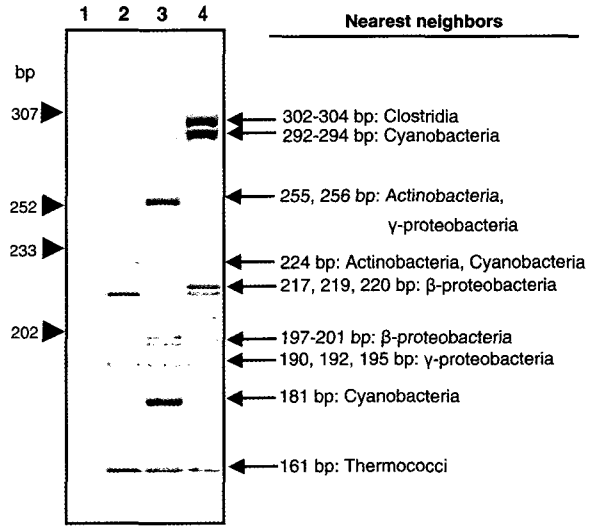

Fig. 1. *Hae*III T-RF profiles of 16S rDNA amplified from the lake samples. Lane: 1, Size marker; 2, Daecheong; 3, Dunpo; 4, Chungju.

DNA fragments used as probes were obtained from the UltraCleanTM 15 kit (MO BIO, CA, USA).

2,3-DHBP Degradation Activity Assay

The degradation activity of 2,3-dihydroxybiphenyl (2, 3-DHBP) was assayed with the high-speed supernatants of the clone cell extracts prepared by sonication. The enzymatic activity was assayed at 23°C in 50 mM potassium phosphate buffer (pH 7.5), containing 330 M 2,3-DHBP, as the 1,2-dioxygenase activity, which was determined by measuring the *meta*-cleavage product (MCP) formation at 434 nm with a spectrophotometer. The relative *meta*-cleavage activities of the clones were determined by measuring the MCP produced from the following substrates: catechol (λ_{max}, 375 nm), 3-methylcatechol (λ_{max}, 388 nm) and 4-methylcatechol (λ_{max}, 382 nm).

Data Analysis

The nucleotide sequences of the *bphC* genes were determined by the Sanger's dideoxy-chain termination method. Sequencing was performed using a ThermoSequenase kit (Part number: US78500, Amersham Life Science, USA) and LongRealR 4200 sequencer. 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. The phylogenetic analysis was performed on these data sets using the PHYLIP (phylogenetic inference package, version 3.6 a2.1) algorithm.

RESULTS AND DISCUSSION

Bacterial Diversity in Lake Waters

The location and environmental conditions of the three lakes are shown in Table 2. In general, Dunpo lake is a small reservoir and much more polluted than the Chungju and Deacheong lakes in terms of chlorophyll-a, total nitrogen, total phosphate and BOD. The water samples taken from these three lakes were analyzed by T-RFLP to examine the diversities of the microbial communities. The *Hae*III T-RF profiles are shown in Fig. 1. The total number of T-RF bands between 150 and 350 bp in size were 36 for Daecheong Lake, 32 for Chungju and 24 for Dunpo. Cyanobacteria and γ-preteobacteria were more dominant in Dunpo Lake than in Chungju and Daecheong Lakes. The 181 bp T-RF band derived from *Micrococcus* and *Synechococcus* were predominately detected in the water of Dunpo Lake. However, the same band was not detected at Chungju Lake, where the 292 and 294 bp bands derived from *Anabaena* predominated. Cyanobacteria were common in eutrophic natural water. Being favored by warm, stable, and nutrient-enriched waters, they might constitute an important part of the

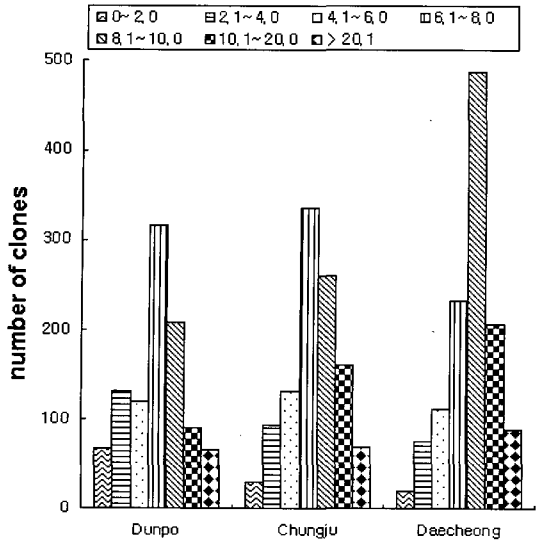

Fig. 2. Size distribution of the inserts included in the metagenomic DNA library.

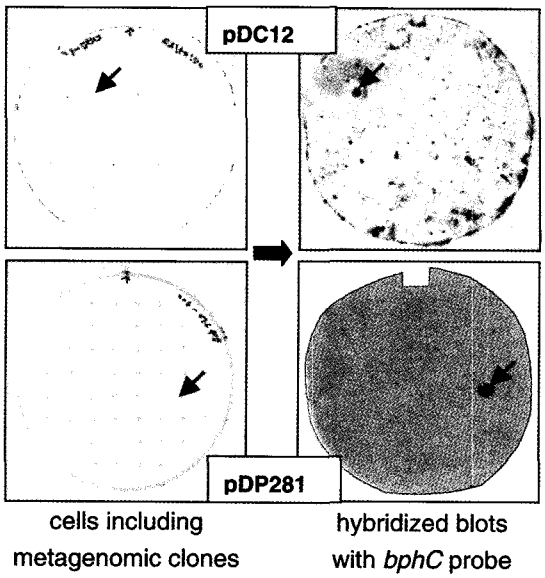

Fig. 3. Detection of *bphC* genes including in the metagenomic clones, pDC12 and pDP281.

phytoplankton community in Dunpo. The abundance of nutrients in Dunpo favored the proliferation of γ-preteobacteria, as Wagner *et al.* [28] reported that these nutrients were strongly selected against β-proteobacteria and to a lesser degree against α-proteobacteria. This result indicated a clear correlation between the abundance of cyanobacteria and γ-preteobacteria and the pollution level of lake water. Therefore, those results provided evidence for low diversity in warm-lake waters in the summer season.

Construction of Metagenomic Library

To access as much genomic information as possible from a pool of microorganisms in the lake water, including those not readily culturable, the SDS-based freezing-thawing method was used to extract and clone the larger DNA fragments. The metagenomic library was constructed with the DNAs partially digested with *Eco*RI, *Bam*HI and *Sac*II in *Escherichia coli* DH10B using the pBACe3.6 vector. Approximately 50% of the clones were examined for inserts; 99% contained insert DNA, with an average insert size of 13~15 kb (Fig. 2). Approximately a 44.0 Mb (3,032 clones) metagenomic DNA library was constructed from lake waters in this study. This approach provided a route to study the phylogenetic, physical and functional properties of the metagenomes.

Identification of *bphC* Gene for 2,3-DHBP Dioxygenase

In order to detect the 2,3-DHBP dioxygenase encoded by the *bphC* gene from the metagenomic libraries of the three lake waters, the transformant *E. coli* strains from each metabolic library were examined for the production of 2,3-DHBP dioxygenase by colony hybridization using the *bphC* specific sequence. A horse-radish peroxidase-labeled 2 kb and 900 bp fragment, containing the *bphC* gene, was used as a probe. Two clones of pDC12, and pDP281 were selected to be hybridized with the probe, as shown in Fig. 3.

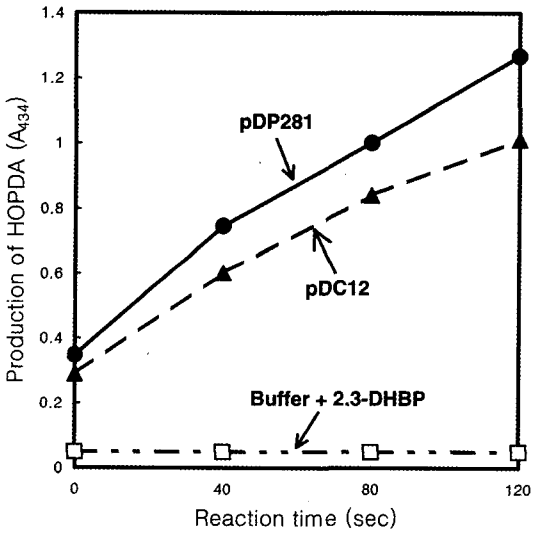

Fig. 4. Degradation of 2,3-DHBP to produce HOPDA by the transformant *E. coli* cells, including the metagenomic clones, pDC12 and pDP281.

The degradation of 2,3-DHBP producing HOPDA by the two transformants with the metagenomic clones is shown in Fig. 4. The transformant *E. coli* harboring pDP281 showed broad substrate activity to 2,3-DHBP, catechol, 3-methylcatechol and 4-methylcatechol (Table 3). However, the *bphC* product of *E. coli* harboring the pDC12 clone showed degradation activity to 2,3-DHBP and catechol only. The 2,3-DHBP dioxygenase produced by pDP281 was very similar to the *bphC* of *Pseudomonas*

Table 3. *meta*-cleavage activities of aromatics by the metagenomic clone cells containing *bphC* gene

Substrates	Optical density of *meta*-cleavage products[a]	
	pDC12	pDP281
2,3-dihydroxybiphenyl	1.487	1.795
Catechol	0.525	0.921
3-methylcatechol	0	1.007
4-methylcatechol	0	0.456

[a] The *meta*-cleavage products produced from the substrates were examined by colorimetric spectrophotometry at the following wavelength: 2, 3-dihydroxybiphenyl, 434 nm; catechol, 375 nm; 3-methylcatechol, 388 nm; 4-methylcatechol, 382 nm.

Table 4. Similarity (%) of amino acid sequences of *bphC* encoding 2,3-DHBP dioxygenase in pDC12 and pDP281 with those from several other bacteria

Strains	GenBank accession No.	Amino acid similarity (%)	
		pDC12	pDP281
Acinetobacter baumannii KF714	AB110457	79.9	36.1
Burkholderia sp. TSN101	D85182	69.7	44.6
Comamonas testeroni KF704	AB110451	66.6	46.9
Pseudomonas aeruginosa KF702	AB110449	99.6	45.5
Pseudomonas pseudoalcaligenes KF707	AB110456	79.9	36.1
Pseudomonas putida KF715	M33813	79.8	36.2
Pseudomonas sp. Cam-1	AY027651	98.5	45.1
Pseudomonas sp. DJ-12	D44550	45.5	99.8
Pseudomonas sp. NCIMB 10643	AF468021	66.7	46.5
Pseudomonas stutzeri	D85129	31.6	35.5

sp. The DJ-12 was similar in terms of substrate specificity [19], but quite different from *bphC* of other *Pseudomonas* strains. The degradation activity of pDC12 to 2,3-DHBP was similar to the narrow substrate range shown by *Pseudomonas pseudoalcaligenes* KF707 [29]. It seems highly likely that the different isoenzymes contribute to the broad substrate range of pDP281, as indicated by McKay *et al.* [30].

Genetic Structure of *bphC*

The genetic structure of *bphC* genes derived from the pDC12 and pDP281 metagenomic clones were analyzed in more detail. The nucleotide sequences of pDC12 and pDP281 were found to have a complete operon reading frame corresponding to *bphC*. The *bphC* genes in pDC12 and pDP281 consisted of 897 bp and 966 bp, respectively, and their corresponding polypeptides were found to be 299 and 322 residues. The G+C contents of the *bphC* gene in both clones were 60.0 and 57.2%, respectively. A promoter-like sequence (-35 and -10 region) was also found at a position 11~12 bp upstream from the start codon of the *bphC* gene.

The amino acid sequences of 2,3-DHBP dioxygenase (*bphC* product) from the two metagenomic clones were aligned with those of the 2,3-DHBP dioxygenases from 12 other bacteria, as shown in Table 4. The *bphC* from pDP281 showed 99.8, 46.9, 46.5, and 45.5% identities to those of the corresponding genes from *Pseudomonas* sp. DJ-12, *Comamonas testeroni* KF704, *Pseudomonas* sp. NCIMB 10643 and *Pseudomonas aeruginosa* KF702, respectively. The *bphC* from pDC12 showed 99.6, 98.5, 79.9, and 66.6% identities to those of the corresponding genes from *Pseudomonas aeruginosa* KF702, *Pseudomonas* sp. Cam-1, *Pseudomonas pseudoalcaligenes* KF707 and *Comamonas testeroni* KF704, respectively.

In terms of the phylogenetic relationship between the 2,3-DHBP dioxygenases from the two metagenomic clones and those from other strains, the *bphC* products from two recombinants were divided into two groups, as shown in Fig. 5. The BphC of pDP281 was closely related to that from *Pseudomonas* sp. DJ-12 [19], and was similar to those from *Pseudomonas pseudoalcaligenes* KF707 and *Pseudomonas aeruginosa* KF702 [29]. These results were

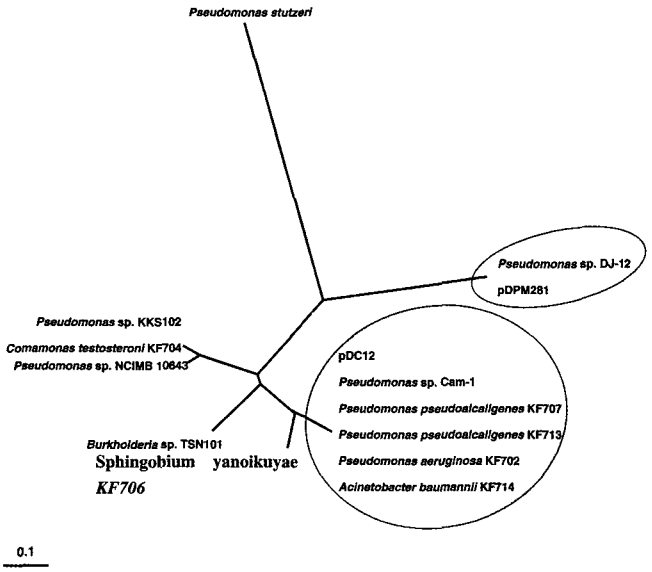

Fig. 5. Phylogenetic trees of the metagenomic clones and other bacteria on the basis of the nucleotide sequences of the *bphC* gene encoding dioxygenase for *meta*-cleavage of aromatic compounds. The bar indicates a 10% difference.

similar to those reported by McKay *et al.* [30] in the grouping of the enzyme activities to 2,3-DHBP.

Among the fourteen 2,3-DHBP dioxygenases aligned, sixty amino acids in the enzyme were shown as conserved residues. Six (three His, one Tyr, and two Glu) of these have been shown to be involved in coordination of active site ferrous ions in extradiol dioxygenases. Histidyl residues have been reported to be particularly critical ligands for coordination of the ferrous ions, which was documented by the site-specific mutagenesis of 2,3-DHBP dioxygenase from *Pseudomonas pseudoalcaligenes* KF707 [29] and the chemical modification of the catechol 2,3-dioxygenase from *Rhodococcus rhodochrous* CTM [31], as well as by the recent analyses of crystal structures of 2, 3-DHBP dioxygenases from *Pseudomonas cepacia* LB400 [23] and *Pseudomonas* sp. KKS102 [18]. The convergence and divergence of essential amino acids in the active sites of extradiol-type dioxygenases correlated well with those of the overall sequence homologies among the enzymes.

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