Emergence of Multifunctional Oxygenase Activities by Random Priming Recombination*

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Biphenyl dioxygenase (Bph Dox) is responsible for the initial dioxygenation of biphenyl. The large subunit (BphA1) of Bph Dox plays a crucial role in determination of substrate specificity of biphenyl-related compounds including polychlorinated biphenyls (PCBs). Functional evolution of Bph Dox of Pseudomonas pseudoalcaligenes KF707 was accomplished by random priming recombination of the bphA1 gene, involving two rounds of in vitro recombination and mutation followed by selection for increased activity in vivo. Evolved Bph Dox acquired novel and multifunctional degradation capabilities not only for PCBs but also for dibenzofuran, dibenzo-p-dioxin, dibenzothiophene, and fluorene, the compounds scarcely attacked by the original KF707 Bph Dox. The modes of oxygenation were angular and lateral dioxygenation for dibenzothiophene, and mono-oxygenation for fluorene. These enzymes also exhibited enhanced degradation abilities for PCB congeners, retaining 2,3-dioxygenase activity and gaining 3,4-dioxygenase activity, depending on the chlorine substitution of PCB congeners. Further mutation analysis revealed that the amino acid at position 376 in BphA1 is significantly involved in the acquisition of multifunctional oxygenase activities and mode of oxygenation.

Bacterial oxygenases are involved in the initial hydroxylation of aromatic hydrocarbons, and they are usually two or three component enzymes. The corresponding subunits share various degrees of homology (1–4). This implies that bacteria have adaptively evolved, by modifying key enzymes, to utilize a variety of aromatic compounds (4). Biphenyl-utilizing bacteria have been widely isolated (5–8). These bacteria have been studied extensively with respect to the degradation of PCBs,1 a family of xenobiotic compounds that is one of the major environmental pollutants. Considerable differences are found in the congener selectivity patterns and in the range of activity of various PCB-degrading bacteria (5, 6, 7, 9). Both the relative rates of primary degradation of PCBs and the mode of the ring attacked are dependent on the bacterial strains (9–11).

Pseudomonas pseudoalcaligenes KF707 and Burkholderia cepacia LB400 exhibit distinct differences in substrate ranges for PCBs (12, 13), despite the fact that these two bph operons are nearly identical in gene organization and the amino acid sequences of the corresponding enzymes (14, 15).

Biphenyl and PCBs are oxidized to the dihydrodiol compound by Bph Dox (Fig. 1). The dihydrodiol is dehydrogenated to the dihydroxy compound by dihydrodiol dehydrogenase (encoded by bphB), and the dihydroxy compound is then meta-cleaved to form 2-hydroxy-6-oxo-6-phenylhexa-2,4-dieneoate (ring meta-cleavage yellow compound) by 2,3-dihydroxybiphenyl dioxygenase (encoded by bphC). Subsequently, the ring meta-cleavage yellow compound is hydrolyzed to benzoic acid by hydrolase (encoded by bphD). Bph Dox is a three-component enzyme consisting of terminal dioxygenase and a short electron transport chain. The former comprises BphA1 (an iron sulfur protein encoded by bphA1) and BphA2 (a small subunit encoded by bphA2). The latter is composed of a ferredoxin (encoded by bphA3) and a ferredoxin reductase (encoded by bphA4). BphA1 and BphA2 are associated as an α2β heterohexamer and catalyze the direct introduction of two atoms of oxygen into the biphenyl ring. BphA1 contains the motif Cys-Xaa-His-Xaa-17-Cys-Xaa-2-His, which forms a Rieske-type [2Fe-2S] cluster. Bph Dox requires Fe(II) for activity, and oxygen activation is supposed to occur at the mononuclear iron center (14). Ferredoxin and ferredoxin reductase act as an electron transfer system from NADH to reduce the terminal dioxygenase.

Among these four subunits, we found that BphA1 is crucially responsible for recognition and binding of substrates and hence for substrate specificity (1, 16). We constructed a variety of chimeric bphA1 genes between KF707 and LB400 using three common restriction sites (17). The results demonstrated that a relatively small number of amino acids in the carboxyl-terminal half of BphA1 are involved in the recognition of the chlorinated ring and the site of dioxygenation and are therefore responsible for the degradation of PCB. We also recombined the two bphA1 genes using DNA shuffling (18). Some clones expressing the evolved Bph Dox exhibited enhanced abilities for PCB degradation.

In this report, we engineered the KF707 bphA1 gene by a method of random priming recombination (19) and obtained some novel Bph Dox that exhibited multifunctional oxygenase activities not only for PCB but also for dibenzofuran, dibenzo-p-dioxin, dibenzothiophene, and fluorene.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmid, and Growth Conditions—The biphenyl-utilizing strain P. pseudoalcaligenes KF707 was grown in basal salt medium as described previously (20). Escherichia coli strains were grown in Luria-Bertani medium or on Luria-Bertani agar medium (1.5% agar). Antibiotics (50 μg/ml ampicillin and 34 μg/ml chloramphenicol) were added when needed to select for the presence of plasmid in E. coli transformants. pJHF18ΔMluI containing disrupted bphA1

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1 The abbreviations used are: PCB, polychlorinated biphenyl; Bph Dox, biphenyl dioxygenase; PCR, polymerase chain reaction; GC-MS, gas chromatography-mass spectrometry.
(bphA1) was constructed as described previously (18). pJH18ΔMluI was used for the replacement of bphA1 with the bphA1 gene variants. The pRPF1000 series plasmids contain bphA1 ( evolved)-bphA2A34BC and were used to assay the production of ring meta-cleavage products from PCBs and bphB-related compounds. The pRPF2000 series plasmids were constructed by removing bphBC genes from pRPF1000 series plasmids by digestion with PpuMI and religation and used for the production of dihydrodiol compounds. pSSF2202T and pSSF2202N were pRPF2002 variants constructed by site-directed mutagenesis. Plasmids pUCARA, which carries the genes carAAcarAd coding for carbazole 1,9-dioxynonogen (21) and pQRI56, which carries the genes nahAaaaAbAcAd coding for naphthalene dioxygenase (22), were provided by Toshio Ohmori (University of Tokyo, Tokyo, Japan).

Preparation of bphA1 DNA—For the template for random priming recombination, the KF707 bphA1 DNA was amplified from plasmid pKT18 (14) using the following oligonucleotide primers: 5’-CCGAAT-3’ overlapping start codon ATG, and the SacI PCR products were digested by screening of the min, followed by 25 cycles (94 °C, 1 min; 52 °C, 1 min; and 72 °C, 1 min).

Function of BphA1 were added (Fig. 2). The polymerase reaction was brought to 95 °C for 3 min, followed by 40 cycles (94 °C, 1 min; 52 °C, 1 min; and 72 °C, 1 min) were run with 2.5 mM each primer, 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 0.1 M MgCl2, 20 mM dithiothreitol, and 5 mM each deoxynucleotide (94 °C, 1 min; 52 °C, 1 min; and 72 °C, 1 min).

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Assay of Degradation Capability and GC-MS Analysis—The recombinant E. coli JM109 cells expressing the original and mutant Bph Dox were grown to logarithmic phase (turbidity of 0.8–1.2 at 600 nm), washed twice in 50 mM phosphate buffer (pH7.5), and resuspended in 20 ml of the same buffer to adjust the turbidity to 1.0. PCBs (Accustandard Inc.) dissolved in dimethyl sulfoxide were added at a final concentration of 20 μg/ml. Biphényl-related compounds dissolved in ethanol were added at a concentration of 50 μg/ml. After being shaken at 200 rpm for predetermined periods (1–8 h), aliquots (1 ml) were centrifuged, and the formation of ring meta-cleavage products from various aromatic compounds was monitored with the supernatants at the following absorption maxima: biphenyl, 434 nm (ε = 33,200 M⁻¹ cm⁻¹); diphenyl methane, 395 nm (ε = 20,200 M⁻¹ cm⁻¹); dibenzofuran, 465 nm (ε = 37,500 M⁻¹ cm⁻¹); and 2,5,4’-trichlorobiphenyl, 385 nm (ε = 211,400 M⁻¹ cm⁻¹). The molar extinction coefficient of those ring meta-cleavage compounds was determined experimentally and used for the calculation of compound formation. The dihydrodiol products from PCBs were derivatized with 100 μg of n-butyliconic acid in 10 μl of acetone-dimethyl formamide (23). The products of dibenzofuran and dibenzoo-p-dioxin were subjected to the trimethylsilylation with N,O-bis-(trimethylsilyl)-acetate. The samples were analyzed by GC-MS (model QP5000; Shimadzu) with a coiled stainless steel column (0.33 mm, inner diameter; 25 m long) packed with methyl silicon CBP1 as described previously (17). Amounts of parks and aliphatic acid derivatives were quantified and corrected by using a standard curve (5, 18).

RESULTS

Random Priming in Vitro Recombination—Random priming recombination was performed by the method of Shao et al. (19). Briefly, the KF707 (40 pmol) was digested with 6.7 pmol of BphA1 random primer (Amersham Pharmacia Biotech). After denaturation at 100 °C for 5 min, 10 μl of 10× reaction buffer (900 mM HEPES, pH 6.6, 0.1 mM MgCl2, 20 mM dithiothreitol, and 5 mM each deoxyxynucleotide triphosphate, PCR buffer, and 20% Q-solution (Qiagen GmbH) with Taq DNA polymerase (Qiagen). The PCR product was digested by EcoRI and inserted at the EcoRI site of pHSG396 (Takara Shuzo) to obtain pRPF707. The EcoRI fragment containing the bphA1 gene was cut from pRPF707, electropherograms on 0.7% agarose gels, and recovered by a DNA purification kit (TOYOBO).

Cloning and Screening of bphA1 Variants—Cloning, expression, and screening of the bphA1 mutant genes were carried out as follows. The PCR products were digested by SacI and BglII and purified by agarose gel electrophoresis. Because the SacI site is present at the 5’-end of bphA1 overlapping start codon ATG, and the BglII site is present at the flanking region between bphA1 and bphA2, the mutants of bphA1 were double-digested with SacI and BglII and ligated at the same site of pJH18ΔMluI, replacing bphA1 with the mutant bphA1. The recombinant plasmids were transformed into E. coli JM109 and plated onto Luria-Bertani agar containing ampicillin and 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). For the first screening, colonies producing the ring meta-cleavage yellow pigment from biphenyl vapor were picked up. Subsequently, positive colonies for biphenyl were checked for the production of yellow compound from dibenzofuran.

Site-directed Mutagenesis—Site-directed mutagenesis was performed by a Quickchange Site-directed Mutagenesis Kit (STRATAGENE) in accordance with the manufacturer’s instructions. Plasmid pSSF1202, which contains bphA1 from pRPF1202 (described below), was used as the template for mutagenesis. This plasmid was amplified by PCR using two complementary oligonucleotides: 5’-CAGCATCCGGCACTTCCTGCAAGCGGC-3’ and 5’-GCTCTCCGGAAGAAGTGCG-GGTTCCCGCGCC-3’ for amino acid change V376E and 5’-CAGACATCCGGCAACCTTCCGCCAGGGCC-3’ and 5’-GCTGTCGGGAAAAGT- GGTTCCCGCGCC-3’ for V376N (the codon of the amino acid to be changed is underlined). After mutations were confirmed by a DNA sequencer (model 4000L; LI-COR), the SacI-BglII fragments were cloned into SacI-BglII-digested pJH18ΔMluI for the functional analyses.
enzyme were added to protect against ablation of oxygenase activity (Fig. 2). On the other hand, Thr at position 376, which could be involved in the substrate specificity and mode of oxygenation (17, 25), was intentionally altered by using random primers that certainly provide random mutation. In the presence of protective primers for the essential sequences of Rieske center and mononuclear iron binding motif, the rate of positive clones was increased to 60%, as compared with 42% in the absence of primers.

The bphA1 variants obtained were digested with SacI and BglII and inserted into just upstream site of bphaA2A3A4BC of the SacI- and BglII-digested pJHF18AmI. Colonies forming the ring meta-cleavage yellow products from both biphenyl and dibenzo-p-dioxin were screened, and the extended function of these evolved enzymes was analyzed with PCBs and biphenyl-related compounds. After the primary round of cycle and selection, E. coli carrying pRPF1113 exhibited enhanced degradation ability for dichlorobiphenyl (Fig. 3C). One additional round of cycle and selection using both pRPF1113 and KF707-bphA1 as template DNA resulted in the emergence of evolved Bph Dox encoded by pRPF1202 and pRPF1217.

Functional Analyses of Bph Dox Variants—E. coli cells expressing the original and evolved Bph Dox were examined in some detail for the degradation of biphenyl-related compounds. All E. coli transformants tested expressed almost the same amounts of Bph Dox as judged by SDS-polyacrylamide gel electrophoresis (data not shown). They were incubated with biphenyl, 4-methylbiphenyl, diphenylmethane, dibenzofuran, and 2,5,4'-trichlorobiphenyl (Fig. 3). E. coli [pRPF1707] expressing the original Bph Dox degraded biphenyl, diphenylmethane, and 2,5,4'-trichlorobiphenyl, forming yellow pigments via 2,3-dihydroxy compounds (2,3-dioxygenation mode) (17). E. coli harboring pRPF1113, pRPF1202, and pRPF1217 exhibited enhanced production of yellow compounds from biphenyl, diphenylmethane, and dibenzofuran to different degrees, as compared with E. coli [pRPF1707] expressing the original Bph Dox. Among these clones, E. coli [pRPF1202] exhibited highest activities of 182% for biphenyl and 190% for diphenylmethane, compared with E. coli [pRPF1707]. More interestingly, these three evolved clones exhibited degradation activities for both dibenzofuran and dibenzo-p-dioxin compounds that were scarcely attacked by the original Bph Dox, as described later.

To examine the mode of oxygenation of these evolved Bph Dox for PCBs, the bphB and bphC genes were deleted from plasmids pRPF1707, pRPF1113, pRPF1202, and pRPF1217, generating pRPF2007, pRPF2113, pRPF2202, and pRPF2221, respectively. Using E. coli cells carrying pRPF2000 series plasmids, the metabolites produced from 2,2'-dichlorobiphenyl, 4,4'-dichlorobiphenyl, 2,5,4'-trichlorobiphenyl, and 2,5,2',5'-tetrachlorobiphenyl were analyzed by GC-MS (Table I). E. coli [pRPF2207] expressing the original KF707 Bph Dox degraded biphenyl, 4,4'-dichlorobiphenyl, and 2,5,4'-trichlorobiphenyl but hardly attacked 2,5,2',5'-tetrachlorobiphenyl as described previously (17). Thus, KF707 Bph Dox recognizes primarily a 4'-chlorinated ring to introduce O2 at the 2,3 position. Bph Dox expressed from pRPF2113, pRPF2202, and pRPF2221 exhibited 2,3-dioxygenase activity for 4,4'-dichlorobiphenyl, as does the original KF707 Bph Dox. On the other hand, the same evolved Bph Dox preferentially introduced O2 at the 3,4 position of the 2,5-dichlorinated ring to 2,5,4'-trichlorobiphenyl but 2,5,2',5'-tetrachlorobiphenyl. For 2,2'-dichlorobiphenyl, the same clones produced 2-chloro-2,3-dihydroxybiphenyl, indicating that the dioxygenase-catalyzed reaction occurred at the 2,3 position of a 2-chlorinated ring, resulting in dechlorination, as does B. cepacia LB400 (23).

Thus, the modes of oxygenation of the evolved Bph Dox from pRPF2113, pRPF2202, and pRPF2221 were varied, depending on the chlorine substitution of PCBs.

Degradation of Dibenzo-furan and Dibenzo-p-dioxin—Bacterial degradation of dibenzofuran and dibenzo-p-dioxin occurs by either angular (21, 26, 27) or lateral (28, 29) attack on the ring system. E. coli cells expressing the original and the evolved Bph Dox were investigated for the degradation of dibenzofuran and dibenzo-p-dioxin. To detect the metabolites, E. coli JM109 [pUCARA] carrying carAaAcAd coding for carbazole 1,9-a dioxygenase and JM109 [pQR156] carrying the genes naAaAhAcAd coding for naphthalene dioxygenase were used, confirming that JM109 [pUCARA] produced cis-cis-2,3,3'-trihydroxyphenyl and cis-cis-2,3,3'-trihydroxybiphenyl ether and that JM109 [pQR156] produced cis,1,2-dihydroxy-1,2-dihydrodibenzofuran and cis,1,2-dihydroxy-1,2-dihydrodibenzo-p-dioxin from dibenzofuran and dibenzo-p-dioxin, respectively (21, 29). E. coli [pRPF2207] scarcely attacks both dibenzofuran and dibenzo-p-dioxin (Table I). On the other hand, E. coli cells carrying pRPF2213, pRPF2202, and pRPF2221 attacked dibenzofuran.
The GC-MS profile of the metabolites from dibenzofuran by *E. coli* [pRPF2202] is presented in Fig. 4. These metabolites peaks were assigned to be monohydroxydibenzofuran (M₁, m/z 256; DF-I), *cis*-1,2-dihydroxy-1,2-dihydrodibenzofuran (M₁, m/z 346; DF-II), and 2,3,2,9-trihydroxydiphenyl (M₁, m/z 418; DF-III). *E. coli* carrying pRPF2113 and pRPF2217 also produced the same compounds (DF-I, DF-II, and DF-III), but to lesser extents. For dibenzo- *p*-dioxin, two major peaks were detected from *E. coli* cells carrying pRPF2113 and pRPF2202. Shown in Fig. 5 is the GC-MS profile of the metabolites from dibenzo- *p*-dioxin by *E. coli* [pRPF2202] that exhibited the highest activity in evolved Bph Dox. These compounds were determined to be monohydroxydibenzo- *p*-dioxin (M⁺, m/z 272; DD-I) and 2,3,2'-trihydroxydiphenyl ether (M⁺, m/z 454; DD-III). One minor peak (M⁺, m/z 360; DD-II) was also detected, which was determined to be *cis*-1,2-dihydroxy-1,2-dihydrodibenzo- *p*-dioxin. Monohydroxy compounds of DF-I and DD-I were considered to be dehydration products of *cis*-1,2-dihydrodiphenyl compounds that can be spontaneously converted to 1-hydroxy and 2-hydroxy compounds in the acid extraction procedure.

**Degradation of Dibenzothiophene and Fluorene**—It was shown that dibenzothiophene and fluorene were converted to dibenzo thiophene-5-oxide and 9-hydroxyfluorene, respectively, by carbazole 1,9 a-dioxygenase (30). No metabolites were detected from dibenzothiophene and fluorene by *E. coli* carrying pRPF2707, pRPF2113, and pRPF2217. On the other hand, one major peak corresponding to dibenzothiophene-5-oxide (M⁺, m/z 200) was detected from *E. coli* [pRPF2202]. The same clone also produced 9-hydroxyfluorene (M⁺, m/z 254) from fluorene (date not shown).

**Sequence Analyses of bphA1 Mutant Genes**—The nucleotide sequences of the evolved *bphA1* of pRPF1113, pRPF1202, and pRPF1217 were determined. The base substitutions in the evolved *bphA1* genes and the amino acid changes are shown in Fig. 3B. The nucleotide sequences of evolved *bphA1* genes indicated that the random recombinations took place successfully. Sequence analysis of the *bphA1* from pRPF1113 revealed that seven bases were changed, resulting in the two amino acid substitutions at Val-172 to Ile (V172I) and Thr-376 to Val (T376V). The variants from the second round of mutagenesis with both pRPF1113 and the original KF707-*bphA1* as the template DNA were also analyzed. Substitutions of V172I in pRPF1217 were maintained from pRPF1113, likewise, some base substitutions were inherited from pRPF1113. One muta-
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TABLE I
Degradation capabilities of original and evolved Bph Dox for biphenyl-related compounds and PCBs

| Substrate* | Biphenyl | Dibenzofuran | Dibenzo-p-dioxin | 2,5,4-Trimethylphenol |
|------------|----------|--------------|-----------------|----------------------|
|            | Mode b | Amount (µg/h) | Mode b | Amount (µg/h) | Mode b | Amount (µg/h) | Mode b | Amount (µg/h) |
| pRPF2202 [I24V, H66Y, K89R, T376V] | 2.3- | 0.01 | 2,3- | 4.2 | 3.4- | 9.0 |
| pSSF2202 [I24V, H66Y, K89R, T376V] | 2.3- | 0.02 | 2,3- | 0.7 | 2.3- | 7.3 |
| pSSF2707N (T376N) | 2.3- | 1.5 | 2,3- | 14.9 | 3.4- | 11.0 |
| E. coli carrying [altered amino acids] | | | | | | | |
| pRPF2707 [wild type (376T)] | 2.3- | 9.1 | 2,3- | 7.0 | 3.4- | 10.0 |
| pSSF2707V (T376V) | 2.3- | 9.5 | 2,3- | 11.0 | 3.4- | 17.0 |
| pSSF2707N (T376N) | 2.3- | 10.5 | 2,3- | 12.0 | 3.4- | 17.0 |

a The concentration of substrate was 30 µg/ml for biphenyl, dibenzofuran, and dibenzo-p-dioxin and 20 µg/ml for 2,5,4-trichlorophenol.

b Mode of dioxygenation: 2,3-, 2,5-dioxynogenase activity; 3,4-, 3,4- dioxygenase activity; angular, angular dioxygenase activity; lateral, cis,2,12-dioxygenase activity.

c Amounts of the degradation for each substrate were estimated by comparing the peak of substrate with that of negative control on GC-MS analysis. The results are expressed as an average ± S.D. of three independent experiments.

Monello et al. (31) reported that PCB-degradative strains fell into two categories according to their degradation abilities. The strains categorized as having broad substrate specificity tend to attack ortho-meta-substituted congeners such as 2,5,2,5'-tetrachlorobiphenyl, which are oxidized at the 3,4 position. This 3,4-dioxygenase activity is relatively rare among PCB-degrading bacteria. However, they had poor activity for double para-substituted congeners such as 4,4'-dichlorobiphenyl. In contrast, strains having a relatively narrow range of PCB substrates, including *P. pseudoalcaligenes* TKF707, were superior with respect to the degradation of 4,4'-dichlorobiphenyl but were unable to degrade 2,5,2,5'-tetrachlorobiphenyl. Between these two categorized PCB-degradative strains, the strains with broad specificity contain Asn at position 376, whereas the strains with narrow specificity contain Thr at this site. It was also reported that chlorobenzene dioxygenase of *Burkholderia* sp. strain PS12, which utilizes 1,2,4,5-tetrachlorobenzene, attacks dibenzo-p-dioxin preferentially by lateral dioxygenation (32). The same enzyme converts dibenzofuran into cis,1,2-dihydroxy,1,2-dihydrobenzofuran as a single metabolite. Dioxygenases from carboxylate-utilizing *Pseudomonas* sp. CA10, naphthalene-utilizing *Pseudomonas* sp. strain NCI11316-4, and 1,2,4,5-tetrachlorobenzene-utilizing *Burkholderia* sp. strain PS12 also have broad substrate ranges (29, 30, 32, 33, 34).

In this study, we have successfully engineered Bph Dox of *P. pseudoalcaligenes* TKF707 by rational enzyme design and random priming mutagenesis. The resulting evolved enzymes exhibited a wider range of oxygenation capabilities not only for PCBs but also for various biphenyl-related compounds. According to the method described by Shao et al. (19), random hexamers were used to generate a large number of short DNA fragments complementary to different sections of the template sequences. Due to base misincorporation and mispriming, these short DNA fragments contain a low level of point mutations over template gene(s). In this work, the point mutations were intentionally introduced at the critical position of Thr-376, which is supposed to be involved in the substrate specificity (17, 25). On the other hand, the essential regions for the activity, i.e., Rieske center and the mononuclear iron binding motif, were protected using the primers annealed with these...
regions (Fig. 2). Two rounds of random priming recombination and selection resulted in the emergence of novel Bph Dox that acquired novel degradation capabilities.

The functional BphA1 exhibited specific point mutations at position 376, in which Thr was changed to Val or Asn. It was previously shown that the change of Thr-376 to Asn permits K89R in the pRPF2202 enzyme were also involved in the emergence of degradation capability together with the change of T376V.

The three-dimensional structure of naphthalene dioxygenase of Pseudomonas sp. NCIB9816-4 was determined and identified amino acids near the active site iron atom in the catalytic domain of the large subunit (35). Analysis of site-directed mutagenesis revealed that Phe-352 appears to play a major role in controlling both the stereochemistry and regioselectivity, suggesting that the novel catalytic ability can be generated by introducing a single mutation or multiple mutations near the active site (36, 37). The amino acid sequence of KF707 BphA1 shows -30% identity with the naphthalene dioxygenase large subunit (NahAc). However, the amino acids of essential regions for its activity are highly conserved (24). Consequently, it is conceivable that the Thr-376 of KF707 BphA1 lies near the active site and plays a role similar to that of Phe-352 of NahAc.

In this evolved enzyme from pRPF2202, the functional importance of Val-376 was further confirmed by site-directed mutagenesis for pRPF2202. The alteration of Val-376 to Thr in pSSF2202T abolished 3,4-dioxygenase activity for PCBs and lateral dioxygenation activity for dibenzofuran and dibenzo-p-dioxin. On the other hand, the alteration of Val-376 to Asn in pSSF2202N retained 3,4-dioxygenase activity for PCBs but hardly showed dioxygenase activity for dibenzo-p-dioxin. pSSF2202T and pSSF2202N also failed to attack dibenzothiophene and fluorene. Thus, Val-376 is significantly important in multifunctional oxygenase activities.

The change of Thr-376 to Val (Bph Dox from pSSF2707V) in the original KF707 Bph Dox also dramatically improved the degradation capacity for various phenyl-related compounds and selection resulted in the emergence of novel Bph Dox that acquired novel degradation capabilities.

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neered for enhanced activities and expanded substrate ranges. Directed evolution can be one of the most effective tools for this purpose.

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