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Subcloning of *bph* Genes from *Pseudomonas testosteroni* B-356 in *Pseudomonas putida* and *Escherichia coli*: Evidence for Dehalogenation during Initial Attack on Chlorobiphenyls

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The *bphA*, *-B*, *-C*, and *-D* genes from *Pseudomonas testosteroni* B-356 were mapped to a 5.5-kb DNA fragment of cloned plasmids pDA1 and pDA2 by use of deletion and insertion mutants of these plasmids. The expression of each of these genes was evaluated in *Escherichia coli* and in *Pseudomonas putida*, and it was found that the *bphC* and *bphD* genes are well expressed in both *E. coli* and *P. putida* cells while the *bphA* and *bphB* genes are very poorly expressed in *E. coli*, even when placed downstream of a *tac* promotor. *P. putida* clones carrying the *bphA* gene were used to study the metabolites produced from 4,4′-dichlorobiphenyl, 2,2′-dichlorobiphenyl, and 2,4′-dichlorobiphenyl. It was shown that dehalogenation of 4-Cl and 2-Cl occurs in the course of the initial oxygenase attack on these molecules, which always occurs on carbons 2 and 3, independently of the positions of the chlorine atoms. Our data also suggest that in the case of polychlorobiphenyl congeners carrying chlorine atoms on both rings, it appears that, depending on the chlorine positions, dioxygenation will occur predominantly on one ring over the other. However, attack of the more resistant ring is not excluded, resulting in multiple conversion pathways.

Polychlorobiphenyls (PCBs) are widespread persistent pollutants. Studies with various microorganisms indicated that low-chlorine-content PCBs are degraded through the pathway illustrated in Fig. 1; the substrate is converted first through cyclic peroxide intermediates into dihydrodiols and then through a dioxygenase-catalyzed reaction into catechol derivatives. The catechol metabolites are further transformed to 2-hydroxy-6-oxo-6-(chlorophenyl)hexa-2,4-dienoic acids, the *meta*-cleavage product (MCP), and ultimately to chlorobenzoic acids (CBAs). *Pseudomonas testosteroni* B-356 is a soil isolate that can degrade several PCB congeners at rates that are affected by the position of chlorine on the molecules. We recently cloned the *bph* genes from this strain (1) and constructed subclones carrying genes for part of the pathway only.

Bacteria carrying the *bph* gene cluster completely degrade monochlorobiphenyls when they are cocultured with bacteria able to degrade CBAs (23). However, in situations in which CBAs accumulate in the growth medium, the *bph* genes are not the only ones operating, since PCBs are then converted into a number of phenolic and acidic metabolites that are not expected as part of the pathway controlled by this gene cluster (14). In the case of monochlorobiphenyls, many transformation pathways involved in the production of these metabolites were recently elucidated by use of *Pseudomonas putida* clones carrying *bph* genes from *P. testosteroni* B-356 (2). Although the metabolites produced during the conversion of monochlorobiphenyls and their mechanism of formation are now well understood, little is known about the metabolites that are generated from PCB congeners carrying chlorine atoms on both rings.

On the basis of the published data, it appears that CBAs are the ultimate metabolites of most PCB congeners. However, the presence of some acidic metabolites with shorter side chains has been used as evidence for 3,4-dihydroxylation of the *ortho*-substituted biphenyl rings in some PCB-degrading strains (4, 15, 16). At this time, it is not clear whether the oxygenase attack on *ortho*-substituted PCB congeners always proceeds through an initial 3,4-dioxygenation of the molecule or whether other types of initial attack could occur in some strains. In the present study, we constructed subclones carrying part of the *bph* gene cluster from *P. testosteroni* B-356, mapped the genes and evaluated their expression in *Escherichia coli* and in *P. putida*, and identified the metabolites produced from dichlorobiphenyls bearing chlorine atoms on both rings by clones carrying the *bphA* gene. Interestingly, our data show that the initial oxygenase attack on chlorobiphenyl (CBP) molecules by the strain B-356 *bphA* gene product can involve dehalogenation of the molecules. The implications of this finding are discussed.

MATERIALS AND METHODS

Bacterial strains and culture media. The two bacterial strains used as host strains in this work were *E. coli* DH1 and *P. putida* KT2440 (3). The parent strain, *P. testosteroni* B-356, has been described previously (1). The minimal medium used was MM 30 (22). LB broth (13) and nutrient broth (Difco, Detroit, Mich.) were also used when required. All CBP congeners were from Ultra Scientific Inc. (Hope, R.I.), except for 4-chlorobiphenyl (4CBP), which was from Aldrich Chemicals (Milwaukee, Wis.). All other chemicals were of the highest purity grade. A 4CBP MCP was produced by use of *E. coli* B-530 carrying plasmid pDA251 as described previously (2). The protein concentration was determined by the Lowry protein assay (21).

Subcloning and other DNA manipulation techniques. The clones used in this paper were subclones derived from pDA1 or pDA2 (1) carrying biphenyl (BP) or PCB degradation genes from *P. testosteroni* B-356. Plasmid pDA1 carries all

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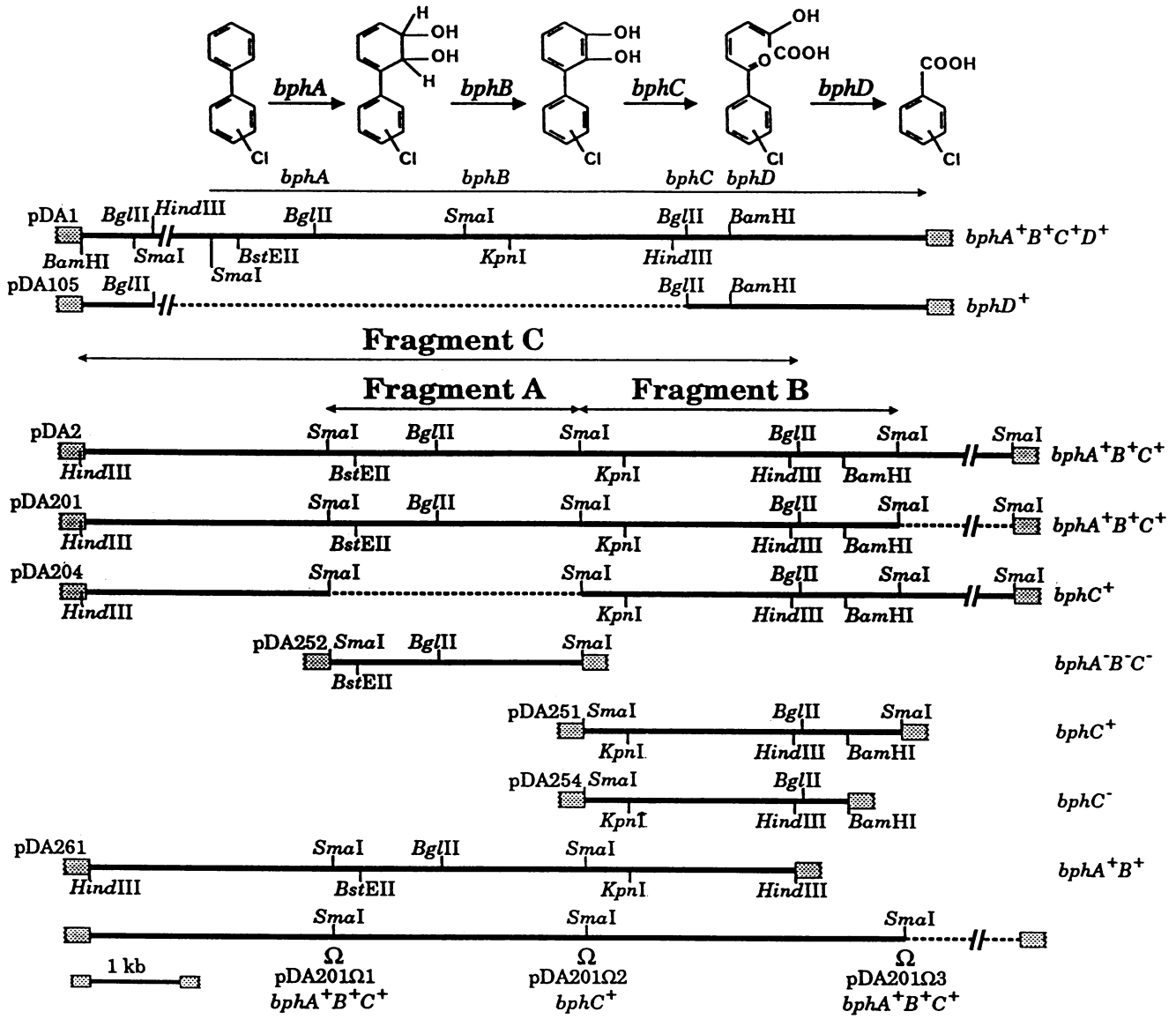

FIG. 1. Biochemical steps involved in the transformation of CBPs into CBAs and organization of the *bphABCD* gene cluster involved in PCB degradation. Symbols: —, insert; ▨, vector.

four genes required to transform CBPs into chlorobenzoic acids (Fig. 1), while plasmid pDA2 carries the *bphA*, *bphB*, and *bphC* genes, which are required to transform CBPs into acidic MCPs (2). Subclones were constructed by introducing endonuclease restriction fragments from pDA1 or pDA2 into either pUC18 or pMMB66 (8). The characteristics of the various plasmids used in this paper are listed in Table 1, and their construction is presented in Fig. 1.

Restriction endonuclease digestions, ligations, and agarose gel electrophoresis were all done by the procedures of Maniatis et al. (13) or Sambrook et al. (18). Plasmid DNA was isolated by the preparation method of Birnboim (5). Transformation of *E. coli* cells was done by the procedure described by Hanahan (10), and transformation of *Pseudomonas* cells was done as described previously (1). Insertion of an omega fragment (17) was performed as described by Frey and Krish (7).

Analysis of B-356 metabolites produced from dichlorobiphenyls. *P. testosteroni* B-356 was grown to the log phase on BP (0.25% [wt/vol]) in MM 30. The cultures were filtered on glass wool to remove BP particles, and the cell density was adjusted by spectrophotometric measurement at 600 nm. The substrate was added as particulate material, and the resting cell suspensions were incubated with agitation at 29°C for 16 to 72 h before analysis. The metabolites were extracted with ethyl acetate at pH 7 and 3. Gas chromatographic (GC) and GC-mass spectral (MS) analyses of trimethylsilyl (TMS)-derivatized metabolites were done as described previously (2).

Oxygen uptake rates for selected substrates were evaluated with oxygen electrodes (YSI model 53 biological oxygen monitor; Yellow Springs Instrument Co., Yellow Springs, Ohio) at 30°C. The reaction mixture contained 2.9 ml of phosphate buffer (20 mM, pH 7.1), 1 ml of resting cell

TABLE 1. Plasmids used in this study

Plasmid	Size (kb)	Genotype or phenotype[a]	Source or reference
pUC18	2.68	Ap^r	20
pPSA842	15.3	Ap^r Sm^r Su^r Tc^r Mob^+ *cos*	19
pMMB66	8.9	Ap^r Sm^r Su^r	8
pMMB66-Ω	10.9	Ap^r Sm^r Su^r Sp^r Mob^+ pMMB66 with insertion	This study
pDA1[b]	36.9	In pPSA842, Ap^r Sm^r Su^r Mob^+ *cos bphA bphB bphC bphD*	1
pDA2[b]	40.2	In pPSA842, Ap^r Sm^r Su^r Mob^+ *cos bphA bphB bphC*	1
pDA201[b]	24.5	In pPSA842, Ap^r Sm^r Su^r Mob^+ *cos bphA bphB bphC*	1
pDA201Ω1[b]	26.5	In pPSA842, Ap^r Sm^r Su^r Sp^r Mob^+ *cos*	This study
pDA201Ω2[b]	26.5	In pPSA842 Ap^r Sm^r Su^r Sp^r Mob^+ *cos bphC*	This study
pDA201Ω3[b]	26.5	In pPSA842, Ap^r Sm^r Su^r Sp^r Mob^+ *cos bphA bphB bphC*	This study
pDA261[b]		In pMMB66Ω, Ap^r Su^r Sp^r *bphA bphB*	This study
pDA262[b]		In pMMB66Ω, Ap^r Su^r Sp^r *bphA bphB*	This study
pDA251[b]		In pUC18, Ap^r *bphC*	This study
pDA252[b]		In pUC18, Ap^r 2.7-kb *Sma*I fragment from pDA2	This study
pDA254[b]		In pUC18, Ap^r, 3-kb *Sma*I-*Bam*HI fragment from pDA2	This study
pDA105[b]		In pPSA842, Ap^r Sm^r Su^r Mob^+ *cos bphD*	This study
pDA204[b]		In pPSA842, Ap^r Smr^r Su^r Mob^+ *cos bphC*	This study

[a] Ap^r, ampicillin resistance; Sm^r, streptomycin resistance; Su^r, sulfathiazole resistance; Sp^r, spectinomycin resistance; Mob^+, can be mobilized; *cos*, phage λ cohesive ends (DNA fragment carrying *cos*); *bphA*, *bphB*, *bphC*, and *bphD*, refer to Fig. 1 for functions (the DNA fragments carrying the respective genes).
[b] See Fig. 1 for construction.

suspension (optical density at 600 nm, 2.0), and 0.1 ml of substrate dissolved in dimethyl sulfoxide to yield a final concentration of 3 mM in the reaction vessel. The *meta*-cleavage enzyme (1,2-dihydroxybiphenyl 2,3-oxygenase) activity was determined spectrophotometrically at 434 nm (9).

RESULTS

Construction of subclones and localization of the *bphA*, *-B*, *-C*, and *-D* genes on a cloned DNA fragment. Deletion mutants (carrying plasmids pDA105, pDA201, and pDA204) and insertion mutants (carrying plasmids pDA201Ω1, pDA 201Ω2, and pDA201Ω3) of pDA1 and pDA2 and subclones in pMMB66 (pDA261) or pUC18 (pDA251, pDA252, and pDA254) were analyzed in *P. putida* KT2440 and in *E. coli* to map the *bphA*, *-B*, *-C*, and *-D* genes. It was previously shown that plasmid pDA201 was the smallest deletion clone from pDA2 (1) capable of transforming CBPs into corresponding MCPs (Fig. 1). Therefore, this subclone carries functional *bphA*, *-B*, and *-C* genes.

P. putida(pDA261) (Fig. 1) produced 2,3-dihydroxy-4′-chlorobiphenyl (2,3-diOH-4′-CBP) from 4CBP and 2,3-dihydroxy-2′-chlorobiphenyl (2,3-diOH-2′-CBP) from 2-chlorobiphenyl, identified from previously published GC-MS data (14). Since the dihydroxy derivatives are the only metabolites produced by *P. putida*(pDA261) from CBPs, the implication is that pDA261 carries only functional *bphA* and *bphB* genes. Therefore, the *bphA* and *bphB* genes are located on restriction fragment C (Fig. 1). Since subclone pDA252, carrying 2.7-kb *Sma*I fragment A from pDA2, did not show any activity at all and since plasmid pDA201Ω1, with an insertion in the leftmost *Sma*I site of fragment A, retained *bphA* and *bphB* activities, the implication is that the *bphA* and *bphB* genes are located in a region covering fragment A plus the *Sma*I-*Hin*dIII section of fragment B. The orientation of the insert toward the *tac* promotor affected the conversion activity, since *P. putida*(pDA262), with the insert in the opposite orientation, was unable to transform CBPs when grown in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG).

Plasmid pDA204 is a deletion mutant of pDA2 having lost *Sma*I fragment A. In MM 30, resting cell suspensions of *P. putida*(pDA204) produced MCP from 2,3-diOH-4′-CBP but was unable to transform 4CBP in either resting cell suspensions or growing cultures. Therefore, pDA204 only carries a functional *bphC* gene, located on 3.7-kb *Sma*I restriction fragment B (Fig. 1). The *bphC* gene was not inactivated by the insertion in the *Sma*I restriction sites of fragment B, since pDA201Ω3 and pDA201Ω1 were still functional. However, the insertion in the central *Sma*I site rendered pDA201Ω2 nonfunctional. Yields of MCPs from hydroxychlorobiphenyls were rather low in *P. putida*(pDA2) and *P. putida*(pDA204) because host strain KT2440 carries enzymes that can transform MCPs through various other routes (2). However, *E. coli* B-547 carrying pDA204 or *E. coli* B-530 carrying pDA251, in which 3.7-kb *Sma*I fragment B was inserted into pUC18, could both efficiently transform 2,3-dihydroxychlorobiphenyls (2,3-diOHCBPs) into the corresponding MCPs, and this bacterium did not further transform MCPs like *P. putida* did. A cell extract (diluted to 40 μg of protein per ml) of *E. coli* B-530 grown in LB broth containing IPTG (0.12 μg/ml) and ampicillin (100 μg/ml) had specific activity toward 2,3-diOH-4′-CBP of 90 nmol of substrate converted per min per mg of protein. The same cell extract concentrated to 4 mg of protein per ml was unable to degrade 3,4-dihydroxybiphenyls, showing that the enzyme only has an affinity for 2,3-dihydroxybiphenyls or 2,3-diOHCBPs. The 3,4-dihydroxybiphenyl attack was evaluated by monitoring substrate disappearance as well as the production of any subsequent metabolite by GC and GC-MS analyses. For these assays, substrate concentrations between 2 and 25 nmol/ml were used. Finally, the ability of *P. putida*(pDA105) to convert MCP into CBA and its inability to transform CBPs or 2,3-diOHCBPs confirmed that only a functional *bphD* gene is located on the 2.2-kb fragment to the left of the *Bam*HI site, which itself is located in the *bphC* gene, as indicated by the loss of the *meta*-cleavage activity in *P. putida*(pDA254) (Fig. 1). The *bphC* gene from pDA105 was expressed in *P. putida* as well as in *E. coli*, since in both cases MCP (50 nmol/ml) was converted into stoichiometric amounts of 4-chlorobenzoic acid within 2 h.

Production of dihydroxylated derivatives of dichlorobiphenyls by using subclones carrying pDA261 (*bphA bphB*). Although the *bphC* and *bphD* genes were well expressed in *E.*

coli, the expression of the *bphA* gene was poor in this organism as compared with its expression in *P. putida*. Hence, resting cell suspensions or growing cultures of *E. coli* DH1(pDA261) carrying the *bphA* and *bphB* genes in the presence of IPTG and independently of temperature (37 or 29°C) produced only trace amounts of 2,3-dihydro-2,3-dihydroxy-4'-chlorobiphenyl from 4CBP, with no detectable amounts of 2,3-diOH-4'-CBP. In contrast, *P. putida* (pDA261) very efficiently cometabolically converted monochlorobiphenyls (0.025 to 0.1% [wt/vol]) into the corresponding 2,3-diOHCBPs, with a conversion rate of 500 μg of 4CBP per h per 100 ml of cell suspension (10^8 cells per ml). The most interesting observation about the initial oxygenase reaction coded by *bphA* genes from *P. testosteroni* B-356 was its ability to dehalogenate the BP ring. When 4,4'-dichlorobiphenyl (4,4'CBP) (0.01 to 0.12% [wt/vol]) was added to a resting cell suspension of strain B-356 in the presence of a surfactant, such as dimethyl sulfoxide (0.1%) or Tween 80 (1%), about 30% of the substrate was transformed into 4-chlorobenzoic acid within 3 days of incubation. However, very few other metabolites were detected in the medium. When 4,4'CBP was fed to *P. putida* KT2440, no metabolite was detected. However, *P. putida*(pDA261) cells incubated for 18 h at 29°C on glucose MM 30 containing 0.12 μg of IPTG and 0.025% (wt/vol) 4,4'CBP produced 2,3-dihydroxy-4,4'-dichlorobiphenyl (2,3-diOH-4,4'-diCBP) and 2,3-diOH-4'CBP in approximately equal amounts. Approximately equal amounts of both metabolites (although at low levels) were also detected after 8 h of incubation. Figure 2A illustrates a GC spectrum of the metabolites produced from 4,4'CBP. From the characteristic ions of its TMS derivative at *m/z* 364 (M^+), *m/z* 349 ($M^+ - 15$), and *m/z* 276 (M^+ − TMS), one metabolite was identified as 2,3-diOH-4'-CBP (Fig. 2C), and the other metabolite, whose TMS derivative had characteristic ions at *m/z* 398 (M^+), *m/z* 383 ($M^+ - 15$), and *m/z* 310 (M^+ − TMS), was identified as 2,3-diOH-4,4'-diCBP (Fig. 2B). Because the 4,4'CBP preparation used in these experiments did not show any detectable trace of 4CBP as a contaminant upon GC-MS analysis, it is likely that 2,3-diOH-4'-CBP is produced by dehalogenation of 4,4'-CBP.

As determined by oxygen uptake measurements, the rate of transformation of 2,2'-dichlorobiphenyl (2,2'CBP) by *P. testosteroni* B-356 was much lower than the rate of transformation of 3,3'-dichlorobiphenyl or 4,4'CBP, with respective values of 2.4, 4.8, and 9.5 nmol of O_2 consumed per min when a 1-ml cell suspension with an optical density at 600 nm of 2 was added to a 3-ml reaction mixture in the electrode cell. However, a metabolite with MS characteristics and retention times identical to those of authentic 2,3-diOH-2'-CBP was detected when 2,2'CBP was fed to *P. putida* (pDA261) (Fig. 3). The host strain, *P. putida* KT2440, was unable to carry out this transformation and, again, the substrate preparation did not show any detectable trace of 2-chlorobiphenyl. Therefore, the oxygenase produced by the *bphA* gene appears to attack the *ortho*-substituted PCB congeners at the 2,3 position, with concomitant dehalogenation.

When 2,4'-dichlorobiphenyl (2,4'CBP) was used as a substrate in a *P. putida*(pDA261) culture growing on glucose, the major metabolite generated was 2,3-diOH-2'-CBP (Fig. 4), suggesting that the substrate was hydroxylated on the ring bearing the chlorine atom in position 4, with concomitant dehalogenation. Another metabolite detected in these cultures had the MS characteristics of dichlorodihydroxybiphenyl, but from the GC-MS data it was not possible to determine the ring of attack. It is, however, most likely that this metabolite is 2,3-dihydroxy-2',4-dichlorobiphenyl(2,3-diOH-2',4-diCBP), since hydroxylation at the 3,4 or 5,6 position of the BP ring has never been detected from 2,2'CBP. It is therefore likely that, as in the case of 4,4'CBP, the oxygenase attack will generate metabolites that are hydroxylated on the *para*-substituted ring, with a concomitant loss of the chlorine atoms.

Further work with strain B-356 showed that 2,4'CBP was converted into both 2-chlorobenzoate and 4-chlorobenzoate, indicating that the *ortho*-substituted BP ring is also attacked, but because 2,3-diOH-4'-CBP is not produced from 2,4'CBP by *P. putida*(pDA261), it is most likely that the *para*-substituted ring is the preferred ring of attack in the initial oxygenase reaction.

Therefore, in the case of PCB congeners carrying chlorine atoms on both rings, it appears that, depending on the chlorine position, dioxygenation will occur predominantly on one ring over the other and that, in some instances, oxygenation will be accompanied by simultaneous dehalogenation. However, attack on the more resistant ring is not excluded, resulting in multiple conversion pathways, such as the one illustrated in Fig. 5.

DISCUSSION

Gene cloning is a convenient tool for the elucidation of biodegradation pathways, since genes for each enzymatic step can be introduced into hosts which have no other catalytic activity for the substrate and which cannot further transform the product of the reaction either. We previously used clones to elucidate the conversion pathways for the MCP produced by the *pbhC* gene product (2). However, many questions still remain to be answered about the initial oxygenase attack on the CBP molecule. For instance, it is still not clear whether the *bphA* gene product is the only one involved in the hydroxylation of all PCB congeners that a single strain can degrade, and we also know very little about the site (or sites) of attack on the molecule when both rings contain chlorine atoms. The isolation of the *bphA* and *bphB* genes from *P. testosteroni* B-356 has allowed us to establish some facts about this significant reaction in strain B-356.

The first important observation about the *bphA* and *bphB* genes is that although they are part of a single gene cluster in strain B-356 which also includes the *bphC* and *bphD* genes, contrary to the other two genes, their expression is very poor in *E. coli*, as compared with that in *Pseudomonas* spp. Even the *tac* promoter on pMMB66 did not favor the expression of the *bphA* and *bphB* genes from *P. testosteroni* B-356 in *E. coli*. Recently, Khan and Walia (12) reported that *P. putida* OU83 2,3-biphenyl dioxygenase genes were expressed in *E. coli* clones at 1/10 the level of expression in the parental strain, while the *meta*-cleavage enzyme activity, on the contrary was 10 times higher in *E. coli* clones than in the parental strain. It is possible that the low water solubility of the substrate prevents its access in *E. coli* cells; however, several other factors may be involved as well. Our data surely suggest that the *bphA* and *bphB* genes could be controlled by a promotor different from the one controlling the expression of the *bphC* and *bphD* genes.

During the course of this work, the *bphA* and *bphB* genes could not be dissociated because we could not find any deletion mutants that accumulated the corresponding dihydrodiol derivatives from CBPs. On the basis of previous work with other bacterial strains (14), we have evidence that dihydrodiols can be extracted from the growth medium when

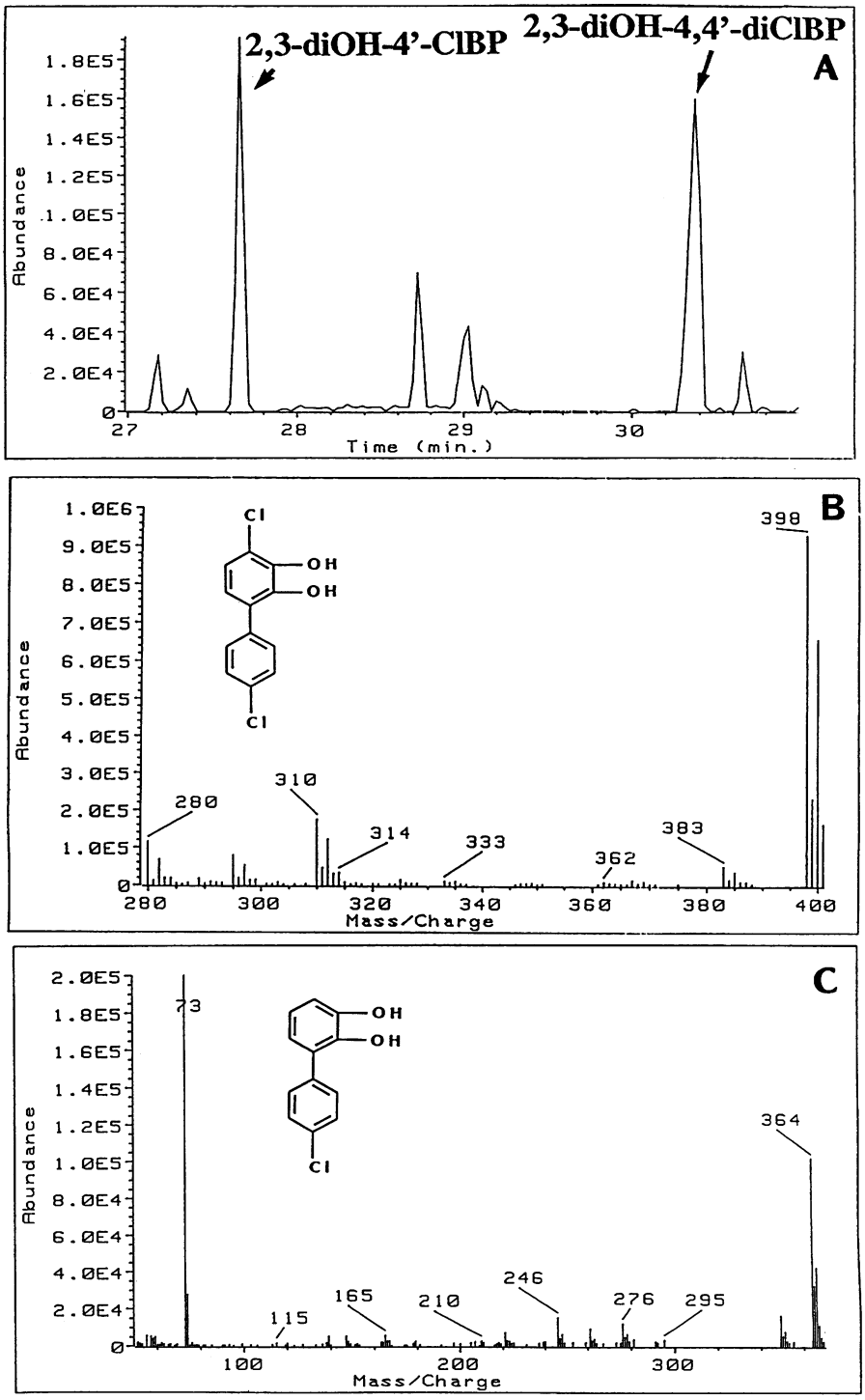

FIG. 2. (A) GC spectra of TMS-derived metabolites produced from 4,4'CBP by *P. putida*(pDA261). The metabolites were extracted from an 18-h-old culture with ethyl acetate at a neutral pH. (B) Mass spectra of the GC peak corresponding to 2,3-diOH-4,4'-diCBP–TMS. (C) Mass spectra of the GC peak corresponding to 2,3-diOH-4'-CBP–TMS.

appropriate care is taken. Therefore, it is possible that an enzymatic system in *P. putida* cells that is not associated with the *bph* gene cluster can transform dihydrodiols into the corresponding dihydroxy derivatives. Since the *bphA* gene is poorly expressed in *E. coli*, it was not possible to use this bacterium to map the *bphA* and *bphB* genes.

However, the dissociation of the *bphA* and *bphB* genes was not necessary to study the initial oxygenase attack on some monochlorobiphenyls and dichlorobiphenyls. The most interesting observation about this attack is the dehalogenation of the *para*-substituted carbon in 4,4'CBP and 2,4'CBP. Khan and Walia (11) previously showed that 4CBP

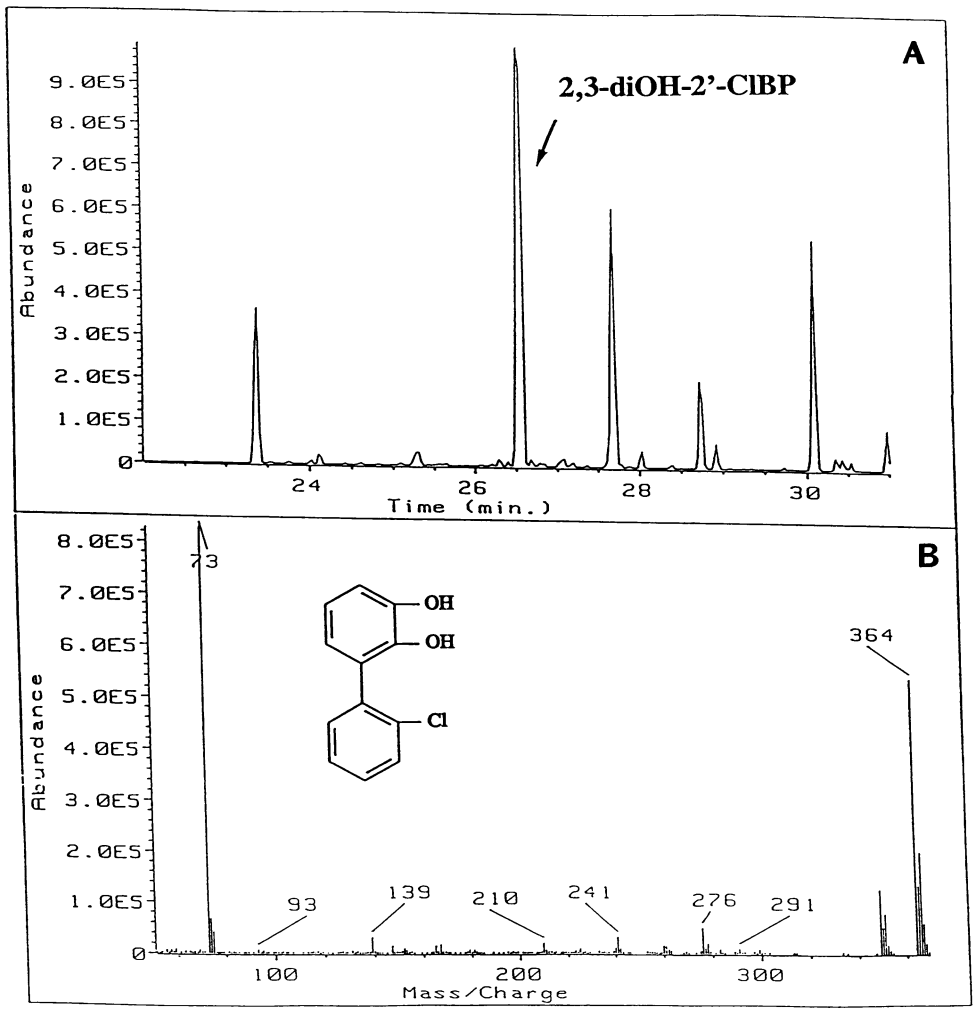

FIG. 3. (A) GC spectra of TMS-derived metabolites produced from 2,2'CBP by *P. putida*(pDA261). The metabolites were extracted from an 18-h-old culture with ethyl acetate at a neutral pH. (B) Mass spectra of the GC peak corresponding to 2,3-diOH-2'-CBP.

is converted into both 4-chlorobenzoic acid and benzoic acid by a *P. putida* OU83 clone that carries all four *bph* genes. Dehalogenation of the *para*-substituted carbon atom appears, from our work, to occur during the course of the initial oxygenase attack on the molecule, since both chlorinated and nonchlorinated dihydroxy derivatives were produced from the CBP molecules that we tested. It is probable that the peroxide generated by the oxygenase attack on carbons 2 and 3 creates an environment that destabilizes the chlorine atom in the *para* position. Because 2,3-diOH-4,4'-diCBP was also detected in the growth medium of *P. putida*(pDA261), it is possible that only part of the destabilized molecule is dehalogenated before hydrogenation of the peroxide occurs. However, the possibility that the *bphA* gene product in strain B-356 is monooxygenase cannot be excluded, since dehalogenation of the *para*-substituted chlorine is also likely to arise as a result of an NIH shift between C—3H and C—4Cl (6). In any case, the generation of two or more products from a single reaction during the enzymatic conversion of xenobiotics is not unusual, particularly when the intermediate is a very reactive molecule, as is often the case with oxygenase reactions (20). For example, the epoxide derived from the monooxygenase reaction is very unstable and is spontaneously hydrolyzed to phenolic derivatives or enzymatically transformed into dihydrodiol derivatives by an epoxide hydrase (20). Thus, in the case of CBP, it is possible that spontaneous dehalogenation of the peroxide or the dihydrodiol derivative could occur before the molecule is enzymatically converted to a more stable form.

The other important observation about the initial attack on CBP by the *bphA* and *bphB* gene products from strain B-356 is that this reaction appears always to occur on carbons 2 and 3, even in the case of *ortho*-substituted CBPs. This observation, made in *P. putida*(pDA261) was corroborated by experimentation in parental strain B-356, in which 2-chlorobenzoate was the only metabolite detected from 2,2'CBP. Any other position of initial oxygenase attack would not have generated CBA. This observation is a major distinction from the BP oxygenase reaction found in *P. putida* LB400, which appears to be able to attack the *ortho*-substituted PCBs in the 3,4 position (16).

Finally, we showed that although, for any given congener, the *bphA* gene product from strain B-356 has a preferred ring of attack which depends on the chlorine substitution, the nonpreferred ring can also be hydroxylated, resulting, for some of the congeners, in a mixture of several metabolites.

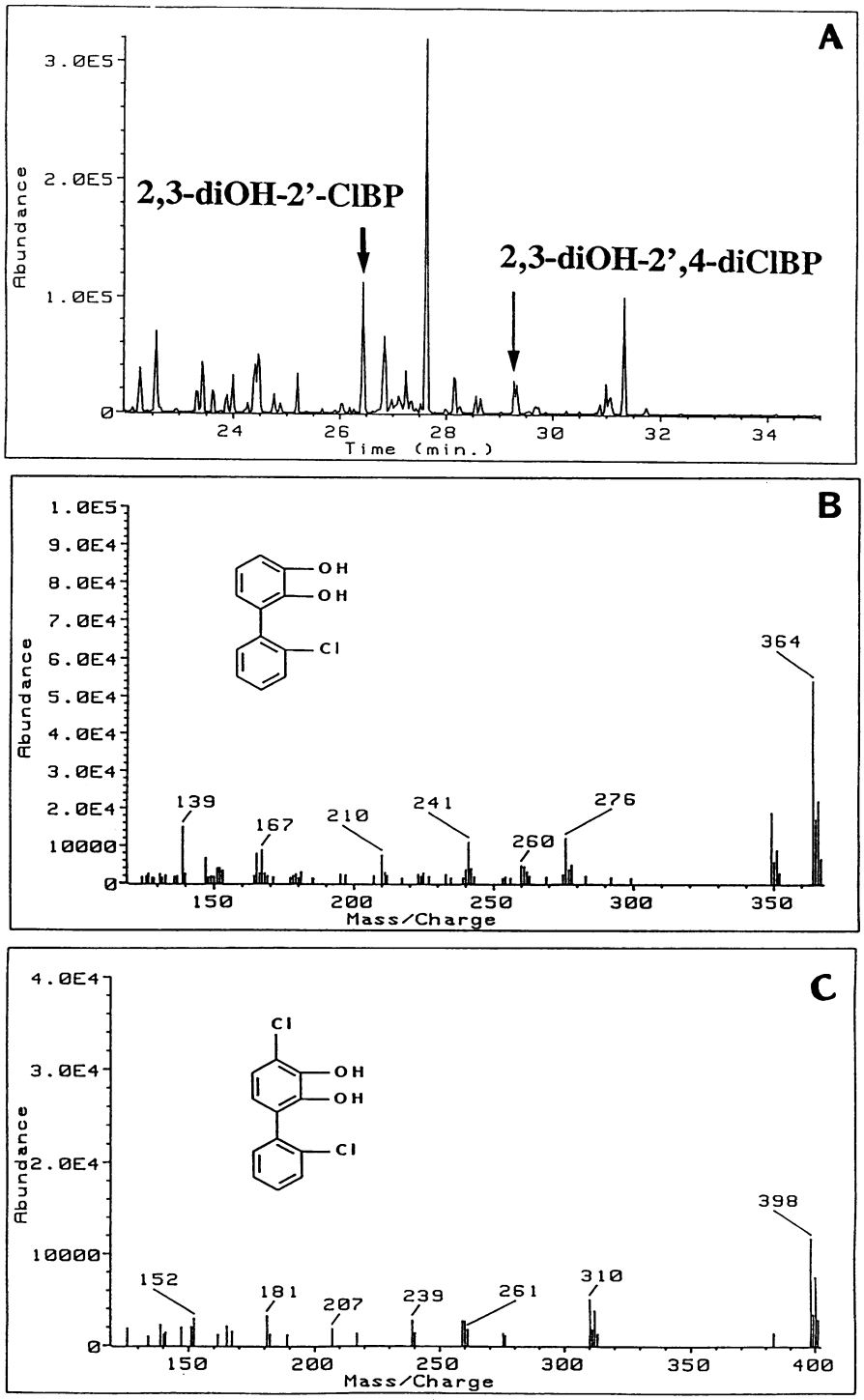

FIG. 4. (A) GC spectra of TMS-derived metabolites produced from 2,4'CBP by *P. putida*(pDA261). The metabolites were extracted from an 18-h-old-culture with ethyl acetate at a neutral pH. (B) Mass spectra of the GC peak corresponding to 2,3-diOH-2,4'-diCBP. (C) Mass spectra of the GC peak corresponding to 2,3-diOH-2'-CBP.

From the perspective of using microorganisms for the remediation of CBPs, this fact could be problematic, since such uncontrolled reactions could generate dead-end compounds that cannot be further processed through the other steps of the pathway. However, the fact that in the case of strain B-356 the oxygenase attack on PCB congeners carrying chlorine atoms on both rings is usually accompanied by *para* or *ortho* dehalogenation of the molecule is very interesting, since it may suggest that the broadening of the enzyme activity toward more-chlorinated PCBs would be sufficient

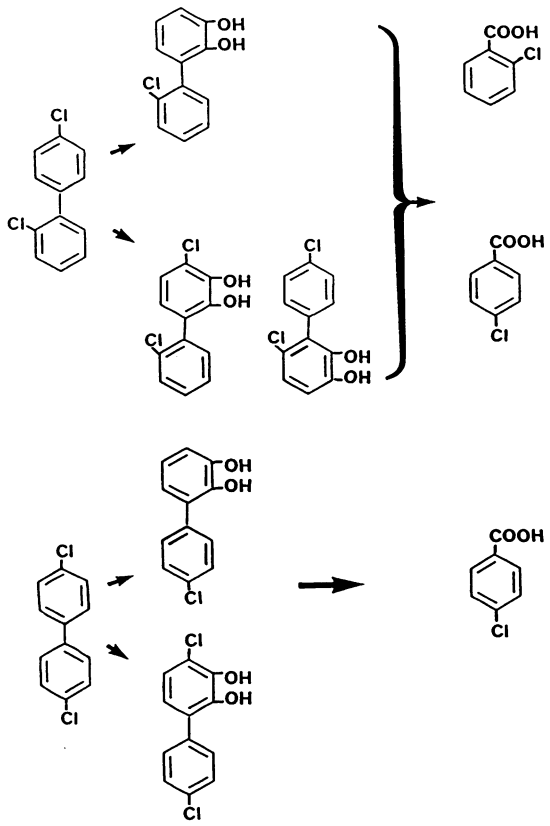

FIG. 5. Proposed patterns of conversion of dichlorobiphenyls by clones carrying the *bphA* gene from *P. testosteroni* B-356.

to improve degradation of the more resistant congeners. Work is now in progress to understand and determine the parameters affecting the mode of the initial oxygenase attack.

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