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Cloning and Phylogenetic Analysis of Two Different *bphC* Genes and *bphD* Gene From PCB-Degrading Bacterium, *Pseudomonas* sp. Strain SY5

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Abstract *Pseudomonas* sp. strain SY5 is a PCB-degrading bacterium [24] that includes two different enzymes (BphC1 and BphC2) encoding 2,3-dihydroxybiphenyl 1,2-dioxygenase and BphD encoding 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase. The *bphC1* and *bphC2* genes were found to consist of 897 bases encoding 299 amino acids and 882 bases encoding 294 amino acids, respectively, whereas the *bphD* gene consisted of 861 bases encoding 287 amino acids. According to a homology search, a 50% and 39% similarity between the *bphC1* and *bphC2* genes at the nucleotide and amino acid level was shown, respectively. The *bphC1* gene showed a 38% and 45% similarity at the amino acid level to *Alcaligenes eutrophus* A5 and *Rhodococcus rhodochrous*, respectively, whereas, *bphC2* showed a 95% and 43% similarity, respectively. A comparison of the deduced amino acid sequence of the *bphD* product of *Pseudomonas* sp. SY5 with that of *A. eutrophus* A5, *Pseudomonas* sp. KKS102, and LB400 showed a sequence identity of 92, 92, and 79%, respectively. Strain SY5 was originally isolated from municipal sewage containing recalcitrant organic compounds and found to have a high degradability of various aromatic compounds [23]. The current study found that strain SY5 had two extradiol-type dioxygenases, which did not hybridize with each other as they had a low similarity, yet a similar structure of evolutionarily conserved amino acids residues for catalytic activity between BphC1 and BphC2 was observed.

Key words: PCBs, SY5, *bphC*, *bphD*, dioxygenase, hydrolase

Polychlorinated biphenyls (PCBs), a family of synthetic organic chemicals, are widely used by many industries due to their chemical stability, thermal stability, and insulating properties. PCB molecules consist of a biphenyl nucleus carrying 1 to 10 chlorines; hence, there are 209 possible PCB congeners based on the different numbers and positions of the chlorines [3, 17, 28]. However, these PCBs can result in serious environmental pollution even when a small quantity is released into the biosphere because they are very strong toxic materials.

The ultimate purpose of the physiological and genetic study of PCB-degrading bacteria is to develop strains harboring a good degradability that can be practically adapted to the environment. The degradation of PCBs is aerobically initiated by the cleavage of the aromatic ring, thus aromatic ring-cleavage enzymes play a very important role in the degradation of PCBs. Ring-cleavage enzymes are divided into two branches, extradiol and intradiol types, and they exhibit quite different characterization [27]. Most of the bacteria highlighted in previous reports degrade PCBs through the *meta*-cleavage pathway, using extradiol-type enzymes [1, 2, 5, 8, 9, 12-15, 18-20, 27, 32, 35].

Seven enzymes are responsible for the complete degradation of PCBs. The first step of the oxidative catabolic pathway is initiated by the insertion of two atoms of oxygen at carbon positions 2 and 3 in the PCB aromatic ring by BphA (biphenyl dioxygenase). The resulting product *cis*-biphenyl 2,3-dihydrodiol is then oxidized to 2,3-dihydroxybiphenyl by BphB (dihydrodiol dehydrogenase). The 2,3-dihydroxybiphenyl is sequentially cleaved by BphC (2,3-dihydroxybiphenyl 1,2-dioxygenase (2,3-DHBD)) at the *meta* position. The resulting *meta*-cleavage product (yellow product), 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA), is then hydrolyzed by BphD (HOPDA hydrolase) to the corresponding benzoic acid. The enzymes BphE, BphF, and BphG are then ultimately converted to a TCA cycle [12, 15, 25, 27]. 2,3-Dihydroxybiphenyl 1,2-dioxygenase (BphC) plays a very important role in the degradation of PCBs by cleaving the aromatic ring, and *bphABCD* genes are usually clustered as an operon [2, 9, 15, 20].

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Many Gram-negative and Gram-positive bacteria harboring the *bphC* gene have been isolated in previous reports [1, 2, 8, 9, 12-15, 18-20, 27, 32, 35]. *Rhodococcus globerulus* P6 [1], a Gram-positive bacteria, was reported to have three different *bphC* genes, whereas *Rhodococcus* sp. strain RHA1 [8] has two *bphC* genes, and in the case of *Rhodococcus erythropolis* TA421 [15], it has seven *bphC* genes designated as *bphC1* to *bphC7*.

The current study isolated two significantly different *bphC* genes coding for 2,3-dihydroxybiphenyl 1,2-dioxygenases, and a *bphD* gene coding for HOPDA hydrolase, from the aerobic PCB-degrading bacterium *Pseudomonas* sp. strain SY5 [24]. In addition, nucleotide and amino acid sequences were determined and analyzed.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

To clone and analyze the *bph* genes involved in PCB degradation, *Pseudomonas* sp. strain SY5 [23, 24], exhibiting the highest degree of PCB degradation among the strains isolated by the current authors, was used. *Escherichia coli* DH5α (*F^- Ø80dlacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK^-mK^+)supE44λ^- thi-1 gyrA relA1*) [7] was used as the recipient strain for the transformation, and the pUC119 vector was used to clone the *bph* genes.

Isolation of DNA and Cloning of Genes

Strain SY5 grown in a C-medium [23] containing PCBs (1,000 ppm) was harvested at the mid-log phase and centrifuged at 10,000 ×g for 10 min. The collected cells were resuspended in 19 ml of a TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) with 80 mg lysozyme, and incubated at 37°C for 10 min. Subsequently, 1 mg of proteinase K was added, then the suspension was incubated again at 37°C for 60 min. SDS [Sodium Dodecyl Sulfate, final conc. 0.5% (v/v)] and 1 mg of proteinase K was added to the reaction for lysis, then the suspension was incubated for a further 30 min. After another addition of SDS [final conc. 1% (v/v)] for a better lysis, the cell suspension was incubated at 50–60°C for 30 min. The NaCl concentration of the solution was adjusted to 0.7 M using 5 M NaCl. Three-and-a-half ml of a CTAB/NaCl solution (CTAB: cetyltrimethyl-ammonium bromide, consisting of 10% CTAB/0.7 M NaCl) was added and the solution was mixed thoroughly for 10 min, then a final incubation at 65°C for 40 min was performed. The solution containing total DNA was extracted three times with phenol/chloroform solution [1:1 (v/v)], and the solution was finally extracted with chloroform/isoamyl alcohol [24:1 (v/v)]. The total DNA was then precipitated by absolute ethanol (–20°C), and collected by spooling onto a glass stick. The collected DNA was dissolved in a TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) [1, 30].

The total DNA was partially digested with the restriction enzyme *Pst*I and electrophoresed on a 1.2% agarose gel. DNA fragments with the size of 2–10 kb were collected using a dialysis bag (Spectra/Por® Membrane, MWCO: 12-14000, Alondra · Gardena, CA, U.S.A.). pUC119 was cleaved at the *Pst*I site and treated with CIAP (calf intestinal alkaline phosphatase). The ligation of the *Pst*I fragments from the total DNA and dephosphorylated *Pst*I site of the pUC119 with T4 ligase was performed under the reaction conditions recommended by the supplier (Takara Shuzo Co., Otsu, Shiga, Japan) [30].

The transformation of *Escherichia coli* DH5α was accomplished using the calcium chloride method [30] and the transformants were plated onto LB agar plates containing ampicillin (final conc. 50 μg/ml), IPTG (isopropyl-β-D-thiogalactopyranoside, final conc. 25 μg/ml), and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, final conc. 20 μg/ml), Thereafter, only the yellow expressed colonies (enzyme-positive transformants) were isolated through treatment with an acetone solution containing 0.05 M 2,3-dihydroxybiphenyl (Wako Chemical Co., Tokyo, Japan) after incubation at 37°C overnight [4].

Analysis of Recombinant Plasmids

The recombinant plasmid DNAs from the transformants were prepared using an alkaline lysis procedure [30]. The plasmids were digested with *Pst*I and their sizes were compared with a DNA molecular size marker (λ-*Hin*dIII size marker). Subsequently, the plasmids were treated with various restriction enzymes belonging to the multiple cloning site of pUC119 for restriction mapping.

Southern hybridization was performed as described by Sambrook *et al.* [30]. The partial digest of the chromosomal DNA was separated on 1.2% agarose gels and hybridized onto Hybond-N nylon membranes (Amersham International PLC. U.K.) according to the instructions of the manufacturer. The detection was carried out with non-radioactive DNA probes. The probes, which were the cloned *bphC* genes from the current study, were labeled using a Dig DNA labeling and detection Kit (Boehringer Mannheim GmbH, Germany) according to the manufacturer's recommendation.

Deletion Mutation and Nucleotide Sequence Determination of Genes

The principles of the construction of deletion mutants were modifications of Henikoff's [10] and Yanisch-Perron's [39] methods. A series of deletion derivatives, whose nucleotide sequences had been determined by the Sanger dideoxy sequencing procedure [31] with a 373A DNA sequencer (Applied Biosystems, Foster City, CA, U.S.A.), was constructed using a kilosequence kit (Takara Shuzo Co., Otsu, Shiga, Japan).

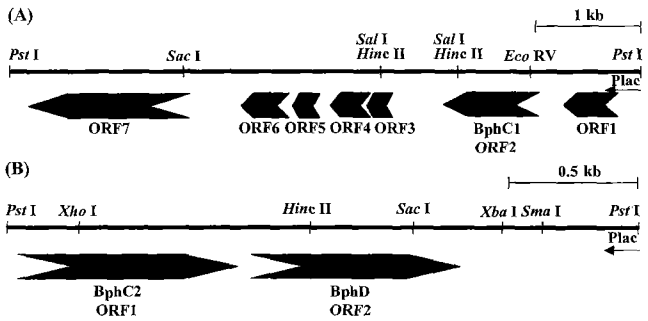

Fig. 1. Gene location in an approximately 6-kb *Pst*I fragment containing *bphC1* from pSY3 (A) and a 2.5-kb *Pst*I fragment containing *bphC2* and *bphD* from pSY4 (B).

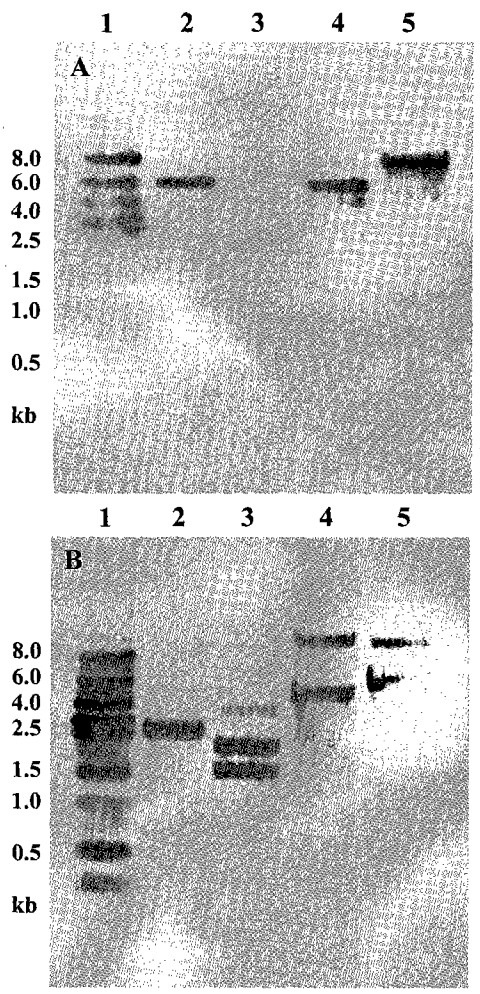

Fig. 2. Southern hybridization of *bphC* genes with total DNA from *Pseudomonas* sp. strain SY5.
(A) Lane 1, molecular weight marker (1 kb DNA Ladder); lanes 2, 3, 4, and 5, chromosomal DNA and pSY3 digested with *Pst*I, *Hin*cII, *Sac*I, and *Sma*I, respectively. The probe used was the approximately 6-kb *Pst*I fragment from pSY3 containing the *bphC1* gene. (B) Lane 1, molecular weight marker (1 kb DNA Ladder); lanes 2, 3, 4, and 5, chromosomal DNA and pSY4 digested with *Pst*I, *Hin*cII, *Sac*I, and *Sma*I, respectively. The probe used was the 2.5 kb *Pst*I fragment from pSY4 containing the *bphC2* gene.

A sequence analysis and homology search relative to other cloned nucleotide and amino acid sequences currently in the GeneBank were performed using the BCM search launcher and NCBI's sequence similarity search tool on the web pages http://dot.imgen.bcm.tmc.edu:9331/ and http://www.ncbi.nlm.nih.gov/BLAST/, respectively.

Phylogenetic Analysis

The alignments of the current BphC1, BphC2, and BphD sequences with other BphC and BphD sequences, respectively, were performed using the CLUSTAL W program [36] on the web page http://www.ddbj.nig.ac.jp, and phylogenetic tree analyses were performed with the TREEVIEW program package [26] using the PHYLIP style tree files produced by the CLUSTAL W program.

Nucleotide Sequence Accession Number

The nucleotide sequences of the *bphC1*, *bphC2*, and *bphD* genes of *Pseudomonas* sp. strain SY5 were assigned nucleotide database accession numbers KS102652, KS102653, and KS102654 for GENENURI, and AF190705, AF190706, and AF190707 for the GeneBank, respectively.

Results and Discussion

Isolation and Cloning of *bph* Genes

The recombinant strain *E. coli* DH5α containing the clone of the *Pseudomonas* sp. strain SY5 chromosomal DNA was screened for the cloned *bph* genes. Those transformants possessing the *bph* genes were selected by treatment with an acetone solution containing 2,3-DHBP. Two positive

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atacgccaagtacaaaaacgaggagatcgtgcatggatatccgtggcctgggttacgtca 1020
                    ORF2 BphC1→  M  D  I  R  G  L  G  Y  V  T
ccgtacgttccagcgacctggcgcaatggcgtcactacgccagccaagtgctgggcatga 1080
  V  R  S  S  D  L  A  Q  W  R  H  Y  A  S  Q  V  L  G  M  M
tggtggtcgaggacgagagcggcgagcgtctgttcctgaagatggacgagcgcccctatc 1140
  V  V  E  D  E  S  G  E  R  L  F  L  K  M  D  E  R  P  Y  R
gcatcctggtgcagcacagcgcccaggatggtttcggcgcctgtggctggaaagtggccg 1200
  I  L  V  Q  H  S  A  Q  D  G  F  G  A  C  G  W  K  V  A  G
gccaggccgccttcgaccaggccgtggccgagctgcacgccgccggcgtggcggtggagc 1260
  Q  A  A  F  D  Q  A  V  A  E  L  H  A  A  G  V  A  V  E  Q
agggcagcgctgagcaagccgcgctgcgccaggtccaggcgctggcgttgttcgccgacc 1320
  G  S  A  E  Q  A  A  L  R  Q  V  Q  A  L  A  L  F  A  D  P
ccgatggcaatcgccacgagctgtactggggcccgcgccaggacttcgcccgcttcgtct 1380
  D  G  N  R  H  E  L  Y  W  G  P  R  Q  D  F  A  R  F  V  S
ccccggtgggcgtgcgcggtttcgtcagcgacgggctgggcatgggccatgtggtcctgc 1440
  P  V  G  V  R  G  F  V  S  D  G  L  G  M  G  H  V  V  L  P
cggcaccgaccttcgatcgctgccgcgatttctatgagcaggtgatggctttggcctgt 1500
  A  P  T  F  D  R  C  R  D  F  Y  E  Q  V  M  G  F  G  L  S
cggacctgatgaaagtgcgcttcaccccggaccctgccgagccggaaaaacgtatccact 1560
  D  L  M  K  V  R  F  T  P  D  P  A  E  P  E  K  R  I  H  F
tcatgcactgcaacaacggccgtcaccactcgctggcgatcttcgaatgcccggtgccca 1620
  M  H  C  N  N  G  R  H  H  S  L  A  I  F  E  C  P  V  P  S
gcggctgcgtgcacatgatggtcgaggtcgcgggcctggaggacgtgggtcgcgccctgg 1680
  G  C  V  H  M  M  V  E  V  A  G  L  E  D  V  G  R  A  L  D
accgtatgcacgccaacggcgtgaagctctccgcgaccctgggccagcacaccaacgacc 1740
  R  M  H  A  N  G  V  K  L  S  A  T  L  G  Q  H  T  N  D  Q
agatgatctccttctacatgaagaccccgtccggtttcgacctggagtacggctgcgacg 1800
  M  I  S  F  Y  M  K  T  P  S  G  F  D  L  E  Y  G  C  D  G
gcctggtggtcgactggagccgccacaccccgttcgaaagcacggtggtcagccagtggg 1860
  L  V  V  D  W  S  R  H  T  P  F  E  S  T  V  V  S  Q  W  G
gccacgacttcagcgtcggccgccaatgaggacaacacccatggataaacagatgaccac 1920
  H  D  F  S  V  G  R  Q  *
```

Fig. 3. Nucleotide and deduced amino acid sequences of the *bphC1* gene of *Pseudomonas* sp. SY5 cloned in pSY3.
Stop codons are indicated by asterisks.

colonies from 2,000 transformants per plate expressed a yellow color, indicating that they possesed the *bphC* gene of *Pseudomonas* sp. strain SY5. These two positive clones were designated as pSY3 and pSY4, respectively.

Analysis of Recombinant Plasmids

The inserted DNA fragments of pSY3 and pSY4 were characterized by digesting with restriction enzymes belonging to the multiple cloning site of pUC119. A detailed physical mapping of pSY3 and pSY4, and their relation with the *bph* genes are shown in Fig. 1. pSY3 and pSY4 had a 6.0 kb and 2.5 kb *Pst*I fragment of DNA in pUC119, respectively.

pSY3 and pSY4 were used as probes to determine whether the plasmids indeed originated from strain SY5. The probes were hybridized with the total DNA of strain SY5. As a result of a Southern hybridization, pSY3 and pSY4 were indeed identified as cloned plasmids from strain SY5 (Fig. 2). However, pSY3 as probes did not hybridize with pSY4 (data now shown), thus suggesting that pSY3 and pSY4 are different plasmids that possess significantly different *bph* genes.

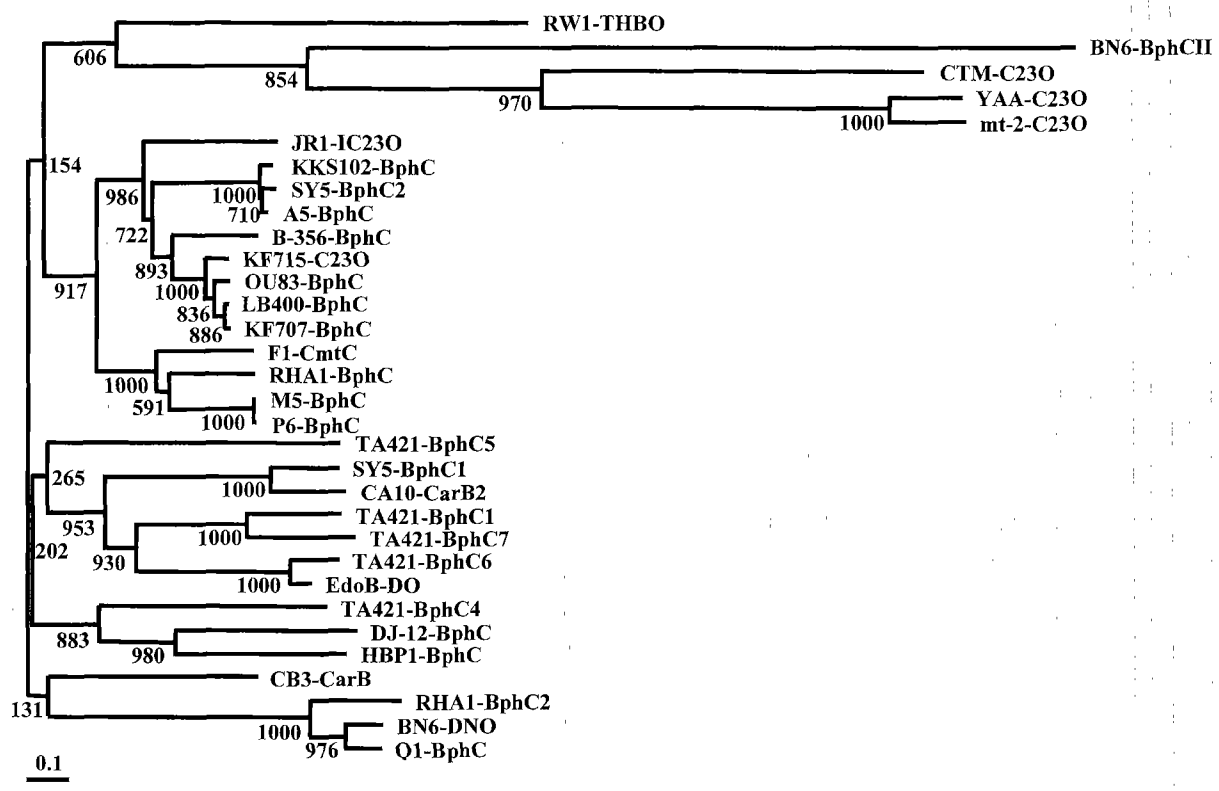

Fig. 4. Phylogenetic tree produced by comparison of amino acid sequences from different extradiol-type dioxygenases.

The tree analysis was performed with the TREEVIEW program package [26] using the PHYLIP style tree files produced by the CLUSTAL W program [36]. Bootstrap values from 1,000 resampling trials are shown under the branches. The scale bar indicates 0.1 substitution per site. The amino acid sequences from the different extradiol-type dioxygenases are as follows: SY5-BphC1 and SY5-BphC2, 2,3-dihydroxybiphenyl 1,2-dioxygenase (BphC) of *Pseudomonas* sp. in this study; KKS102-BphC, BphC of *Pseudomonas* sp. KKS102 (accession number P17297); DJ-12-BphC, BphC of *Pseudomonas* sp. DJ-12 (accession number D44550); CA10-CarB2, carbazole dioxygenase of *Pseudomonas* sp. (accession number D89065); OU83-BphC, BphC of *Pseudomonas putida* OU83 (accession number X91876); LB400-BphC, BphC of *Pseudomonas* sp. LB400 (accession number P47228); KF707-BphC, BphC of *Pseudomonas pseudoalcaligenes* KF707 (accession number P08695); JR1-IC23O, 3-isopropylcatechol 2,3-dioxygenase of *Pseudomonas* sp. JR1 (accession number U53507); KF715-C23O, catechol 2,3-dioxygenase of *Pseudomonas putida* KF715 (MEDLINE accession 90130279); mt-2-C23O, catechol of 2,3-dioxygenase of *Pseudomonas putida* mt-2 (accession number P06622); F1-CmtC, 3-methylcatechol 2,3-dioxygenase of *Pseudomonas putida* F1 (accession number P13453); HBP1-BphC, BphC of *Pseudomonas azelaica* HBP1 (accession number U73900); RW1-THBO, 2,2',3-trihydroxybiphenyl dioxygenase of *Sphingomonas* sp. RW1 (accession number X72850); BN6-BphCII, BphC of *Sphingomonas* sp. BN6 (accession number U38978); CB3-CarB, carbazole dioxygenase of *Sphingomonas* sp. CB3 (accession number AF060489); BN6-DNO, 1,2-dihydroxynaphthalene dioxygenase of *Sphingomonas* sp. BN6 (accession number U65001); Q1-BphC, BphC of *Sphingomonas paucimobilis* Q1 (accession number P11122); CTM-C23O, catechol 2,3-dioxygenase of *Rhodococcus rhodochrous* CTM (MEDLINE accession 94236236); RHA1-bphC, BphC of *Rhodococcus* sp. RHA1 (accession number D32142); M5-BphC, BphC of *Rhodococcus* sp. M5 (accession number U27591); P6-BphC, BphC of *Rhodococcus globerulus* P6 (accession number P47231); TA421-BphC1, TA421-BphC4, TA421-BphC5, TA421-BphC6, and TA421-BphC7, represent BphC1, BphC4, BphC5, BphC6, and BphC7 of *Rhodococcus erythropolis* TA421 (accession number D88013, D88016, D88017, D88018, and D88019, respectively); edoB-DO, extradiol dioxygenase of *Rhodococcus rhodochrous* (accession number AJ003244); RHA1-BphC2, bphC2 of *Rhodococcus* sp. RHA1 (accession number D76438); YAA-C23O, catechol 2,3-dioxygenase of *Acinetobacter* sp. YAA (accession number AB008831); A5-BphC, BphC of *Ralstonia eutropha* A5 (accession number X97984); B-356-BphC, BphC of *Commamonas testosteroni* B-356 (accession number U91936).

Determination of Nucleotide and Deduced Amino Acid Sequences of the Cloned Genes

The nucleotide sequence of the approximately 6 kb DNA fragment contained in pSY3, the larger of the two plasmids, was determined as shown in Fig. 3. The nucleotide and deduced amino acid sequences of ORF2 showed a sequence similarity to a known BphC enzyme encoding 2,3-dihydroxybiphenyl 1,2-dioxygenase, however, the other ORFs did not match with the Bph enzymes. The deletion mutation containing ORF2 exhibited BphC enzyme activity, which was detected by an acetonic spray assay for biphenyl metabolism [23]. Thus, ORF2 was designated as *bphC1*, and was composed of 897 bp (299 amino acids) with an ATG start and TGA stop codon. The G+C content of the *bphC1* gene was 66.3%.

A phylogenetic tree analysis of the extradiol dioxygenases in this study and from other previous reports was carried out (Fig. 4). The deduced amino acid sequence of *bphC1* from strain SY5 exhibited a 74% and 45% identity with those of the extradiol dioxygenase from *Pseudomonas* sp. CA10 [32] and *Rhodococcus rhodochrous* [16], respectively. In the cases of *bphC6* from *Rhodococcus erythropolis* TA421 [15], *bphC* from *Pseudomonas putida* OU83 [12], and *bphC1* from *R. erythropolis* TA421 [15], there was a sequence identity of 43, 42, and 42%, respectively.

The nucleotide sequence of the 2.5 kb DNA fragment, the smaller of the two plasmids contained in pSY4, was determined as shown in Fig. 5. The nucleotide and deduced amino acid sequences of ORF1 and ORF2 showed a sequence similarity to a known BphC enzyme encoding 2,3-dihydroxybiphenyl 1,2-dioxygenase and BphD enzyme encoding 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase, respectively. The ORF2 was located 36 bp downstream of the ORF1. The deletion mutation containing ORF1 exhibited BphC enzyme activity, which was detected by an acetonic spray assay for biphenyl metabolism [23]. ORF1 and ORF2 were designated as *bphC2* and *bphD*, respectively. The *bphC2* gene was composed of 882 bp (294 amino acids) and the *bphD* gene of 861 bp (287 amino acids), both with an ATG start and TGA stop codon. The G+C content of the *bphC2* and *bphD* genes was 64.1% and 60.3%, respectively.

As shown in Fig. 4, at the amino acid sequence level, BphC2 from strain SY5 exhibited a 95% and 94% identity with those of the 2,3-DHBD from *Alcaligenes eutrophus* A5 [21] and *Pseudomonas* sp. KKS102 [14], respectively. In the case of the *bphC* gene product of *Pseudomonas* sp. LB400 [11], *Pseudomonas putida* OU83 [12], and *Rhodococcus* sp. RHA1 [20], and *bphC4* of *Rhodococcus erythropolis* TA421 [15], BphC2 showed a sequence identity of 65, 64, 48, and 37%, respectively. As a result of the similarity analysis with other extradiol-type dioxygenases, *bphC2* exhibited a high identity with Gram-negative bacteria and low identity with Gram-positive bacteria.

```
cctgcagctttcataaccaaggagaagacatgagcatcgaacgtttgggctacctcggct   60
                ORF1  bphC2→  M  S  I  E  R  L  G  Y  L  G  F
tcgccgtccaggatgtacccgcctgggaccaatttctgaccaagagcgtgggtttgatgg   120
  A  V  Q  D  V  P  A  W  D  Q  F  L  T  K  S  V  G  L  M  A
cttcgggttcggctggcgacgcttcgctgtaccgggccgatcagcgtgcttggcgcatcg   180
  S  G  S  A  G  D  A  S  L  Y  R  A  D  Q  R  A  W  R  I  A
ccgtgcagccgggcgaactcgacgacctggcctacgcaggcttggaagtggatggcgccg   240
  V  Q  P  G  E  L  D  D  L  A  Y  A  G  L  E  V  D  G  A  A
ccgcgctcgagcgcatggccgacaagctgcgccaggcaggggtggccttcacccgcggtg   300
  A  L  E  R  M  A  D  K  L  R  Q  A  G  V  A  F  T  R  G  D
acgaagcgctcatgcagcatcgcaaagtcatgggcctgttgtgcctgcaagacccgtacg   360
  E  A  L  M  Q  H  R  K  V  M  G  L  L  C  L  Q  D  P  Y  G
gtctgtcgcttgagatttactacggcccggcagaaaccttcgaccagccctttcctgccca   420
  L  S  L  E  I  Y  Y  G  P  A  E  T  F  D  Q  P  F  L  P  S
gcgctcccgtgtcgggcttcgtcacgggcgaccagggcatcgggcatttcgtgcgctgcg   480
  A  P  V  S  G  F  V  T  G  D  Q  G  I  G  H  F  V  R  C  V
tcccgacaccgccaaggcgatggagttctacaccgaagtgctgggctttgtgctgtccg   540
  P  D  T  A  K  A  M  E  F  Y  T  E  V  L  G  F  V  L  S  D
acatcatcgacatccagatggggccggaaatgagcgtgcccgcgcacttcctgcactgca   600
  I  I  D  I  Q  M  G  P  E  M  S  V  P  A  H  F  L  H  C  N
atgggcgccaccacacgatcgccctggctgccttccaatcccaagcgcatccaccatt   660
  G  R  H  H  T  I  A  L  A  A  F  P  I  P  K  R  I  H  H  F
tcatgctgcaagccaacaccatcgatgacgtgggctatgccttcgatcggctggacgcgg   720
  M  L  Q  A  N  T  I  D  D  V  G  Y  A  F  D  R  L  D  A  A
ccgggcgcatcacttccctgctcgggcgtcacaccaacgaccatacgatctccttctatg   780
  G  R  I  T  S  L  L  G  R  H  T  N  D  H  T  I  S  F  Y  A
ccgacacgccgtcgcccatgatcgaagtcgagttcggctggggaccgcgcacggtggatt   840
  D  T  P  S  P  M  I  E  V  E  F  G  W  G  P  R  T  V  D  S
cctcctggacggtggtgcgccacaaccgcaccgccatgtggggtcacaagtcggtacgcg   900
  S  W  T  V  V  R  H  N  R  T  A  M  W  G  H  K  S  V  R  G
gccagcgctgagccgcgtcattcatctagttttttcaggaaattcccatgtcagaactca   960
  Q  R  *                        ORF2  bphD→  M  S  E  L  N
acgaaagcacgacgagcaaattcgtcaccatcaacgaaaaaggcctctccaacttccgca   1020
  E  S  T  T  S  K  F  V  T  I  N  E  K  G  L  S  N  F  R  I
ttcacctcaacgatgcaggcgagggcgaagcggtaatcatgctgcacggaggcggaccgg   1080
  H  L  N  D  A  G  E  G  E  A  V  I  M  L  H  G  G  G  P  G
gagcgggggggtggagcaactactaccgcaacatcggccccttcgtcaaggccggttacc   1140
  A  G  G  W  S  N  Y  Y  R  N  I  G  P  F  V  K  A  G  Y  R
gcgtgatccttcaggacgcgccgggcttcaacaagtccgacaccgtcgtgatggacgagc   1200
  V  I  L  Q  D  A  P  G  F  N  K  S  D  T  V  V  M  D  E  Q
agcgcggcttagtcaacgcgcgctcggtcaaagggatgatggatgtactgggcatcgaaa   1260
  R  G  L  V  N  A  R  S  V  K  G  M  M  D  V  L  G  I  E  K
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  A  H  L  V  G  N  S  M  G  G  A  G  A  L  N  F  A  L  E  Y
acccggagcgcaccggcaagctcatcctcatggggccgggcggattgggcaacagcctgt   1380
  P  E  R  T  G  K  L  I  L  M  G  P  G  G  L  G  N  S  L  F
tcactgcgatgcccatggaaggaatcaagctgctgtttaagctctacgccgagccttcgc   1440
  T  A  M  P  M  E  G  I  K  L  L  F  K  L  Y  A  E  P  S  L
tcgacacgctcaagcagatgctcaacgtcttcctgttcgaccagagcctgatcaccgacg   1500
  D  T  L  K  Q  M  L  N  V  F  L  F  D  Q  S  L  I  T  D  E
aactggtgcaaggacgctgggccaacatccagcgcaaccccgagcacctgaagaacttcc   1560
  L  V  Q  G  R  W  A  N  I  Q  R  N  P  E  H  L  K  N  F  L
tcttgagctcgcaaaagttgccactttcgtcctgggacgtgtcgccgcgcatgggcgaga   1620
  L  S  S  Q  K  L  P  L  S  S  W  D  V  S  P  R  M  G  E  I
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  K  A  K  T  L  V  T  W  G  R  D  D  R  F  V  P  L  D  H  G
gcctcaagctggtcgcgaacatgccggatgcgcagttgcacgtcttcccgcgctgtggtc   1740
  L  K  L  V  A  N  M  P  D  A  Q  L  H  V  F  P  R  C  G  H
actgggcgcaatgggagcatgcggacgccttcaatcggctgacgctggacttcctagcca   1800
  W  A  Q  W  E  H  A  D  A  F  N  R  L  T  L  D  F  L  A  N
acggctgagccccccatttccttttttccgcacggaagcaaatctgttggccgctttcccg   1860
  G  *
```

Fig. 5. Nucleotide and deduced amino acid sequences of the *bphC2* and *bphD* genes of *Pseudomonas* sp. strain SY5 cloned in pSY4.
Stop codons are indicated by asterisks.

A phylogenetic tree analysis of the hydrolases obtained in this study and from other previous reports was carried out (Fig. 6). A comparison of the deduced amino acid sequence of *bphD* of *Pseudomonas* sp. SY5 with those of *Alcaligenes eutrophus* A5 [21], *Pseudomonas* sp. KKS102 [14], *Pseudomonas* sp. LB400 [11], *Pseudomonas putida* KF715 [9], *Pseudomonas* sp. DJ-12 [13], and *Rhodococcus erythropolis* TA421 [15] showed a sequence identity of 92, 92, 79, 78, 52, and 37%, respectively. In the case of *etbD1* of *Rhodococcus* sp. RHA1 [38], a sequence identity of 36% was found. The *bphD* product of *Pseudomonas* sp. SY5 showed a high sequence identity with the Gram-negative Pseudomonad.

According to an alignment of the deduced amino acid sequences of the *bphC*s of *Pseudomonas* sp. strain SY5 with those of other extradiol-type dioxygenases, 47

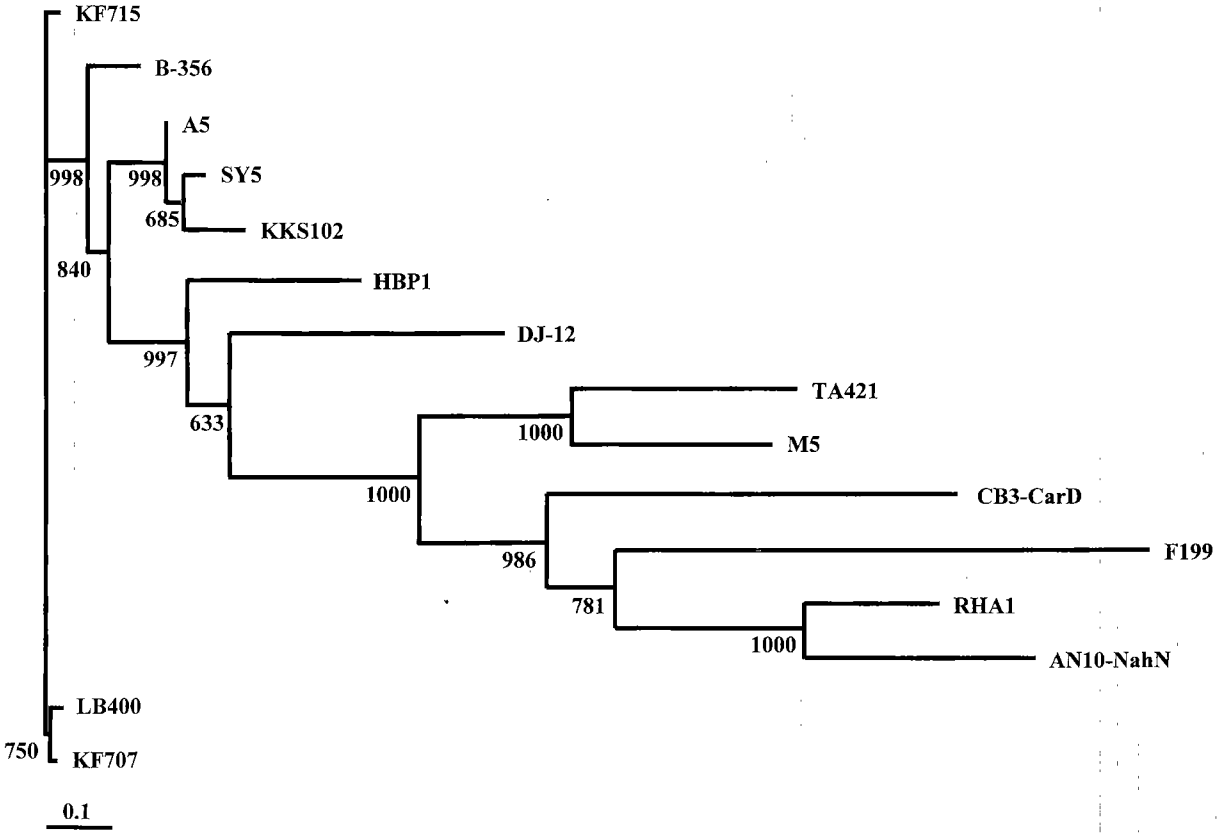

Fig. 6. Phylogenetic tree produced by comparison of amino acid sequences from the different hydrolases.
The tree analysis was performed with the TREEVIEW program package [26] using the PHYLIP style tree files produced by the CLUSTAL W program [36]. Bootstrap values from 1,000 resampling trials are shown under the branches. The scale bar indicates 0.1 substitution per site. The amino acid sequences from the different hydrolases are as follows: SY5, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid hydrolase (BphD) of *Pseudomonas* sp. in this study; KKS102, *Pseudomonas* sp. KKS102 (accession number P17548); DJ-12, *Pseudomonas* sp. DJ-12 (accession number D44550); LB400, *Pseudomonas* sp. LB400 (accession number P47229); KF707, *Pseudomonas pseudoalcaligenes* KF707 (accession number D85851.1); KF715, *Pseudomonas putida* KF715 (MEDLINE accession 90130279); HBP1, *Pseudomonas azelaica* HBP1 (accession number U73900); CB3-CarD, carbazole degradation hydrolase of *Sphingomonas* sp. CB3 (accession number AF060489.1); RHA1, *Rhodococcus* sp. RHA1 (accession number AB004320); M5, *Rhodococcus* sp. M5 (accession number U44891.1); TA421, *Rhodococcus erythropolis* TA421 (accession number D88016); A5, *Ralstonia eutropha* A5 (accession number X97984); B-356, *Commamonas testosteroni* B-356 (accession number L34338.1); F199, *Sphingomonas aromaticivorans* F199 (accession number AF079317); AN10-NahN, hydroxymuconic semialdehyde hydrolase of *Pseudomonas stutzeri* AN10 (accession number AF039534.1).

amino acids marked by asterisks were evolutionarily conserved (Fig. 7). When *bphD* was aligned with other hydrolases, 64 amino acids were found to be identical (Fig. 8).

All the aligned extradiol-type dioxygenases, including the BphCs from this study, had amino acid residues related to the binding of ferrous ion. Furthermore, they also had substrate binding residues related with the formation of hydrogen bonds with iron-ligating amino acids. Five ligands, one for Glu, two for His, and two for water molecules, coordinated with ferrous ion, were previously identified in the crystal structure of 2,3-DHBD [6, 22, 33]. BphC1 of *Pseudomonas* sp. strain SY5 had two His and one Glu well-conserved residues corresponding to His^{146}, His^{214}, and Glu^{265}, respectively. Also, two His residues corresponding to His^{146} and His^{210} and one Glu residue corresponding to Glu^{261} were well conserved in BphC2.

The substrates dihydroxylated ring can be tucked into a pocket defined by ferrous ion and the side chains of several residues, one Asn, one Asp, two His, one Phe, one Met, and one Tyr in the crystal structure, as shown in previous reports [6, 22]. BphC1 was found to have well-conserved Asn^{248}, Asp^{249}, His^{199}, His^{246}, Phe^{190}, Met^{251}, and Tyr^{255} residues. Asn^{243}, Asp^{244}, His^{195}, His^{241}, Phe^{187}, and Tyr^{250} residues were well conserved in BphC2, yet the Met residue was substituted with Thr^{246} in the BphC2 of strain SY5 and BphC of strains A5 and KKS102 (Fig. 7).

According to the comparison of *bphC1* and *bphC2* from strain SY5, they were found to be significantly different, exhibiting a 50% and 39% homology at the nucleotide and amino acid sequence level, respectively. Also, as a result of a Southern hybridization, *bphC1* did not hybridized with *bphC2*.

The *bph* genes of Gram-negative bacteria forming an operon are located adjacent to each other. As such, the *bph*

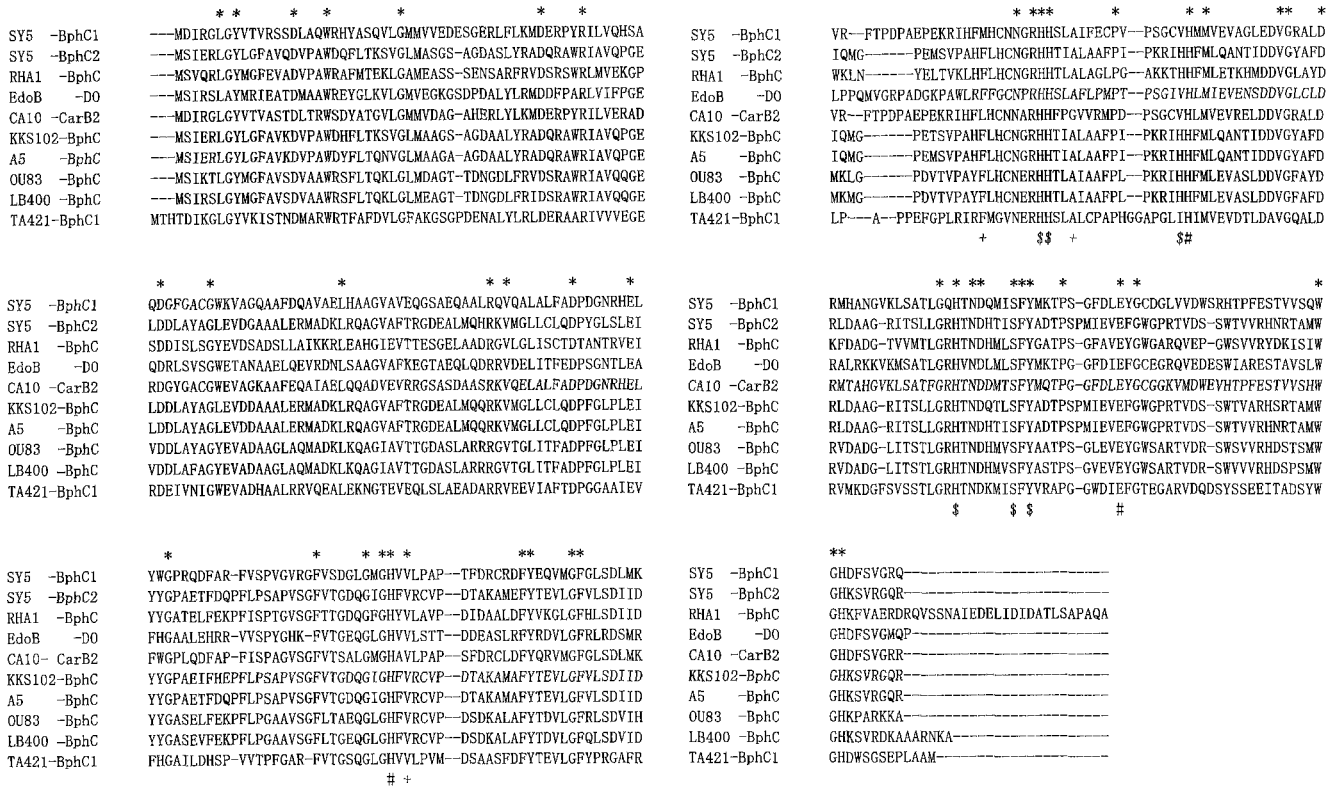

Fig. 7. Comparison of amino acid sequences of extradiol-type dioxygenases.
SY5 [24], CA10 [32], KKS102 [14], and LB400 [11]; *Pseudomonas* sp., OU83 [12]; *Pseudomonas putida*, A5 [21]; *Alcaligenes eutrophus*, TA421 [15]; *Rhodococcus erythropolis*, RHA1 [20]; *Rhodococcus* sp., EdoB [16]; *Rhodococcus rhodochrous*. The amino acid residues indicated by asterisks have identical positions in all the aligned sequences. The ferrous ion binds the amino acid residues indicated by #. The substrate-binding site is marked with +. Hydrogen bonds with ligands are marked as $.

genes in this study would appear to be an original *bph* operon of a Gram-negative bacteria, because *bphC2* and *bphD* from pSY4 are located close to each other. Accordingly, the *bphA1 A2 A3 A4*, *bphB* genes have a high possibility of being located either in front of or behind the *bphC2 and bphD* genes.

In contrast, the ORFs in front of and behind the *bphC1* gene from pSY3 did not match with the other *bph* genes from the GeneBank search. The homology research showed that the *bphC2* gene had high identities with those of other Gram-negative bacteria, whereas the *bphC1* gene had low identities. Conversely, the *bphCl* gene exhibited higher identities with those of other Gram-positive bacteria than the *bphC2* gene. Also, the *bphC1* gene showed a low homology and did not hybridize with the *bphC2* gene. Therefore, it would appear that the *bphC1* gene may be an external gene derived from a gene transfer.

The Gram-negative bacteria *Pseudomonas* sp. CA10 [32] and *Sphingomonas* sp. BN6 [29] have been reported to have two genes coding the *meta*-cleavage dioxygenases of PCB-degrading bacteria. In addition, two 2,3-dihydroxybiphenyl 1,2-dioxygenases (*bphCs*) that play a significant role in degrading PCBs from the Gram-negative bacterium *Pseudomonas* sp. SY5 were characterized in the current study. This is significant for PCB degradation as both Gram-positive and Gram-negative bacteria can universally harbor multiple genes encoding 2,3-dihydroxybiphenyl 1,2-dioxygenases.

Examples of multiple gene-coding systems for degrading recalcitrant compounds in Gram-positive bacteria have been previously reported [1, 8, 15], and many plasmids and transposons harboring degradation genes of aromatic compounds have also been reported [15, 18, 37]. Springael *et al.* [34] reported the transfer and transposition of these catabolic genes on the basis that plasmid can be transferred via transformation or conjugation. Thus, it is likely that multiple gene-coding systems may be developed for catabolic pathway evolution for the degrading xenobiotic compounds in the stressed environment via horizontal gene transfer and catabolic gene assembly [15].

Municipal sewage contains organic compounds that are either readily or laboriously degraded by microorganisms, thus there is a high possibility of the presence of microbial populations that can degrade the recalcitrant organic compounds in sewage. Strain SY5 was isolated from municipal

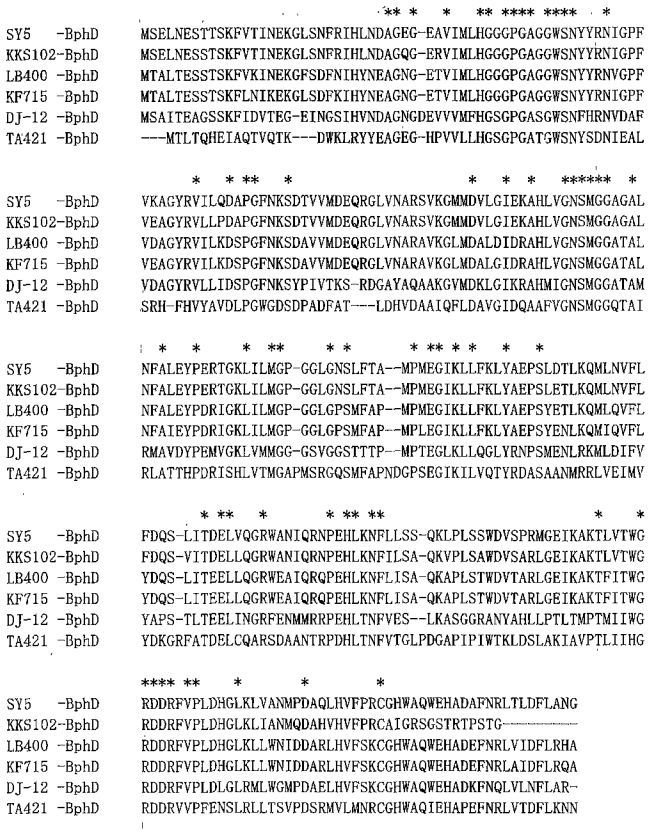

Fig. 8. Comparison of amino acid sequences of hydrolases.
SY5 [24], KKS102 [14], LB400 [11], and DJ12 [13]; *Alcaligenes eutrophus*, KF715 [9]; *Pseudomonas putida,* TA421 [15]; *Rhodococcus erythropolis*. The amino acids residues indicated by asterisks have identical positions in all the aligned sequences.

sewage containing recalcitrant organic compounds and exhibits a high degradability of various aromatic compounds [23]. Also, the current study established that strain SY5 had two extradiol-type dioxygenases. These dioxygenases did not hybridize with each other as they had a low similarity, yet a similar structure of evolutionarily conserved amino acid residues for catalytic activity was found between BphC1 and BphC2. These results suggested that there was a very high possibility that strain SY5 might either have accepted an external gene via gene transfer or modulated itself for survival in the stressed environment after adaptation, similar to the gene evolution involved in the catabolic pathway for recalcitrant compounds in Gram-positive bacteria.

Based on results of this study, our future study will focus in confirming differences of substrate specificity and functional activity between purified BphC enzymes. Also, we will verify whether gene transfer and/or gene modulation of the *bphC1* gene can occur and analyze the change of specific functions in interaction of catabolic genes with various substrates, since strain SY5 could degrade various aromatic compounds [23].

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