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Functional Analyses of a Variety of Chimeric Dioxygenases Constructed from Two Biphenyl Dioxygenases That Are Similar Structurally but Different Functionally

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The biphenyl dioxygenases (BP Dox) of strains *Pseudomonas pseudoalcaligenes* KF707 and *Pseudomonas cepacia* LB400 exhibit a distinct difference in substrate ranges of polychlorinated biphenyls (PCB) despite nearly identical amino acid sequences. The range of congeners oxidized by LB400 BP Dox is much wider than that oxidized by KF707 BP Dox. The PCB degradation abilities of these BP Dox were highly dependent on the recognition of the chlorinated rings and the sites of oxygen activation. The KF707 BP Dox recognized primarily the 4′-chlorinated ring (97%) of 2,5,4′-trichlorobiphenyl and introduced molecular oxygen at the 2′,3′ position. The LB400 BP Dox recognized primarily the 2,5-dichlorinated ring (95%) of the same compound and introduced O_2 at the 3,4 position. It was confirmed that the BphA1 subunit (iron-sulfur protein of terminal dioxygenase encoded by *bphA1*) plays a crucial role in determining the substrate selectivity. We constructed a variety of chimeric *bphA1* genes by exchanging four common restriction fragments between the KF707 *bphA1* and the LB400 *bphA1*. Observation of *Escherichia coli* cells expressing various chimeric BP Dox revealed that a relatively small number of amino acids in the carboxy-terminal half (among 20 different amino acids in total) are involved in the recognition of the chlorinated ring and the sites of dioxygenation and thereby are responsible for the degradation of PCB. The site-directed mutagenesis of Thr-376 (KF707) to Asn-376 (LB400) in KF707 BP Dox resulted in the expansion of the range of biodegradable PCB congeners.

Recent studies revealed that oxygenases involved in the initial oxidation of aromatic hydrocarbons are multicomponent enzymes and that their corresponding subunits show various degrees of homology (14, 26, 27, 31). This implies that soil bacteria have adaptively evolved, by modifying key enzymes, to utilize a variety of aromatic compounds which are present in the environment. Biphenyl-utilizing bacteria are ubiquitously distributed and can be isolated from various environmental samples (3, 8, 29) including intestine of termite (5), suggesting that they are involved in the degradation of plant lignin at the final stage, together with other aromatic degraders (24). Biphenyl-utilizing bacteria have been extensively studied with respect to the degradation of polychlorinated biphenyls (PCB), which are known to be serious environmental pollutants. These studies revealed considerable differences in the congener selectivity patterns and in the range of activity of various PCB-degrading bacteria (9). It was also demonstrated that both the relative rates of primary degradation of PCB and the choice of the ring attacked were dependent on the bacterial strains (1, 2, 4, 16). *Pseudomonas pseudoalcaligenes* KF707 was isolated from soil near a biphenyl-producing factory in Kitakyushu, Japan (12, 15). This strain showed a narrow range of degradable PCB congeners (7, 17). *Pseudomonas* sp. strain LB400 (referred to as *Pseudomonas cepacia* LB400 in a recent publication [19]) was isolated from a PCB-contaminated site in New York State (3, 4). It was shown that LB400 metabolized PCB via both 2,3-dioxygenation and 3,4-dioxygenation, depending on the chlorine substitution of PCB, and therefore that LB400 had a much greater degradation range of PCB congeners than did KF707. Of 19 PCB congeners tested, 17 were degraded by LB400 while only 8 were attacked by KF707 (7, 17). It was noted, however, that KF707 was superior to LB400 with respect to the degradation of 4,4′-chlorobiphenyl (CB) (double *para*-substituted congener), but the same strain was unable to degrade 2,5,2′,5′-CB (*ortho-meta*-substituted congener). LB400, on the other hand, degraded 2,5,2′,5′-CB quickly but degraded 4,4′-CB poorly. It is of particular interest to note that the biphenyl catabolic *bph* operons of these two strains are nearly identical in terms of gene organization and nucleotide sequences (6, 22, 23, 30). Biphenyl dioxygenases (BP Dox) are multicomponent enzymes comprising four subunits: a large subunit of terminal dioxygenase (encoded by *bphA1*), a small subunit of terminal dioxygenase (encoded by *bphA2*), ferredoxin (encoded by *bphA3*), and ferredoxin reductase (encoded by *bphA4*) (23). (The naming of some *bph* genes for LB400 differs from that for KF707 [Fig. 1], but we used here the KF707 nomenclature for the corresponding LB400 *bph* genes to avoid confusion.) The identities of these components between KF707 and LB400 are as follows: BphA1, 95.6%; BphA2, 99.5%; BphA3, 100%, and BphA4 100% (Fig. 1). Other enzymes such as dehydrogenase (BphB), ring *meta*-cleavage dioxygenase (BphC), and hydrolase (BphD) are also nearly identical. Haddock et al. (18) showed that purified LB400 BP Dox had a remarkable ability to oxidize PCB congeners that contained up to four chlorine substituents by introducing two hydroxy groups at either the 2,3 or 3,4 position and that dechlorination of *ortho*-substituted congeners such as 2,2′-CB, 2,3′-CB, and 2,5,2′-CB was taking place. Erickson and Mondello (7) showed that the exchange of four amino acids of LB400 BphA1 for the corresponding amino acids of KF707 BphA1 enhanced the capability to degrade PCB. Since LB400 BphA1 differs from KF707 BphA1 at 20 positions, including 19 amino acid substitutions and one glycine deletion in KF707 BphA1 (Fig. 2), we constructed a variety of chimeric BphA1s to investigate how such amino acid differences in these BphA1s

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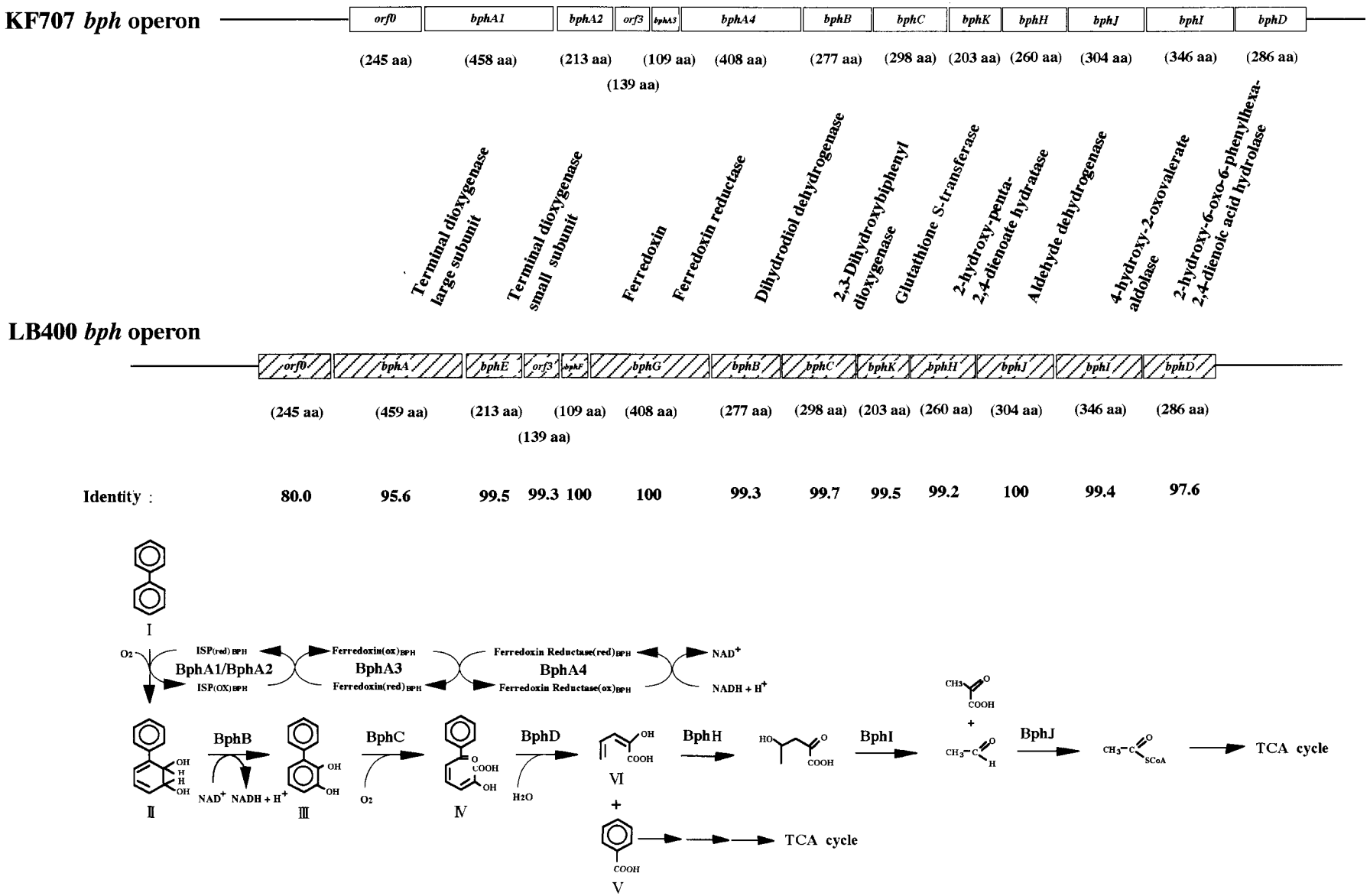

FIG. 1. Biphenyl catabolic pathway for *P. pseudoalcaligenes* KF707 and *P. cepacia* LB400 and organization of their *bph* operons. Compounds: I, biphenyl; II, 2,3-dihydroxy-4-phenylhexa-4,6-diene (dihydrodiol compound); III, 2,3-dihydroxybiphenyl; IV, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (*meta*-cleavage compound); V, benzoic acid; VI, 2-hydroxy-penta-2,4-dienoate. Enzymes: BphA1-BphA2-BphA3-BphA4, biphenyl dioxygenase; BphB, dihydrodiol dehydrogenase; BphC, 2,3-dihydroxybiphenyl dioxygenase; BphK, glutathione *S*-transferase; BphH, 2-hydroxy-penta-2,4-dienoate hydratase; BphJ, acetaldehyde dehydrogenase (acylating); BphI, 4-hydroxy-2-oxovalerate aldolase; BphD, *meta*-cleavage compound hydrolase. Identities of the amino acids of the corresponding proteins of KF707 and LB400 are presented as percent values in the middle of the figure.

FIG. 2. Comparison of amino acid sequences between KF707 BphA1 and LB400 BphA1. Asterisks indicate different amino acids and deletions. Arrows indicate the common restriction sites of *Sal*I, *Sca*I, and *Acc*III within the *bphA1* genes.

affect the initial oxygenation abilities of BP Dox for PCB congeners.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains and plasmids used in this study are listed in Table 1. The biphenyl-utilizing strain *P. pseudoalcaligenes* KF707 was isolated in Kitakyushu, Japan (15), and *P. cepacia* LB400 (3, 4) was isolated in New York State and was provided by the Research and Development Center of the General Electric Company. The strains were grown in a basal salts medium (15) supplemented with 0.2% biphenyl as a sole source of carbon and energy for PCB degradation. *E. coli* strains were grown in Luria-Bertani (LB) medium (32) or on LB agar medium (1.5% agar). Antibiotics were added when needed in order to select for the presence of a plasmid in the recombinant strains as follows: gentamicin, 20 μg/ml for *E. coli* strains and 30 μg/ml for *Pseudomonas* strains; and ampicillin, 50 μg/ml for *E. coli* strains.

Plasmids. pHKF11 was constructed by exchanging the *orf0* of KF707 with that of LB400; the gene was amplified by PCR as described below. pCKF11 was constructed from pKTF18 by removing the 1.3-kb *Ppu*MI fragment containing parts of the *bphB* and *bphC* of KF707. pCKF12 was constructed as follows. The 5.2-kb *Eco*RI-*Sac*I fragment containing *bphA2A3A4BC* (LB400) was cut from pGEM455 and inserted into pUC118 to construct pGEF30. The LB400 *bphA1* was amplified by PCR as described below and was inserted upstream of the *bphA2* of pGEF30, from which the *Ppu*MI fragment containing parts of *bphB* and *bphC* was removed to construct pCKF12. pCKF series plasmids (pCKF101 to -108) contain various chimeric *bphA1* genes constructed by replacing the common restriction fragments between the KF707 *bphA1* and the LB400 *bphA1* (see Fig. 5). pSKF series plasmids contain KF707 *bphA1* (pSKF11 to -15) or chimeric *bphA1* (pSKF101 to -108), in which certain nucleotides were mutagenized site specifically (see Table 2).

DNA sequencing and amplification of DNA. The DNA sequences including *orf0* (ca. 1.0 kilobase pairs [kb]) and the *bphX* region (ca. 3.5 kb) of KF707 were determined by the dideoxy termination method with a DNA sequencer (Applied Biosystems Ltd., model 373A). PCR was performed with a total volume of 50 μl which contained PCR buffer (Takara Shuzo, Co. Ltd. Kyoto, Japan), 50 ng of plasmid as a DNA template, 100 μM (each) deoxyribonucleotide triphosphate, 1 μM (each) oligoprimer, and 0.5 U of *Taq* DNA polymerase. pGEM453 was used as a DNA template for the amplification of *orf0* (LB400) and *bphA1* (LB400). Amplification of DNA was carried out for 25 cycles under the following conditions: denaturation, 93°C for 1 min; primer annealing, 55°C for 90 s; and primer extension, 72°C for 90 s.

The *bphA1* (LB400) gene was amplified by using the following oligoprimers: 5′-CCGAATTCAAGGAGACGTTGAATC**ATG**-3′ for the upstream sequence of *bphA1* (LB400; the *Eco*RI site is underlined and the start codon is in boldface) and 3′-TCTAGACAGTTGGCCTTCTTAAGTT-5′ for the downstream sequence (the *Eco*RI site is underlined). The *orf0* (LB400) was amplified by using the following oligoprimers: 5′-TTTGAATTCGAGCTC**ATG**AATGCGAGAACTCC-3′ for the upstream sequence of *orf0* (LB400; the *Eco*RI site is underlined, the *Sac*I site is underlined with a broken line, and the start codon is in boldface) and 3′-CCTCTGCAATTTAGGTACTCGAGCTTAAGAGA-5′ for the downstream sequence (the *Eco*RI site is underlined, and the *Sac*I site is underlined with a broken line). The amplified DNAs of *bphA1* (LB400) and *orf0* (LB400) were purified by SUPREC-02 (Takara Shuzo) and sequenced.

Site-directed mutagenesis. Site-directed mutagenesis was performed by employing a U.S.E. mutagenesis kit (Pharmacia Biosystems, Co., Ltd.) according to the manufacturer's instructions. The mutagenesis oligonucleotides were synthesized by a DNA synthesizer (model 392; Applied Biosystems, Inc.) and are presented in Table 2. Plasmid pCKF11 was used as a mutagenesis template for pSKF11 to -15, and plasmid pCKF102 was used as a mutagenesis template for pSKF101 to -108. Successful mutations were identified by DNA sequencing.

Native PAGE and SDS-PAGE. *E. coli* strains containing the chimeric *bphA1* genes were grown to late logarithmic phase and harvested by centrifugation at 12,000 × *g* for 10 min. The cells were washed with 10 mM Tris-HCl buffer (pH 6.8), resuspended in the same buffer, and then subjected to sonication (Tosho Electric. Ltd.; model UCD-130) to disrupt the cells. The cell debris was removed by centrifugation at 88,000 × *g* for 1 h. The cell extracts were mixed with the same volume of 10 mM Tris-HCl buffer (pH 6.8)–20% glycerol–0.02% bromophenol blue and subjected to polyacrylamide gel electrophoresis (PAGE; NPG520-E gel; 5 to 20% gradient; Atto Corp., Tokyo, Japan). Proteins were detected by staining with a 0.25% mixture of Coomassie brilliant blue R-250 (Sigma, St. Louis, Mo.) in methanol-water-acetic acid (5:2:1). For sodium dodecyl sulfate (SDS)-PAGE, cell extracts were treated with 2.5% SDS, heated for 10 min at 100°C, and subjected to PAGE with a 12.5% PAGE gel.

PCB degradation by resting cells and GC-MS analysis. The *Pseudomonas* cells and the recombinant *E. coli* cells were grown to logarithmic phase (turbidity of 0.8 to 1.2 at 600 nm), washed twice in 50 mM sodium phosphate buffer (pH 7.5), and resuspended in 5 ml of the same buffer to adjust the turbidity to 1.0 at 600 nm. PCB dissolved in ethanol was added at a concentration of 20 μg/ml. After being shaken at 200 rpm for 20 h at 30°C, the entire incubation mixture was transferred to a 100-ml separatory funnel and extracted with the same volume (5 ml) of ethylacetate. One milliliter of extract was evaporated to dryness with a gentle stream of nitrogen gas and was derivatized with 100 μg of *n*-butylboronic acid (Tokyo Kasei Co. Ltd., Tokyo, Japan) in 10 μl of acetone-dimethyl formamide (1:1) (25). The samples were analyzed by gas chromatography-mass spectrometry (GC-MS) (Shimadzu Co. Ltd.; model QP5000) with a coiled-capillary glass column (0.25-mm inner diameter, 30 m long) packed with methylsilicon DB1. Helium was used as a carrier gas at a flow rate of 50 ml/min. The column temperature on the GC was increased from 140 to 300°C at the rate of 15°C/min. The electron impact masses were measured at a 70-eV ionization potential. Relative percentages of products were calculated from integrated total ion peak areas.

Nucleotide sequence accession numbers. The sequences obtained in this study have been submitted to DDBJ, EMBL, and GenBank and have been assigned accession no. D85852 and D85853.

RESULTS AND DISCUSSION

Nucleotide sequences of *orf0* and *bphX* regions in *P. pseudoalcaligenes* KF707. We have previously sequenced *bphA1A2A3A4BC* and *bphD* in *P. pseudoalcaligenes* KF707 (10, 20, 30). A little later, *bph* sequences of *P. cepacia* LB400 were published by other workers (6, 22, 23). These sequences covered the region upstream of *bphA1* (*orf0*) and the 3.5-kb *bphX* region (termed the *bphK*, *bphH*, *bphJ*, and *bphI* region in LB400) located between *bphC* and *bphD*. Since we intended to analyze the difference in PCB degradation capability between KF707 and LB400 in this study, we sequenced the remaining regions, which include the *orf0* (accession number, D85852) and *bphX* (accession number, D85853) regions. We found one open reading frame (*orf0*) upstream of *bphA1* and four genes within the *bphX* region. The identity of amino acid sequences

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevent description	Source or reference
Strains		
P. pseudoalcaligenes KF707	Wild type, BP^+	15
P. cepacia LB400	Wild type, BP^+	3
E. coli JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac proAB*) [F′ *proAB lacI*^{q} *DM15 traD36*]	Takara Shuzo Co.
Plasmids		
pUC19	Ap^r	31
pUC118	Ap^r	31
pKTF18	*bphA1A2A3A4BC* (KF707) in pUC118	29
pGEM453	*orf0 bphA1* (LB400) in pUC118	6
pGEM455	*bphA2A3A4BCKHJID* in pUC118	6
pGEF30	*bphA2A3A4BC* in pUC118	This study
pHKF11	*orf0* (LB400) *bphA1A2A3A4* (KF707) in pUC118	This study
pCKF11	*bphA1A2A3A4* (KF707) in pUC118	This study
pCKF12	*bphA1A2A3A4* (LB400) in pUC118	This study
pCKF101	*bphA1* I (KF707)/II, III, IV (LB400)[a] *A2A3A4* in pUC118	This study
pCKF102	*bphA1* I, II (KF707)/III, IV (LB400) *A2A3A4* in pUC118	This study
pCKF103	*bphA1* I, II (KF707)/III (LB400)/IV (KF707) *A2A3A4* in pUC118	This study
pCKF104	*bphA1* I,II,III (KF707)/IV (LB400) *A2A3A4* in pUC118	This study
pCKF105	*bphA1* I,II,III (LB400)/IV (KF707) *A2A3A4* in pUC118	This study
pCKF106	*bphA1* I,II (LB400)/III, IV (KF707) *A2A3A4* in pUC118	This study
pCKF107	*bphA1* I (LB400)/II, III, IV (KF707) *A2A3A4* in pUC118	This study
pCKF108	*bphA1* I (KF707)/II (LB400)/III, IV (KF707) *A2A3A4* in pUC118	This study
pCKF201	*bphA2A3A4* (KF707) in pUC118	This study
pSKF11	pCKF11 (Met 283 Ser)[b]	This study
pSKF12	pCKF11 (Ser 324 Thr)	This study
pSKF13	pCKF11 (Val 325 Ile)	This study
pSKF14	pCKF11 (Thr 340 Ile)	This study
pSKF15	pCKF11 (Thr 376 Asn)	This study
pSKF101	pCKF102 (Glu 303 Asp)	This study
pSKF102	pCKF102 (Gly 313 deletion)	This study
pSKF103	pCKF102 (Val 320 Phe)	This study
pSKF104	pCKF102 (Thr 325 Ser)	This study
pSKF105	pCKF102 (Thr 335 Ala)	This study
pSKF106	pCKF102 (Phe 336 Ile)	This study
pSKF107	pCKF102 (Asn 338 Thr)	This study
pSKF108	pCKF102 (Thr 325 Ser, Ile 326 Val)	This study

[a] *bphA1* I (KF707)/II, III, IV (LB400), fragment I of the *bphA1* gene of strain KF707 plus fragments II, III, and IV of the *bphA1* gene of strain LB400.
[b] Met 283 Ser, substitution of Ser for Met at position 283.

of the Orf0 proteins of KF707 and LB400 was 80%, which was less than those for other proteins of the two strains (Fig. 1). The four proteins whose genes are within the *bphX* region were confirmed to be glutathione *S*-transferase (encoded by *bphK*), 2-hydroxy-penta-2,4-dienoate hydratase (encoded by *bphH*), acetaldehyde dehydrogenase (acylating) (encoded by *bphJ*), and 4-hydroxy-2-oxovalerate aldolase (encoded by *bphI*). Although the function of glutathione *S*-transferase in the metabolism of biphenyl and PCB has not been clarified yet, the other three enzymes are involved in the conversion of 2-hydroxy-penta-2,4-dienoate to acetyl coenzyme A (data not shown). The amino acid sequences of these four proteins were nearly identical (more than 99%) with those of the corresponding proteins of LB400 (22) (Fig. 1).

Metabolism of 2,5,4′-CB by KF707 and LB400. It has been shown that *P. pseudoalcaligenes* KF707 oxidizes a narrow range of PCB congeners and that, in contrast, *P. cepacia* LB400 oxidizes a much wider range of PCB congeners, which include penta- and hexachlorobiphenyls (7, 17). Since there was a significant difference in the degradation of 4,4′-CB and 2,5,2′,5′-CB between these two strains (7, 17), we carefully investigated the metabolism of 2,5,4′-CB, which possesses both 2,5- and 4′-chlorinated rings, by using resting cells. A large amount of a *meta*-cleavage yellow compound (3-chloro-2-hydroxy-6-oxo-6-[2′,5′-dichlorophenyl]hexa-2,4-dienoic acid) was produced in 2 h by KF707 resting cells, and this compound accumulated as the predominant compound for further incubation (Fig. 3). A small amount of 2,5-dichlorobenzoic acid was also detected by GC-MS analysis. On the other hand, a small amount of *meta*-cleavage compound was produced by LB400. Instead, a 3,4-dihydrodiol compound (*cis*-3,4-dihydroxy-2,5-dichloro-1-[4′-chlorophenyl]cyclohexa-1,5-diene) with a retention time of 11.66 min predominated in the reaction mixture. These results strongly indicate that KF707 BP Dox recognizes primarily a 4′-chlorinated ring and binds to introduce O_2 at the 2,3 position (Fig. 4), while LB400 BP Dox recognizes a 2,5-dichlorinated ring instead of a 4′-chlorinated ring and introduces O_2 at the 3,4 position. The results obtained from the degradation of 2,5,4′-CB were also applicable to the degradations of 4,4′-CB and 2,5,2′,5′-CB. KF707 attacked 4,4′-CB, producing a large amount of a *meta*-cleavage yellow compound (3-chloro-2-hydroxy-6-oxo-6-[4′-chlorophenyl]hexa-2,4-dienoic acid) and a small amount of 4-chlorobenzoic acid via 2,3-dioxygenation. However, KF707 was unable to attack 2,5,2′,5′-CB at all. On the other hand, LB400 attacked 2,5,2′,5′-CB quickly, producing a large amount of a 3,4-dihydrodiol compound (*cis*-3,4-dihydroxy-2,5-dichloro-1-[2′,5′-chlorophenyl]cyclohexa-1,5-diene). These results were in good agreement with those obtained by Haddock et al. with the

TABLE 2. Oligoprimers used for site-directed mutagenesis[a]

Amino acid change	Oligoprimers used
LB400 sequence → KF707 sequence	
Glu 303 Asp	5'-GCGCTGTTCCGCAAGGTCCGGCAGCCGGACCC-3' (Asp)
Gly 313 deletion	5'-GCGTCGAACCGGCATGGTGTGCCCCAGGCG-3' (deletion)
Val 320 Phe	5'-CATGTGCTGGCCGAACATGCGTCGAACCGG-3' (Phe)
Thr 325 Ser	5'-AGGTCGGGAAGATGCCTCATGTGCTGGCCGA-3' (Ser)
Thr 335 Ala	5'-CCGGATGTTGTTGAAGGCGGGCAGGAATGAACA-3' (Ala)
Phe 336 Ile	5'-GATCCGGATGTTGTTGATTGGTGGGCAGGAATGA-3' (Ile)
Asn 338 Thr	5'-GGTGCCAGATCCGGATGGTGTTGAAGGTGGGCA-3' (Thr)
Thr 325 Ser Ile 326 Val	5'-AGGTCGGGAAGACGCTCATGTGCTGGCCGA-3' (Val Ser)
KF707 sequence → LB400 sequence	
Met 283 Ser	5'-CACCGCCATGAGTGAGCCCGGCTCGTCGAC-3' (Ser)
Ser 324 Thr	5'-GGTCGGCGAAGACCGTCATGTGCTGGCCGAC-3' (Thr)
Val 325 Ile	5'-AGGTCGGGAAGATTGCTCATGTGCTGGCC-3' (Ile)
Thr 340 Ile	5'-CGCGGGTGCCAGATCCGGATGGTGTTGA-3' (Ile)
Thr 376 Asn	5'-CCTGCGGAGAAGTTGCGGATGTTGTGCC-3' (Asn)

[a] Boxes indicate mutagenized nucleotides; the arrow indicates the deletion site.

purified LB400 BP Dox in which 99% of the metabolite was 3,4-dihydrodiol. LB400, however, hardly attacked 4,4′-CB.

Role of *orf0* and *bphA1* in the degradation of PCB. Although the role of *orf0* upstream of *bphA1* in the metabolism of biphenyl and PCB is unknown, the identity of amino acid sequences between KF707 Orf0 and LB400 Orf0 has been determined and is relatively low (80.0%) compared with those for other proteins (Fig. 1). First, we examined the degradation of 4,4′-CB, 2,5,4′-CB, and 2,5,2′,5′-CB by *Escherichia coli* harboring pHKF11 containing hybrid genes *orf0* (LB400) and *bphA1A2A3A4* (KF707). The results showed that *orf0* was not directly responsible for BP Dox activity in terms of PCB degradation capability (data not shown). Second, we replaced the *bphA1* of KF707 with that of LB400. *E. coli* cells expressing BphA1 (LB400) and BphA2A3A4 (KF707) (pCKF12) exhibited PCB degradation capability identical to that of the cells expressing the original LB400 BP Dox. Thus, 3,4-dioxygenation activity was observed for 2,5,4′-CB and 2,5,2′,5′-CB, but no degradation was observed for 4,4′-CB.

Construction of chimeric *bphA1* genes. Since it was found that a large subunit (KF707 BphA1 or LB400 BphA1) of terminal dioxygenase was critically involved in substrate specificity, we were interested to learn which amino acids (among 20 different ones) are responsible for PCB-degrading ability. For this purpose, we first exchanged the corresponding restriction fragments within the two *bphA1* genes (Fig. 5). Since three common restrictions sites (*Sal*I at 836 bp from the start codon of the 1,377-bp KF707 *bphA1*, *Sca*I at 883 bp, and *Acc*III at 1,017 bp) and, additionally, a 5′-vector-borne *Eco*RI site and a 3′-vector-borne *Eco*RI site could be used, four common DNA fragments were generated. We constructed various chimeric *bphA1* genes with the KF707 and LB400 *bphA1* genes by using these four fragments without any frameshifts (Fig. 5). Fragments I, II, III, and IV encoded 8, 2, 8, and 2 different amino acids, respectively, which included one missing amino acid in the protein encoded by fragment II (LB400 Gly-315 is missing in KF707 BphA1). These chimeric *bphA1* genes were then inserted just upstream of *bphA2A3A4* (KF707) in pCKF201. Expression of chimeric BphA1 and the association with BphA2 (KF707) were confirmed to be heterohexamer by native PAGE (data not shown) and SDS-PAGE (Fig. 6), respectively.

Degradation of 4,4′-CB, 2,5,4′-CB, and 2,5,2′,5′-CB by chimeric BP Dox. *E. coli* cells expressing the original and various chimeric BP Dox were examined for the degradation of 4,4′-CB, 2,5,4′-CB, and 2,5,2′,5′-CB (Table 3). *E. coli* cells expressing the original KF707 BP Dox (pCKF11) introduced O_2 preferentially at a 4′-chlorinated ring of 2,5,4′-CB, producing 2′,3′-dihydrodiol (97%), and at a 2,5-chlorophenyl ring, producing

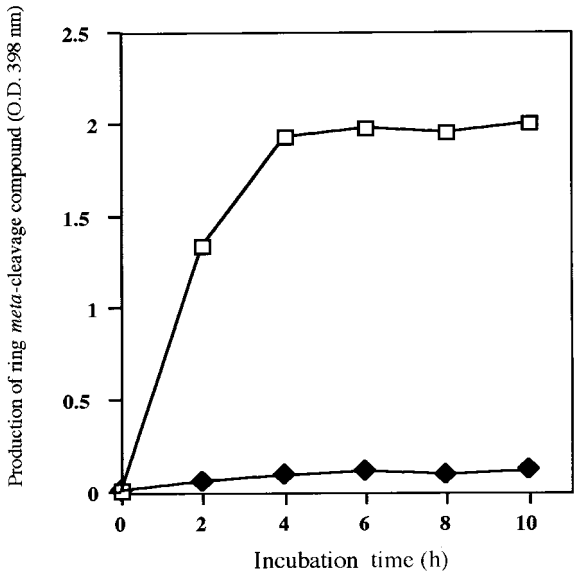

FIG. 3. Production of the ring *meta*-cleavage compound from 2,5,4′-CB by *P. pseudoalcaligenes* KF707 and *P. cepacia* LB400. Resting cells grown with biphenyl were suspended in 50 mM phosphate buffer (pH 7.5) to get an optical density of 1.0 at 660 nm and were incubated with 2,5,4′-CB (20 μg/ml) with shaking at 30°C. The production of the ring *meta*-cleavage compound was measured for the supernatant at 398 nm. □, KF707; ◆, LB400.

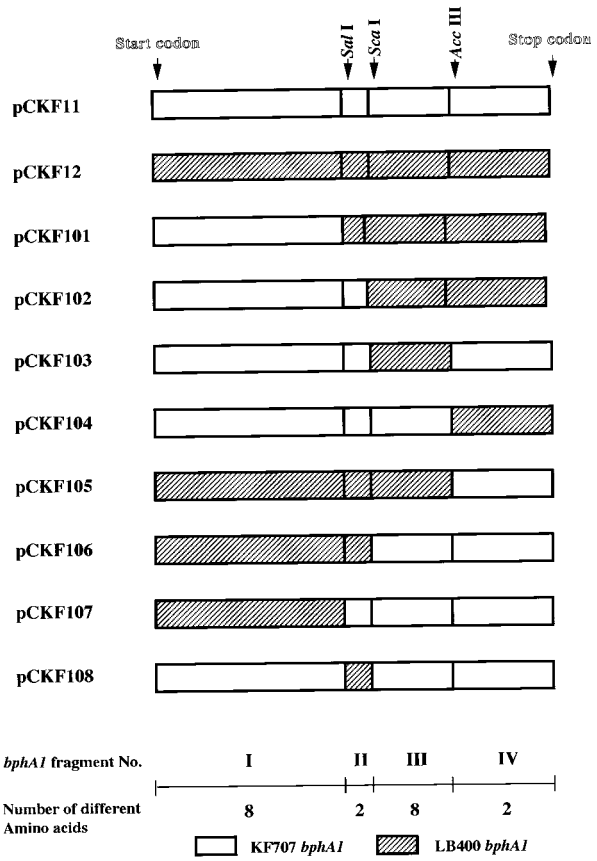

FIG. 5. Construction of chimeric BphA1 between *P. pseudoalcaligenes* KF707 and *P. cepacia* LB400. Common and unique restriction sites of *Sal*I, *Sac*I, and *ACC*III are indicated for the *bphA1* genes; cleavage at these sites generates four fragments, labeled I, II, III, and IV. The numbers of different amino acids within these fragments are indicated at the bottom.

3,4-dihydrodiol (3%). The same cells attacked 4,4′-CB, producing 2,3-dihydrodiol. No metabolite was obtained from 2,5,2′,5′-CB. In contrast to this, *E. coli* cells expressing the original LB400 BP Dox (pCKF12) primarily recognized a 2,5-chlorinated ring of 2,5,4′-CB, introduced O_2 at the 3,4 position (95%), and produced a small amount of 2′,3′-dihydrodiol (5%). The same cells hardly attacked 4,4′-CB but quickly converted 2,5,2′,5′-CB to 3,4-dihydrodiol. These results were comparable to those obtained from the wild-type KF707 and LB400 resting cells.

The chimeric BP Dox expressed from pCKF101 and pCKF102 (Fig. 5), in which fragments II, III, and IV or fragments III and IV of KF707 *bphA1* were replaced by the corresponding fragments of LB400, showed degradation capacity nearly identical to that of the original LB400 BP Dox, so that 3,4-dihydrodiol was the major product from 2,5,4′-CB (more than 95%). No metabolite was obtained from 4,4′-CB, but a large amount of 3,4-dihydrodiol was obtained from 2,5,2′,5′-CB with these cells. *E. coli* cells harboring pCKF103, in which only fragment III (KF707) was replaced by the corresponding fragment of LB400, produced both 2′,3′-dihydrodiol and 3,4-dihydrodiol from 2,5,4′-CB at the ratio of 80:20. The same cells exhibited a wider-range degradation capacity, so that 2,3-dihydrodiol from 4,4′-CB and 3,4-dihydrodiol from

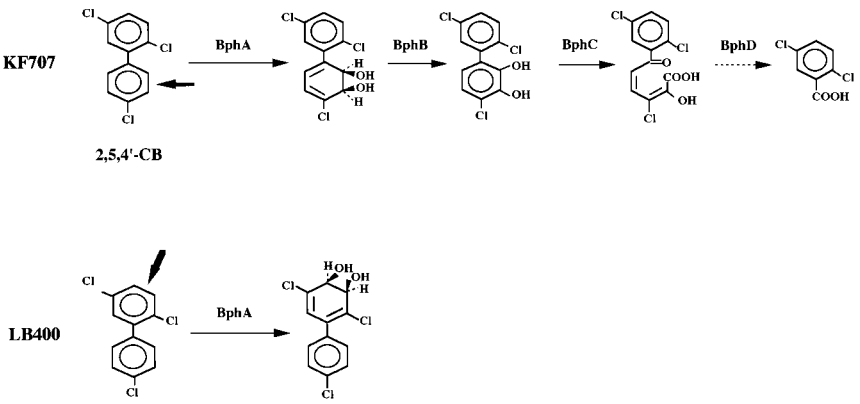

FIG. 4. Metabolism of 2,5,4′-trichlorobiphenyl by *P. pseudoalcaligenes* KF707 and *P. cepacia* LB400. KF707 attacks preferentially the 4′-chlorinated ring (as indicated by the thick arrow) and introduces O_2 at the 2′,3′ position. LB400 attacks the 2,5-chlorinated ring and introduces O_2 at the 3,4 position.

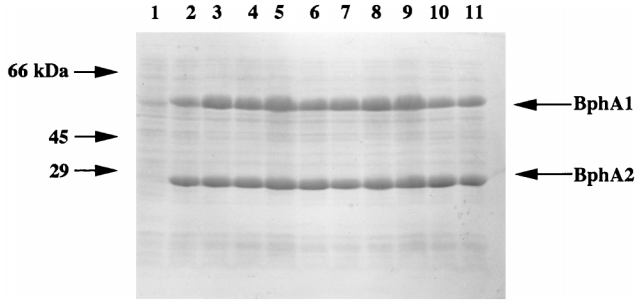

FIG. 6. SDS-PAGE of chimeric biphenyl dioxygenases expressed by *E. coli* cells carrying the plasmids listed below. The sizes of molecular markers are indicated at the left. Lanes: 1, pUC118; 2, pCKF11; 3, pCKF12; 4, pCKF101; 5, pCKF102; 6, pCKF103; 7, pCKF104; 8, pCKF105; 9, pCKF106; 10, pCKF107; 11, pCKF108.

2,5,2′,5′-CB were obtained. *E. coli* cells harboring pCKF104 in which fragment IV (KF707) was replaced by that of LB400 preferentially recognized the 2,5-chlorinated ring of 2,5,4′-CB and introduced O_2 at the 3,4 position (92%) and at the 2′,3′ position (8%) of the 4′-chlorinated ring. The same cells converted both 4,4′-CB and 2,5,2′,5′-CB to the same extent as cells harboring pCKF103. *E. coli* cells harboring pCKF105 in which fragments I, II, and III (KF707) were replaced by those of LB400 showed both 3,4-dioxygenation (64%) and 2′,3′-dioxygenation (36%) capability for 2,5,4′-CB. The same cells converted 4,4′-CB via 2,3-dioxygenation and 2,5,2′,5′-CB via 3,4-dioxygenation. Thus, BP Dox expressed from pCKF105 possessed a wide-range oxygenation ability. The chimeric BP Dox derived from pCKF106, in which fragments I and II were replaced by those of LB400, showed degradation capability similar to that of the original KF707 BP Dox, indicating that the eight-amino-acid difference in fragment I is not significant for the relative PCB degradation capability of KF707 BP Dox and LB400 BP Dox. *E. coli* cells harboring pCKF107, in which fragment I (KF707) was replaced by that of LB400, exhibited almost no activity for these three PCB congeners. The same cells failed to attack even biphenyl, despite the fact that no mutation occurred in the nucleotide sequence in the chimeric *bphA1* gene and the fact that a significant amount of protein was detected by native and SDS-PAGE. The reason why this combination lost its enzymatic activity remains to be elucidated. The BP Dox produced from pCKF108, in which fragment II (KF707) was replaced with that of LB400, showed almost the same activity as that of the original KF707 BP Dox, as in the case of pCKF106.

Site-directed changes of amino acid sequences. Since it was found that amino acid residues within the C-terminal half of BphA1 were responsible for substrate binding ability and determined the site of oxygen activation of BP Dox for PCB, we attempted to change amino acid residues within BphA1. Site-directed mutagenesis, with the nucleotide oligomers listed in Table 2, was applied to pCKF102, in which fragments III and IV of KF707 *bphA1* were replaced by those of LB400 *bphA1* since the chimeric BP Dox expressed from this plasmid showed activity almost identical to that of the original LB400 BP Dox. In this experiment all primers were designed to change amino acid residues of LB400 BphA1 to those of KF707 BphA1. The results obtained were as follows. The following alterations did not significantly change the degradation capability and the site of dioxygenation: Glu-303 to Asp (pSKF101), Gly-313 deletion (pSKF102), Val-320 to Phe (pSKF103), Thr-325 to Ser (pSKF104), Thr-335 to Ala (pSKF105), Phe-336 to Ile (pSKF106), Asn-338 to Thr (pSKF107), and Thr-325 to Ser and Ile-326 to Val (pSKF108). Site-directed mutagenesis was also conducted for pCKF11 carrying the original KF707 *bphA1*. No significant change in enzymatic activity was observed when the following alterations were carried out: Ser-324 to Thr (pSKF12), Val-325 to Ile (pSKF13), and Thr-340 to Ile (pSKF14). However, most interestingly, the alteration of Thr-376 (KF707) to Asn (LB400) (pSKF15) in the original KF707 BP Dox resulted in a remarkable expansion of degradation capability. Thus, *E. coli* cells harboring pSKF15 degraded three PCB congeners tested as follows. 4,4′-CB was degraded to 2,3-dihydrodiol, 2,5,4′-CB was degraded preferentially to 3,4-dihydrodiol (96%) and poorly to 2′,3′-dihydrodiol (4%), and

TABLE 3. Sites of dioxygenation of various modified BP Dox for 4,4′-CB, 2,5,4′-CB and 2,5,2′,5′-CB

Plasmid	Substrate	Degradation pattern		
		Site of dioxygenation	Accumulated metabolite	Relative yield[a] (%)
pCKF11	4,4′-CB	2,3	2,3-dihydrodiol	
	2,5,4′-CB	2′,3′	2′,3′-dihydrodiol	97
		3,4	3,4-dihydrodiol	3
	2,5,2′,5′-CB		No metabolite	
pCKF12	4,4′-CB		No metabolite	
	2,5,4′-CB	2′,3′	2′,3′-dihydrodiol	5
		3,4	3,4-dihydrodiol	95
	2,5,2′,5′-CB	3,4	3,4-dihydrodiol	
pCKF101	4,4′-CB		No metabolite	
	2,5,4′-CB	2′,3′	2′,3′-dihydrodiol	5
		3,4	3,4-dihydrodiol	95
	2,5,2′,5′-CB	3,4	3,4-dihydrodiol	
pCKF102	4,4′-CB		No metabolite	
	2,5,4′-CB	2′,3′	2′,3′-dihydrodiol	4
		3,4	3,4-dihydrodiol	96
	2,5,2′,5′-CB	3,4	3,4-dihydrodiol	
pCKF103	4,4′-CB	2,3	2,3-dihydrodiol	
	2,5,4′-CB	2′,3′	2′,3′-dihydrodiol	80
		3,4	3,4-dihydrodiol	20
	2,5,2′,5′-CB	3,4	3,4-dihydrodiol	
pCKF104	4,4′-CB	2,3	2,3-dihydrodiol	
	2,5,4′-CB	2′,3′	2′,3′-dihydrodiol	8
		3,4	3,4-dihydrodiol	92
	2,5,2′,5′-CB	3,4	3,4-dihydrodiol	
pCKF105	4,4′-CB	2,3	2,3-dihydrodiol	
	2,5,4′-CB	2′,3′	2′,3′-dihydrodiol	36
		3,4	3,4-dihydrodiol	64
	2,5,2′,5′-CB	3,4	3,4-dihydrodiol	
pCKF106	4,4′-CB	2,3	2,3-dihydrodiol	
	2,5,4′-CB	2′,3′	2′,3′-dihydrodiol	97
		3,4	3,4-dihydrodiol	3
	2,5,2′,5′-CB		No metabolite	
pCKF108	4,4′-CB	2,3	2,3-dihydrodiol	
	2,5,4′-CB	2′,3′	2′,3′-dihydrodiol	98
		3,4	3,4-dihydrodiol	2
	2,5,2′,5′-CB		No metabolite	
pSKF15	4,4′-CB	2,3	2,3-dihydrodiol	
	2,5,4′-CB	2′,3′	2′,3′-dihydrodiol	4
		3,4	3,4-dihydrodiol	96
	2,5,2′,5′-CB	3,4	3,4-dihydrodiol	

[a] The relative yield was calculated from the integrated total ion peak areas.

2,5,2′,5′-CB was also degraded to 3,4-dihydrodiol. The PCB degradation ability of *E. coli* (pSKF15) was almost comparable to that of *E. coli* cells expressing the original KF707 BP Dox (pCKF11) for 4,4′-CB and that of *E. coli* cells expressing the original LB400 BP Dox (pCKF12) for 2,5,4′-CB and 2,5,2′,5′-CB. Thus, only one amino acid change resulted in a great expansion of PCB degradation capability. Another interesting mutation was the change of Met-283 (KF707) to Ser (LB400) in the original KF707 BP Dox. This mutant BP Dox totally lost enzymatic activity for an unknown reason.

The results obtained here may allow us to draw the following general conclusions: (i) fragment I containing eight different amino acids in BphA1 is not significantly involved in the functional difference between KF707 and LB400 BP Dox; (ii) the replacement of fragment III or IV of KF707 BP Dox by that of LB400 BP Dox relaxes restrictions on the recognition of PCB congeners, thereby expanding the range of biodegradable PCB congeners and also changing the sites of oxygen activation; (iii) the single change of Thr-376 to Asn (LB400) in KF707 BP Dox permits it to recognize a 2,5-dichlorinated ring as well as a 4′-chlorinated ring and to introduce O_2 at the 3,4 position as well as at the 2,3 position, as shown for pSKF15; (iv) the site-directed change of Met-283 to Ser (LB400) in the KF707 BP Dox (pSKF11) totally eliminates enzymatic activity.

In conclusion, our study demonstrated that a relatively small number of amino acid changes in BphA1 resulted in great changes of PCB degradation capability in terms of the recognition of the chlorinated ring of PCB and the site of dioxygenation. This may reflect the fact that PCB degradation capabilities are greatly varied among PCB-degrading bacteria, despite the fact that the *bph* genes have high homology with one another (9, 11, 25). In vitro shuffling between the *bphA1* genes of KF707 and LB400 by using PCR is currently under way to get more combinations of chimeric *bphA1* genes. These approaches may provide a basic understanding of how microorganisms gain the novel catabolic activities for various aromatic compounds (13, 21, 25, 27) and may lead to breeding strains with enhanced degradation capabilities for environmental pollutants (28).

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