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# Enhanced Biodegradation of Polychlorinated Biphenyls after Site-Directed Mutagenesis of a Biphenyl Dioxygenase Gene

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**Biphenyl dioxygenase catalyzes the first step in the aerobic degradation of polychlorinated biphenyls (PCBs). The nucleotide and amino acid sequences of the biphenyl dioxygenases from two PCB-degrading strains (*Pseudomonas* sp. strain LB400 and *Pseudomonas pseudoalcaligenes* KF707) were compared. The sequences were found to be nearly identical, yet these enzymes exhibited dramatically different substrate specificities for PCBs. Site-directed mutagenesis of the LB400 *bphA* gene resulted in an enzyme combining the broad congener specificity of LB400 with increased activity against several congeners characteristic of KF707. These data strongly suggest that the BphA subunit of biphenyl dioxygenase plays an important role in determining substrate selectivity. Further alteration of this enzyme can be used to develop a greater understanding of the structural basis for congener specificity and to broaden the range of degradable PCB congeners.**

Polychlorinated biphenyls (PCBs) are a group of synthetic compounds composed of biphenyl molecules containing from 1 to 10 chlorines. The vast majority of PCBs in the environment are derived from commercial mixtures (e.g., Aroclors) which contain 60 to 80 different congeners (20). Bacteria able to aerobically degrade PCBs are relatively common; however, in most cases these organisms have narrow congener specificity and are able to degrade only a small number of lightly chlorinated PCBs (1, 2, 5, 6, 14, 21, 22). In contrast, some strains, such as *Pseudomonas* sp. strain LB400, are capable of degrading a broad spectrum of PCB congeners, up to and including some hexachlorobiphenyls (3).

We have previously demonstrated that differences in congener specificity between bacteria reflect differences in the *bph* genes that encode the PCB-degrading enzymes. By Southern hybridization, a variety of PCB-degrading strains lacking the broad substrate specificity of LB400 were shown to contain *bph* genes that were significantly different from those of LB400 (24). *Alcaligenes eutrophus* H850, a strain with congener specificity nearly identical to that of LB400, was found to contain very similar *bph* genes.

The major pathway for PCB degradation is initiated by the insertion of two atoms of oxygen at carbon positions 2 and 3 by biphenyl dioxygenase (9, 11, 17). Biphenyl dioxygenase is a multicomponent enzyme (4, 11, 23), and the genes encoding these components in *Pseudomonas* sp. strain LB400 (4) and *Pseudomonas pseudoalcaligenes* KF707 (23) have been sequenced. Through comparisons of these sequences and the degradative competences of LB400 and KF707, we now report that distinctly different substrate specificities for PCBs can result from relatively few differences in nearly identical sets of *bph* genes. This information has allowed us to identify and modify a region of the biphenyl dioxygenase that affects which PCB congeners are degraded.

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## MATERIALS AND METHODS

**Bacterial strains.** *Pseudomonas* sp. strain LB400 is a PCB-degrading bacterium that was isolated from PCB-contaminated soil in upstate New York (3). *P. pseudoalcaligenes* KF707 is a PCB-degrading bacterium that was isolated near a biphenyl-manufacturing facility in Japan (7). KF707 was obtained from the Fermentation Research Institute (Agency of Industrial Science and Technology, Tsukuba, Ibaraki, Japan) as strain FERM P-8297. FM7415 is a derivative of KF707 and carries the PCB degradation genes of LB400 on plasmid pGEM415 ($Kn^r$), in addition to the KF707 PCB degradation genes on the chromosome (this paper). FM4560 is *Escherichia coli* TB1(pGEM456) (15) and contains the *bphAEFGBC* genes from LB400 that encode the first three enzymes in the PCB degradation pathway. BDE335-5 is *E. coli* TB1(pGEM456-335). pGEM456-335 is a derivative of pGEM456 resulting from site-directed mutagenesis of *bphA* (this paper).

**Culture conditions.** LB400 and KF707 were grown in PAS minimal medium (2) with biphenyl added as the sole carbon source (melted and deposited on the bottom of the flask prior to the addition of media). FM7415 was grown in PAS plus biphenyl, with the addition of kanamycin (40 μg/ml). FM4560 and BDE335-5 were grown in PAS supplemented with 0.042% proline, 52.5 μg of thiamine per ml, 0.4% succinate, and 200 μg of ampicillin per ml. Cells were grown at 30°C to an optical density at 615 nm of 1.0 prior to harvesting for resting-cell assays.

**Sequence analysis.** The nucleotide sequences were retrieved from the GenBank data base under the file names PSEBPHA (LB400, accession no. M86348) (4) and PSEBPHABC (KF707, accession no. M83673) (23). The amino acids sequences were retrieved from the translated GenBank data base under the names PSEBPHA_2, _3, _5, and _6 (LB400) and PSEBPHABC_1, _2, _4, and _5 (KF707), corresponding to each of the biphenyl dioxygenase subunits. The sequences were compared by using the LaserGene package

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TABLE 1. Comparison of the genes and gene products of LB400 and KF707 biphenyl dioxygenase

| Genes | Subunit | No. of nucleotides | % Nucleotide conservation | No. of amino acids encoded | % Amino acid conservation |
|---|---|---|---|---|---|
| *bphA*, *bphA1* | Terminal dioxygenase large subunit | 1,380 (1,377)[a] | 96.3 | 459 (458)[a] | 95.6 |
| *bphE*, *bphA2* | Terminal dioxygenase small subunit | 567 | 99.6 | 188 | 99.5 |
| *bphF*, *bphA3* | Ferredoxin | 330 | 100 | 109 | 100 |
| *bphG*, *bphA4* | Reductase | 1,227 | 100 | 408 | 100 |

[a] Initial values apply to *bphA*; numbers in parentheses apply to *bphA1*.

from DNASTAR (Madison, Wis.). Initial comparison of the ferredoxin and reductase subunits showed single amino acid differences in each. Reexamination of the LB400 sequencing gels showed errors in the published LB400 sequences at positions 4184 and 4185 (TC correct) and 5315 and 5316 (CG correct). These corrections rendered the nucleotide and amino acid sequences of LB400 and KF707 identical for these subunits.

**Resting-cell assays.** PCB degradative competence was determined by resting-cell assays (2). Bacterial cells were pelleted and resuspended in 50 mM $KPO_4$ (pH 7.5) to an optical density at 615 nm of 1.0. One milliliter of cell suspension was transferred to an 8-ml vial, and one of two PCB congener mixes (mix 1B or 2B) was added to a final concentration of 0.5 μM for each congener. Control vials contained cells that had been inactivated with 0.7% perchloric acid prior to the addition of the PCBs. The cell and PCB mixture was shaken for 24 h at 30°C and then extracted with 4 ml of 1:1 ether-hexane. PCB analysis was performed by gas chromatography with electron capture detection on a DB-1 column (J & W Scientific, Inc., Folsom, Calif.) (30 m by 0.25 mm [inner diameter]). The percent degraded for each congener was calculated relative to the acid-killed control, and the results on the individual mixes were then combined for presentation.

**Site-directed mutagenesis.** Site-directed mutagenesis was performed by using the Transformer kit (CLONTECH Laboratories, Inc., Palo Alto, Calif.) according to the manufacturer's instructions, with the exception that the oligonucleotides were in approximately 200-fold molar excess to the template. The selection and mutagenesis oligonucleotides were obtained from Operon Technologies, Inc. (Alameda, Calif.). pGEM456 was used as the mutagenesis template, and successful mutations were identified by DNA sequencing.

## RESULTS AND DISCUSSION

Biphenyl dioxygenase is believed to play a critical role in determining PCB congener specificity, as it catalyzes the first step in the oxidative pathway (conversion of the PCB to a chlorinated dihydrodiol). We compared the sequences of the *Pseudomonas* sp. strain LB400 and *P. pseudoalcaligenes* KF707 biphenyl dioxygenases to determine their relationship, with the hope of gaining insight into the structure-function relationship of these enzymes.

The published dioxygenase sequences of LB400 and KF707 have a region of approximately 4,750 bp in common, with the overall sequence similarity in excess of 97% identical nucleotides. Structurally, both dioxygenases are multicomponent enzymes comprising at least four subunits, which correspond to related components that make up the toluene dioxygenase of *Pseudomonas putida* F1 (4, 23, 25). In LB400 and KF707, these components have been designated BphA and BphA1 (large subunit of the terminal dioxygenase), BphE and BphA2 (small subunit of the terminal dioxygenase), BphF and BphA3 (ferredoxin), and BphG and BphA4, (reductase), respectively. Table 1 summarizes a comparison of the nucleotide and amino acid sequences of these components. The nucleotide sequence conservation varies from 96.3 to 100%, indicating that these genes are very closely related. Even the comparison of *bphA* and *bphA1*, the least conserved of these genes, shows long stretches of identical sequence. With the exception of two differences in the first 10 nucleotides, all of the differences are clustered within a 421-bp region. This gives identical regions of 699 bp near the amino terminus and 250 bp at the carboxy terminus (data not shown). The degree of similarity between the dioxygenases of LB400 and KF707 and the absence of silent mutations in the conserved regions suggest that these two sequences are very recently diverged.

As expected from the nucleotide sequence analysis, the amino acid sequences of the biphenyl dioxygenase subunits from LB400 and KF707 also show a high degree of conservation. BphE differs from BphA2 at a single amino acid, while BphF (ferredoxin) and BphG (reductase) are identical to the homologous KF707 proteins. LB400 BphA differs from KF707 BphA1 at 20 positions, including 19 amino acid substitutions and one glycine deletion in BphA1 (Fig. 1). All but one of these differences are clustered within a 141-amino-acid region located between residues 237 and 377. The BphA protein is proposed to be a Rieske iron sulfur protein (10, 19) with the [2Fe-2S] cluster organized by two cysteine-histidine pairs at amino acids 100, 102, 120, and 123 (4). These residues are located within the longest conserved region, from residues 5 to 236.

In order to determine whether the amino acid differences in LB400 and KF707 biphenyl dioxygenase affect the function of the enzymes, we compared the degradative competence of these strains against two mixtures of PCB congeners in resting-cell assays (Table 2). LB400 and KF707 were found to have distinctly different PCB-degrading abilities in that the range of congeners degraded by LB400 is much greater than that of KF707. Of the 19 PCB congeners present in the two mixtures, 17 are degraded by LB400 while only 8 are attacked by KF707. The congeners degraded by KF707 were more lightly chlorinated, with none containing greater than four chlorines. Against the double-*para*-substituted PCBs 4,4′-chlorobiphenyl and 2,4,4′-chlorobiphenyl, KF707 had greater activity than did LB400, indicating that the differences in congener specificity are not due to generally reduced levels of degradative activity. It is of particular interest that KF707 was unable to degrade 2,5,2′,5′-chlorobiphenyl, a congener readily degraded by LB400 but resistant to most other PCB-degrading bacteria. Oxidation of 2,5,2′,5′-chlorobiphenyl by LB400 involves a 3,4-dioxygenase activity that results in the formation of a chlorinated 3,4-dihydrodiol (17). There is preliminary evidence that

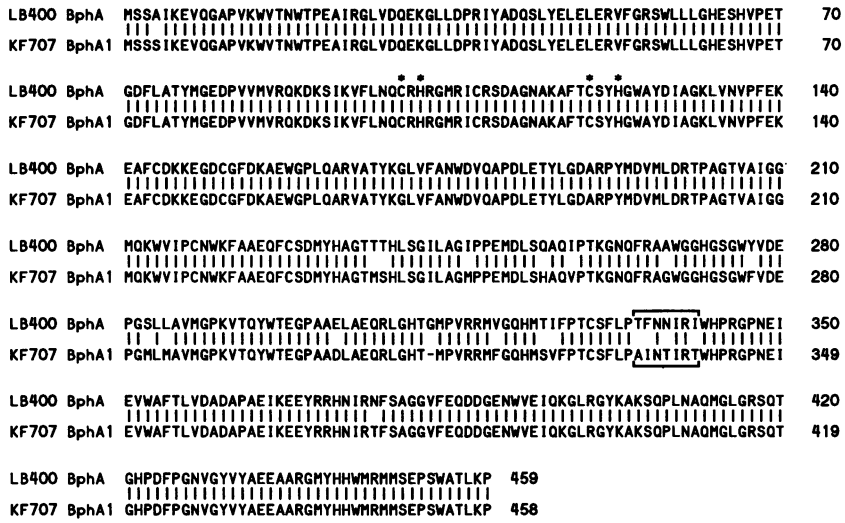


FIG. 1. Alignment of the deduced amino acid sequences of *Pseudomonas* sp. strain LB400 BphA and *P. pseudoalcaligenes* KF707 BphA1. The alignment was generated by using the ALIGN program of the LaserGene software package (DNASTAR). The sequences were obtained from the translated GenBank data base, release 73, under the names PSEBPHA_2 and PSEBPHABC_1, respectively. Identical amino acids are indicated by vertical lines. The cysteine and histidine residues thought to organize the Rieske-type [2Fe-2S] cluster are indicated by asterisks. The brackets indicate the location of the four amino acid differences that were targeted for site-directed mutagenesis.

biphenyl dioxygenase is responsible for both the 2,3- and 3,4-dioxygenase activities. In strains of LB400 containing mutated or deleted biphenyl dioxygenase genes, both activities are lost simultaneously (16). In addition, both activities are present in recombinant strains containing the *bphAEFG* genes (15). The absence of 3,4-dioxygenase activity in KF707 is another indication of significant differences between the two dioxygenases.

The differences in the congener selectivity of LB400 and KF707 could reflect functional differences between their respective biphenyl dioxygenase enzymes or be the result of host-related factors such as membrane permeability. To address this question, we transferred pGEM415, a wide-host-range plasmid carrying 11.6 kb of LB400 DNA encoding the genes for the biphenyl dioxygenase, dihydrodiol dehydrogenase, and catechol dioxygenase, into KF707 via conjugation using the narrow-host-range mobilizing vector R64drd11. pGEM415 was constructed by partial *Eco*RI digestion of pGEM410 (15) followed by reclosure. We then tested the congener specificity of the KF707 transconjugant, FM7415, using resting-cell assays with PCB mixtures 1B and 2B as previously described. The congener specificity displayed by FM7415 was much broader than that of the wild-type KF707 and was similar to that of LB400 (Table 2). This suggests that substrate specificity differences between LB400 and KF707 biphenyl dioxygenase are related to the structural differences in the dioxygenases and not the strain background.

TABLE 2. PCB degradation by LB400 and KF707 in resting-cell assays

| Congener | % Depletion by: | | |
|---|---|---|---|
| | LB400 | KF707 | FM7415 |
| 2,3 | 100 | 100 | 100 |
| 2,2′ | 100 | 5 | 100 |
| 2,4′ | 100 | 100 | 100 |
| 2,5,2′ | 100 | 0 | 100 |
| 2,5,4′ | 94 | 83 | 100 |
| 2,3,2′,3′ | 94 | 60 | 85 |
| 2,3,2′,5′ | 96 | 0 | 84 |
| 2,5,2′,5′ | 95 | 0 | 84 |
| 2,5,3′,4′ | 83 | 0 | 77 |
| 2,4,5,2′,5′ | 73 | 0 | 32 |
| 2,3,4,2′,5′ | 58 | 0 | 28 |
| 2,4,5,2′,3′ | 38 | 0 | 20 |
| 4,4′ | 15 | 100 | 100 |
| 2,4,4′ | 45 | 93 | 88 |
| 2,4,3′,4′ | 16 | 24 | 34 |
| 2,4,2′,4′ | 38 | 0 | 30 |
| 3,4,3′,4′ | 0 | 0 | 0 |
| 2,4,5,2′,4′,5′ | 18 | 0 | 10 |
| 2,4,6,2′,4′ | 0 | 0 | 0 |

Since the amino acid sequences of BphE, BphF, and BphG and their respective KF707 homologs are identical or nearly so, it is likely that the 19 differences within the 141-amino-acid region of BphA and BphA1 are responsible for differences in substrate specificity between LB400 and KF707 biphenyl dioxygenases. If this is the case, then conversion of the LB400 sequence to the KF707 sequence in this region should alter the LB400 dioxygenase so that its congener specificity is more like that of KF707. Alternatively, conversion of some of the differences could result in an enzyme with characteristics of both these dioxygenases. Through the use of site-directed mutagenesis, several modifications in the LB400 biphenyl dioxygenase sequence were made and tested for their effects on the congener specificity of the enzyme. Alterations were made to pGEM456, a recombinant plasmid containing the LB400 genes for biphenyl dioxygenase (*bphAEFG*), dihydrodiol dehydrogenase (*bphB*), and dihydroxybiphenyl dioxygenase (*bphC*). It has previously been reported that this plasmid, when transformed into *E. coli*, confers PCB-degradative capability similar to that of LB400 (15). The mutagenesis primer was 5′-ATTCCTGCCCGCCATCAACACCATCCGGACCTG GCACCCG-3′, with the underlined nucleotides conferring the amino acid changes. The selection primer was 5′-TCGA CTCTAGCGGCCGCCCGGGTACC-3′, which eliminated

TABLE 3. PCB degradation by *E. coli* containing the modified biphenyl dioxygenase

| Congener | % Depletion by: | | |
|---|---|---|---|
| | FM4560 | KF707 | BDE335-5 |
| 2,3 | 100 | 100 | 100 |
| 2,2′ | 100 | 5 | 100 |
| 2,4′ | 100 | 100 | 100 |
| 2,5,2′ | 100 | 0 | 97 |
| 2,5,4′ | 94 | 83 | 94 |
| 2,3,2′,3′ | 87 | 60 | 89 |
| 2,3,2′,5′ | 87 | 0 | 87 |
| 2,5,2′,5′ | 86 | 0 | 82 |
| 2,5,3′,4′ | 75 | 0 | 77 |
| 2,4,5,2′,5′ | 56 | 0 | 73 |
| 2,3,4,2′,5′ | 15 | 0 | 15 |
| 2,4,5,2′,3′ | 20 | 0 | 63 |
| 4,4′ | 13 | 100 | 100 |
| 2,4,4′ | 33 | 93 | 87 |
| 2,4,3′,4′ | 10 | 24 | 35 |
| 2,4,2′,4′ | 14 | 0 | 19 |
| 3,4,3′,4′ | 0 | 0 | 5 |
| 2,4,5,2′,4′,5′ | 15 | 0 | 15 |
| 2,4,6,2′,4′ | 0 | 0 | 0 |

unique *Bam*HI and *Xba*I sites in the pUC18 polylinker of pGEM456 and created a unique *Not*I site. This mutagenesis procedure altered a region of the LB400 *bphA* gene encoding a block of four amino acid differences with KF707 at positions 335, 336, 338, and 341. The region of amino acids 335 to 341 in LB400 BphA (TFNNIRI) was converted to the corresponding KF707 sequence (AINTIRT) at the underlined positions. The resulting plasmid, pGEM456-335, was transformed into *E. coli* TB1 to construct the strain BDE335-5. This mutant strain was tested for PCB-degradative ability by resting-cell assays in comparison with FM4560 [*E. coli* TB1(pGEM456)] (15). As shown in Table 3, alterations in the BphA subunit of the LB400 biphenyl dioxygenase significantly modified the PCB congener specificity of the enzyme. The result is a novel dioxygenase combining the broad substrate specificity of LB400 with the superior ability of KF707 to degrade some double-*para*-substituted congeners.

Multicomponent dioxygenases play a crucial role in the bacterial degradation of naturally occurring and xenobiotic compounds (12, 18). By catalyzing the incorporation of two hydroxyl groups into the aromatic ring, these enzymes increase the reactivity of these compounds, making them susceptible to enzymatic ring fission reactions. Many highly chlorinated compounds (including numerous PCB congeners) are resistant to aerobic biodegradation because of the inability of bacterial dioxygenases to accept them as substrates. It is therefore important to develop a greater understanding of dioxygenase structure and the factors that influence congener specificity.

The biphenyl dioxygenases of strains LB400 and KF707 exhibit dramatic differences in PCB substrate range despite nearly identical amino acid sequences. Similar comparisons have been reported by D. T. Gibson and coworkers (8). The differences in biphenyl dioxygenase activity result from amino acid differences in the large subunit of the terminal dioxygenase (the *bphA* gene product) and are clustered within a 141-amino-acid region. The remainder of the proteins are essentially identical, lacking even silent nucleotide changes in their genes. With no obvious constraints to cause the strong nucleotide conservation, this suggests that the two proteins have recently diverged and have accumulated amino acid differences in one major region.

We have shown that it is possible to modify the PCB congener specificity of the biphenyl dioxygenase by site-directed mutagenesis within this divergent region of the BphA subunit. These mutations have yielded an enzyme with the strengths of both the KF707 and LB400 biphenyl dioxygenases, combining the broad congener specificity of LB400 with high activity against several double-*para*-substituted congeners from KF707. These results suggest that it may be possible to expand the range of biodegradable PCB congeners. This would greatly facilitate the development of effective bioremediation processes for sites contaminated with the more highly chlorinated PCB mixtures that currently resist oxidation by the best available PCB-degrading bacteria. Dioxygenase modifications may also result in increased activity levels on a variety of currently degradable congeners. Since the multicomponent dioxygenases that catalyze the degradation of diverse compounds such as PCBs, toluene, benzene, and trichloroethylene are closely related in structure and function (4, 13, 23, 25), it may be possible to apply the results of these studies toward understanding and improving the degradation of a broad range of environmental pollutants.

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