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Cloning of a Gene Cluster Encoding Biphenyl and Chlorobiphenyl Degradation in *Pseudomonas pseudoalcaligenes*

KENSUKE FURUKAWA* AND TOSHITSUGU MIYAZAKI

Fermentation Research Institute, Agency of Industrial Science and Technology, Tsukuba, Ibaraki 305, Japan

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A gene cluster encoding biphenyl- and chlorobiphenyl-degrading enzymes was cloned from a soil pseudomonad into *Pseudomonas aeruginosa* PAO1161. Chromosomal DNA from polychlorinated biphenyl-degrading *Pseudomonas pseudoalcaligenes* KF707 was digested with restriction endonuclease *Xho*I and cloned into the unique *Xho*I site of broad-host-range plasmid pKF330. Of 8,000 transformants tested, only 1, containing the chimeric plasmid pMFB1, rendered the host cell able to convert biphenyls and chlorobiphenyls to ring *meta* cleavage compounds via dihydrodiols and dihydroxy compounds. The chimeric plasmid contained a 7.9-kilobase *Xho*I insert. Subcloning experiments revealed that the genes *bphA* (encoding biphenyl dioxygenase), *bphB* (encoding dihydrodiol dehydrogenase), and *bphC* (encoding 2,3-dihydroxybiphenyl dioxygenase) were coded for by the 7.9-kilobase fragment. The gene order was *bphA-bphB-bphC*. The hydrolase activity, which converted the intermediate *meta* cleavage compounds to the final product, chlorobenzoic acids, and was encoded by a putative *bphD* gene, was missing from the cloned 7.9-kilobase fragment.

Polychlorinated biphenyls (PCBs) have become serious environmental pollutants. Their toxicity, bioconcentration, and persistence have been well documented. It has been shown that some biphenyl-utilizing bacteria are able to cometabolize various PCB components (1, 3, 5, 11, 12). The biodegradability and catabolic pathways of PCBs have been extensively studied (14–16). A major catabolic pathway of PCBs has been proposed and is presented in Fig. 1 (14, 16). Molecular oxygen is introduced at the 2,3-position of the nonchlorinated or less-chlorinated ring to produce a dihydrodiol (Fig. 1-II) by the action of a biphenyl dioxygenase (product of gene *bphA*). The dihydrodiol is then dehydrogenated to a 2,3-dihydroxybiphenyl (Fig. 1-III) by a dihydrodiol dehydrogenase (product of gene *bphB*). The 2,3-dihydroxybiphenyl is then cleaved at the 1,2-position by a 2,3-dihydroxybiphenyl dioxygenase (product of gene *bphC*). The *meta* cleavage compound (a chlorinated derivative of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate) (Fig. 1-IV) is hydrolyzed to the corresponding chlorobenzoic acid (Fig. 1-V) by a hydrolase (product of gene *bphD*). Thus, at least four enzymes are believed to be involved in the oxidative degradation of PCBs to chlorobenzoic acids.

The involvement of plasmids in PCB degradation has been suggested for some bacterial strains, such as *Klebsiella pneumoniae* (18), *Acinetobacter* sp. (10, 25), and *Alcaligenes* sp. (25), but those plasmids have not yet been characterized. Moreover, the enzymes and their corresponding genes also have not been isolated or characterized.

In this report we describe the cloning and expression of three genes (*bphA*, *bphB*, and *bphC*) involved in biphenyl and PCB catabolism from *Pseudomonas pseudoalcaligenes*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *P. pseudoalcaligenes* KF707 was isolated from soil near a biphenyl manufacturing plant by enrichment cultures with biphenyl as the sole carbon source. Plasmid pKF330 (12.6 kilobases [kb]) was obtained from K. Timmis, University of Geneva, Switzerland. Plasmid pKF330 is derived from pKT230 (2), but it contains an additional small *Pst*I fragment (0.7 kb) derived from RSF1010. Strain KF707 and transformants with *bph* genes were grown in a defined medium (pH 7.0) containing (in grams per liter): K_2HPO_4, 4.3; KH_2PO_4, 3.4; $(NH_4)_2SO_4$, 2.0; $MgCl_2$, 0.16; $MnCl_2 \cdot 4H_2O$, 0.001; $FeSO_4 \cdot 7H_2O$, 0.0006; $CaCl_2 \cdot 2H_2O$, 0.026; and $Na_2MoO_4 \cdot 2H_2O$, 0.002. Substrate (biphenyl or succinate) was added at a concentration of 1 g/liter. For agar plating medium (1.5% agar; Wako Chemical Co., Tokyo), biphenyl was provided as a vapor by placing crystals on the lid of a petri dish. The dish was sealed with polyethylene tape. LB broth containing (per liter) tryptone (Difco Laboratories), 10 g; yeast extract, 5 g; and NaCl, 5 g, pH 7.0, was used as a rich medium. Streptomycin was added to the medium at 300 μg/ml.

Cloning experiments. Chromosomal DNA from strain KF707 was prepared essentially as described by Marmur (21). Plasmid pKF330 and its hybrids with *bph* genes were isolated from *P. aeruginosa* by the method of Birnboim and Doly (4). Restriction enzyme *Xho*I and T4 DNA ligase were supplied by Takara Shuzo Co., Kyoto, and used as recommended by the manufacturer.

P. aeruginosa PAO1161 was transformed as described by Bagdasarian and Timmis (2). Transformants were selected on basal salts agar medium containing succinate (1 g/liter) and streptomycin. Clones expressing *bph* genes were identified by spraying colonies with 2,3-dihydroxybiphenyl solution (1 g/liter). Positive clones quickly turned yellow by forming the *meta* cleavage compound (14).

Hybridization of endonuclease-generated fragments. Hybridization experiments were performed by transferring DNA from agarose gels to nitrocellulose filter paper (26). Hybridization with ^{32}P-labeled, nick-translated DNA was performed as described by Southern (26).

Enzyme assays and oxygen uptake experiments. Strain KF707 and the transformants with *bph* genes were grown as described above, washed once with 0.05 M phosphate buffer

* Corresponding author.

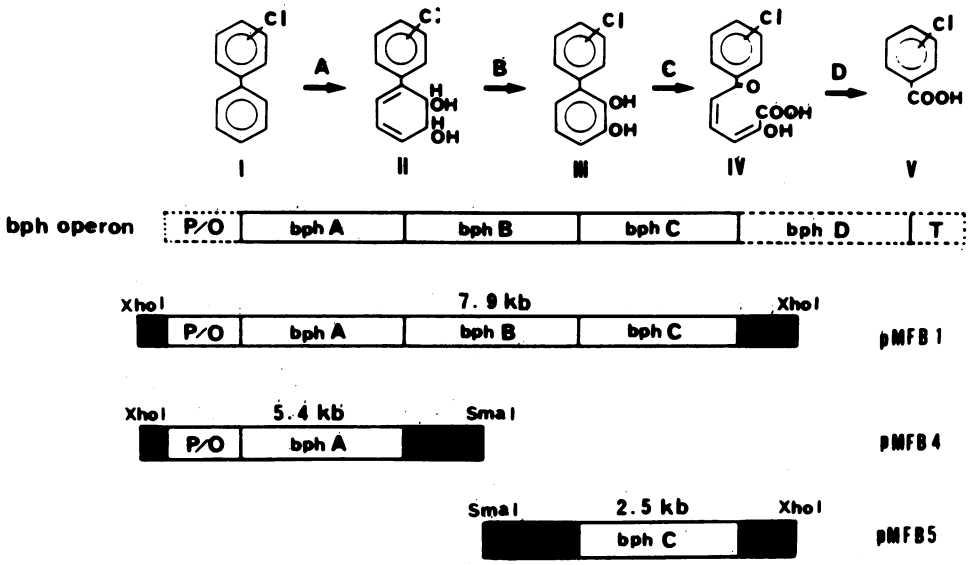

FIG. 1. Catabolic pathway for degradation of biphenyl and chlorobiphenyls and proposed gene organization of *bphABCD* operon in *P. pseudoalcaligenes* KF707. (Top) Compounds: I, biphenyl; II, 2,3-dihydroxy-4-phenylhexa-4,6-diene (dihydrodiol compound); III, 2,3-dihydroxybiphenyl; IV, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (*meta* cleavage compound); V, benzoic acid. Enzyme activities: A, biphenyl dioxygenase; B, dihydrodiol dehydrogenase; C, 2,3-dihydroxybiphenyl dioxygenase; D, *meta* cleavage compound hydrolase. (Bottom) P/O, Putative promoter-operator region; T, putative transcriptional terminator. *bphD* has not yet been cloned, as indicated by the broken line. The structures of plasmids pMFB1, pMFB4, and pMFB5 are shown.

(pH 7.5) containing 10% ethanol and 10% glycerol, and suspended in the same buffer. The cells were disrupted with a French pressure cell (Ohtake Co., Tokyo) and centrifuged at 28,000 × *g* for 30 min. Supernatant fluids were used as cell extracts.

2,3-Dihydroxybiphenyl dioxygenase was assayed by measuring the formation of the *meta* cleavage compound at 434 nm (13) after addition of substrate (2,3-dihydroxybiphenyl; Wako Chemical Co., Tokyo). The molar extinction coefficient at 434 nm (ϵ_{434}) used for this compound was calculated to be 22,000 (13).

Hydrolase activity involved in the conversion of the *meta* cleavage compound to benzoic acid was assayed by measuring the decrease in absorbance at 434 nm. The *meta* cleavage compound used as substrate was produced from 2,3-dihydroxybiphenyl by using resting cells of *P. aeruginosa* KF271 (Table 1), which carries plasmid pMFB5 containing *bphC*.

Protein content was determined by using a protein assay kit as recommended by the supplier (Bio-Rad Laboratories). Rates of oxygen uptake were measured polarographically with a Gilson oxygraph as previously described (10).

PCB degradation, GC-MS analysis, and thin-layer chromatography. The PCBs used in this study were 4-chlorobiphenyl, 2,3-, 3,4-, and 2,4′-dichlorobiphenyls, and 2,4,5- and 2,4,4′-trichlorobiphenyls. Resting cells of strain KF707 grown on biphenyl and transformants with *bph* genes grown in LB broth containing streptomycin were examined for their ability to degrade PCBs. The methods of incubation and extraction have been described previously (14). Trimethylsilyl derivatives of PCB metabolites were analyzed by gas chromatography-mass spectrometry (GC-MS) (model JMS D300; JEOL Ltd.) with a coiled glass column (1 m by 4-mm internal diameter) packed with silicon OV1 (at 2% on 80/100-mesh Chromosorb G). Helium was used as a carrier gas at a flow rate of 20 ml/min. The column temperature on GC was increased from 140 to 250°C at a rate of 8°C/min. The electron impact MS were measured at a 70-eV ionization

TABLE 1. Bacterial strains used

Strain	Plasmid	Relevant genotype or phenotype[a]	Source or reference
P. pseudoalcaligenes			
KF707	pKF707	BP$^+$	This work
P. aeruginosa			
PAO1161		*leu hsdR hsdM*	B. W. Holloway
KF205	pKF330	*leu hsdR hsdM* Km^r Sm^r	PAO1161 carrying pKF330
KF257	pMFB1	*leu hsdR hsdM* Sm^r	PAO1161 carrying pMFB1
KF258	pMFB2	*leu hsdR hsdM* Sm^r	PAO1161 carrying pMFB2
KF259	pMFB3	*leu hsdR hsdM* Sm^r	PAO1161 carrying pMFB3
KF260	pMFB4	*leu hsdR hsdM* Sm^r	PAO1161 carrying pMFB4
KF271	pMFB5	*leu hsdR hsdM* Sm^r	PAO1161 carrying pMFB5

[a] BP$^+$, Ability to grow on biphenyl; Km^r, resistance to kanamycin; Sm^r, resistance to streptomycin; *leu*, requirement for leucine; *hsdR*, host restriction activity; *hsdM*, host modification activity.

potential, 300-μA trap current, and 200°C ion source temperature.

Precoated plates of Silica Gel 60 F_{254} (Merck Inc.) were used for analytical thin-layer chromatography. The solvent system used for analysis of metabolites was benzene-dioxane-acetic acid, 90:20:4. Spots were visualized under UV irradiation.

RESULTS

Identification of strain KF707 and its mode of PCB degradation. A biphenyl-assimilating bacterium, strain KF707, was isolated from soil. It was assigned to the species *P. pseudoalcaligenes* by the following criteria: rod shaped (0.8 by 1.6 μm), motile with a single polar flagellum, gram negative, oxidase positive, catalase positive, strict aerobe, growth at 41°C, optimum growth temperature 30 to 35°C, no pigmentation, metabolism always respiratory and never fermentative, growth on MacConkey agar, no growth on maltose, sucrose, or xylose, arginine dihydrolase positive, lysine decarboxylase negative, ornithine carboxylase negative, urease negative, *o*-nitrophenyl-β-D-galactopyranoside cleavage negative, did not produce indole, reduced nitrate, did not denitrify, did not hydrolyze starch, did not deaminate phenylalanine, and utilized citrate.

Resting cells that had been grown on biphenyl were able to degrade PCBs such as 4-chloro-, 2,3-, 3,4-, 2,4′-dichloro-, and 2,4,5- and 2,4,4′-trichlorobiphenyl. A chlorinated benzoic acid corresponding to each PCB component was detected by GC-MS except for 2,4,4′-trichlorobiphenyl, which was converted to its *meta* cleavage compound which accumulated in the reaction mixture (data not shown). Degradative activity could be induced by adding biphenyl or 4-chlorobiphenyl, but not benzoic acid or 4-chlorobenzoic acid. Oxygen uptake for biphenyl and 4-chlorobiphenyl, enzyme activity of dihydroxybiphenyl dioxygenase, and *meta* cleavage compound hydrolase activity were all induced by growth on biphenyl (data not shown). The pathway for catabolism of PCBs in strain KF707 was the same as the major catabolic pathway observed in other biphenyl-utilizing bacterial strains (1, 3, 5, 12–14).

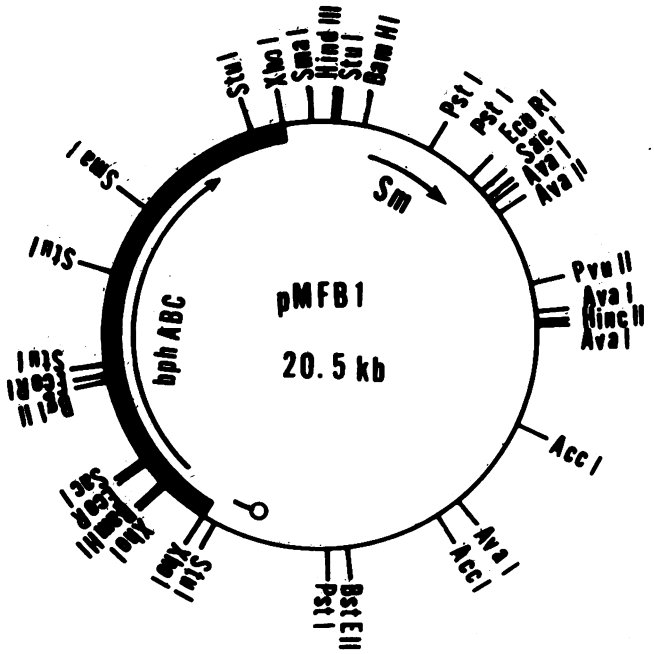

FIG. 2. Schematic presentation of plasmid pMFB1, carrying the *bph* gene cluster. The thick line (7.9 kb) contains the cloned *bph* genes derived from KF707 chromosomal DNA. The thin line shows DNA derived from the pKF330 vector (12.6 kb). The small circle indicates the promoter region of the kanamycin resistance determinant of pKF330. The arrow inside the *bph* region indicates the direction of transcription.

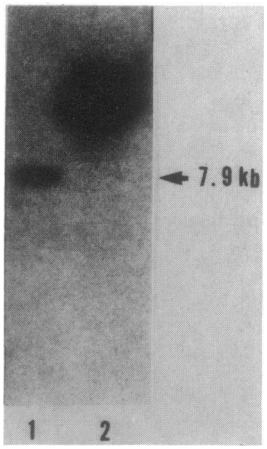

FIG. 3. Southern blot hybridization of *bph* genes of KF707 chromosomal DNA. *Xho*I-digested KF707 chromosomal DNA (lane 1) and *Xho*I-digested pKF330 (lane 2) were hybridized with ^{32}P-labeled pMFB1 plasmid DNA; pKF330 did not hybridize to KF707 chromosomal DNA (data not shown). The arrow indicates the 7.9-kb segment in KF707 chromosomal DNA.

Cloning of *bph* genes. Purified genomic DNA from *P. pseudoalcaligenes* KF707 was digested with restriction endonuclease *Xho*I and ligated to *Xho*I-digested plasmid pKF330. The ligation mixture was transformed into *P. aeruginosa* PAO1161, and streptomycin-resistant transformants were selected on LB agar containing streptomycin. Transformant colonies were sprayed with a solution containing 2,3-dihydroxybiphenyl. One streptomycin-resistant colony among about 8,000 colonies quickly turned yellow, indicating the conversion of 2,3-dihydroxybiphenyl to 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid. This clone was grown in LB broth containing streptomycin, and its plasmid DNA was isolated. The hybrid plasmid, pMFB1, was 20.5 kb in size and contained a 7.9-kb DNA insert in the unique *Xho*I site of pKF330, (Fig. 2). Southern blot experiments (Fig. 3) confirmed that the 7.9-kb *Xho*I fragment was derived from KF707 genomic DNA. Strain KF707 harbors one plasmid (pKF707, about 50 kb), but the cloned 7.9-kb *Xho*I fragment did not hybridize to *Xho*I-digested pKF707 DNA, confirming that the 7.9-kb *Xho*I fragment came from the chromosome of strain KF707. Transformant KF257 containing plasmid pMFB1 was capable of catabolizing biphenyl directly to the *meta* cleavage compound. However, the *meta* cleavage compound formed was not hydrolyzed to benzoic acid by the same cells. These results indicate that the 7.9-kb *Xho*I fragment contains *bphA* (biphenyl dioxygenase gene), *bphB* (dihydrodiol dehydrogenase gene), and *bphC* (2,3-dihydroxybiphenyl dioxygenase gene), but not *bphD* (*meta* cleavage compound hydrolase gene) (Fig. 1). These observations were confirmed by the enzyme assays and the subcloning experiments described below.

The enzyme levels of 2,3-dihydroxybiphenyl dioxygenase and the *meta* cleavage compound hydrolase were measured

TABLE 2. 2,3-Dihydroxybiphenyl dioxygenase and the *meta* cleavage compound hydrolase in cell extracts

Strain	Growth medium or addition	Activity (U/g of protein)	
		2,3-Dihydroxybiphenyl dioxygenase	*meta* cleavage cmpd hydrolase
P. pseudoalcaligenes KF707	Succinate	<10	10
	Succinate with biphenyl	1,140	890
P. aeruginosa KF257(pMFB1)	Luria broth with streptomycin	2,060	<10
	Luria broth with streptomycin and biphenyl	2,160	<10

in cell extracts of the wild-type strain KF707 and the transformant KF257(pMFB1) (Table 2). The apparent enzyme activity of 2,3-dihydroxybiphenyl dioxygenase in the KF257(pMFB1) crude extract was about two times higher than that in the biphenyl-induced wild-type strain KF707. However, the *meta* cleavage compound hydrolase was not detected in extracts of transformant cells. Adding biphenyl remarkably increased both 2,3-dihydroxybiphenyl dioxygenase and the *meta* cleavage compound hydrolase activity in *P. pseudoalcaligenes* KF707, but not in the transformant KF257(pMFB1). These observations suggest that *bph* genes inserted in the *Xho*I site of pKF330 may be transcribed by readthrough from the kanamycin resistance promoter.

pMFB1-mediated degradation of PCB. Strain KF257 (pMFB1) was examined for the degradation of some PCB congeners such as 4-chloro-, 3,4-dichloro-, 2,4'-dichloro-, and 2,4,5-trichlorobiphenyl. All PCB components tested as well as biphenyl were converted to yellow *meta* cleavage compounds. These *meta* cleavage compounds were confirmed to be chlorinated derivatives of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate by GC-MS analysis. Mass spectra of the trimethylsilyl derivatives of the dihydroxy compound and *meta* cleavage compound from 2,4,5-trichlorobiphenyl are shown in Fig. 4.

Subcloning of *bph* genes. The restriction map of pMFB1 containing *bph* genes is shown in Fig. 3. A variety of derivative plasmids were constructed from pMFB1 (Fig. 5). pMFB2 was constructed by eliminating the small *Xho*I fragment (0.8 kb) from pMFB1. Strain KF258(pMFB2) catabolized biphenyl to the *meta* cleavage compound. Strain KF259(pMFB3), containing the same 7.2-kb DNA sequence as pMFB2 but in opposite orientation, also catabolized biphenyl to the *meta* cleavage compound, but to a lower extent. pMFB4 was constructed by eliminating the small *Sma*I fragment (2.5 kb) of pMFB1 (Fig. 5). pMFB5 was constructed by subcloning the small *Sma*I fragment of pMFB1 into the unique *Sma*I site of pKF330 vector.

Biphenyl catabolism mediated by pMFB4 and pMFB5. Plasmids pMFB4 and pMFB5 were transformed into strain PAO1161, and the transformants were examined for the catabolism of biphenyl. Strain KF260(pMFB4) was cultured in LB broth with streptomycin, and the washed cells were incubated with biphenyl in phosphate buffer (pH 7.5). The incubation mixture was extracted with ethyl acetate at pH 1. The concentrated ethyl acetate extracts were subjected to thin-layer chromatography and GC-MS. Two major spots of biphenyl products were detected on the thin-layer chromatogram (data not shown), and subsequent GC-MS analysis revealed that the two major compounds were both monohydroxybiphenyls (M^+ as a trimethylsilyl derivative, m/z 242; M^+-CH_3, m/z 227 as the base peak; M^+-$2CH_3$, m/z 212; M^+-OTMS, m/z 153) with different retention times. These results indicate that biphenyl was converted to the dihydrodiol compound by pMFB4-carrying cells since the dihydrodiol can be easily converted to monohydroxy compounds in acid extraction procedures. In this case the products observed were 2-hydroxybiphenyl and 3-hydroxybiphenyl. The pMFB4-carrying cells could not attack 2,3-dihydroxybiphenyl. In contrast, strain KF271(pMFB5) could not attack biphenyl. No metabolite could be detected by thin-layer chromatography or GC-MS. On the other hand, the same cells converted 2,3-dihydroxybiphenyl to the yellow *meta* cleavage compound. The cell extracts also catalized the conversion of 2,3-dihydroxybiphenyl to the *meta* cleavage compound, indicating the presence of 2,3-dihydroxybiphenyl dioxygenase. However, the same cell extracts did not contain dihydrodiol dehydrogenase. The dihydrodiol compound produced by resting cells of KF260(pMFB4) was converted to the *meta* cleavage compound via 2,3-dihydroxybiphenyl by the cell extracts of strain KF258(pMFB2), but the same compound was not metabolized by the cell extract of strain KF271(pMFB5), indicating the lack of the dihydrodiol dehydrogenase.

These results clearly indicate that both pMFB2 and the parent plasmid pMFB1 contain *bph* genes encoding the conversion of biphenyl to the *meta* cleavage compound: *bphA* (biphenyl dioxygenase), *bphB* (dihydrodiol dehydrogenase), and *bphC* (2,3-dihydroxybiphenyl dioxygenase). pMFB4 contains only *bphA*, and pMFB5 contains only *bphC*. It is also evident that the unique *Sma*I site on the cloned *bph* gene cluster is within the *bphB* gene.

Level of 2,3-dihydroxybiphenyl dioxygenase. The level of 2,3-dihydroxybiphenyl dioxygenase was measured in cell extracts from strains KF257(pMFB1), KF258(pMFB2), KF259(pMFB3), KF260(pMFB4), and KF271(pMFB5) (Table 3). The enzyme level was highest in KF258(pMFB2). Strain KF259(pMFB3), in which *bph* genes are inserted in opposite orientation, had 65% of the enzyme activity found in KF258(pMFB2). No 2,3-dihydroxybiphenyl dioxygenase activity was observed in pMFB4-carrying KF260 cells.

DISCUSSION

In the present study we have cloned biphenyl catabolism genes (*bphA*, *bphB*, and *bphC*) from the chromosomal DNA of *P. pseudoalcaligenes* KF707. The gene order on the chromosome was found to be *bphA-bphB-bphC* by subcloning experiments. Gene *bphD* has not yet been isolated, but it is likely that *bphABCD* is organized by a common regulatory unit forming an operon as shown in Fig. 1, since the *bph* genes were highly inducible.

Genes for catabolic pathways are often highly clustered. Clustering of catabolic genes in *P. putida* has been found with plasmids such as TOL (specifying xylene-toluene catabolism), NAH (specifying naphthalene catabolism), and OCT (specifying *n*-alkane catabolism). The genes of TOL (pWWO) are organized into two regulatory units, the *xylABC* operon and the *xylDEGF* operon (9, 17, 20, 28). The naphthalene oxidation genes of plasmid NAH7 are organized in two operons: *nahABCDEF*, coding for the conversion of naphthalene to salicylate, and *nahGHIJK*, coding for the oxidation of salicylate (8, 29). The *n*-alkane oxidation genes

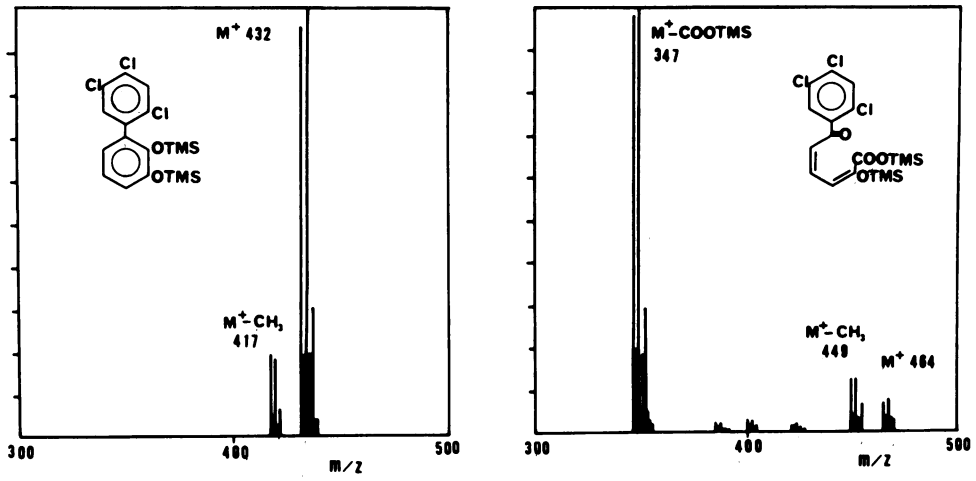

FIG. 4. Mass spectra of the intermediates derived from 2,4,5-trichlorobiphenyl by KF257(pMFB1). The intermediates of the dihydroxy compound (left) and the *meta* cleavage compound (right) derived from 2,4,5-trichlorobiphenyl were treated with bistrimethylsilylacetamide to make the trimethylsilyl (TMS) derivatives.

on the plasmid OCT are also organized in an *alkBAC* operon (22). The catabolism genes of TOL, NAH, and OCT plasmids are thought to be positively regulated. The genes for the degradation of 3-chlorobenzoic acid, which are present in the 110-kb plasmid pAC27, are clustered in one region as judged by molecular cloning experiments with a broad-host-range vector, pLAFR1 (7). A clustering of genes of the mandalate and benzoate pathways has been shown in *P. aeruginosa* by using the transducing phage F116 (19).

In pMFB2, *bphABC* is located downstream of the kanamycin resistance promoter of pKF330 (Fig. 5), so that the *bph* genes are most likely transcribed by readthrough from that promoter. Addition of biphenyl to the growth medium was not effective as an inducer of 2,3-dihydroxybiphenyl

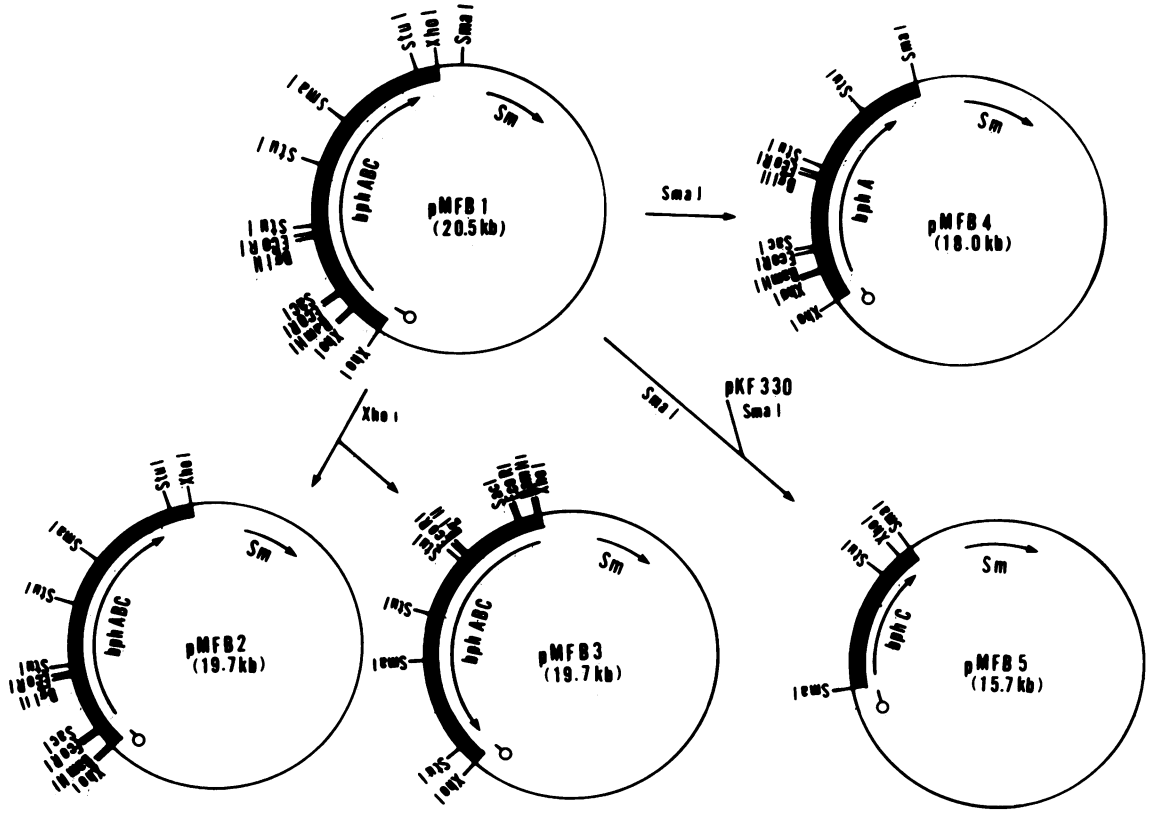

FIG. 5. Subcloning of *bph* genes. The arrows inside the *bph* region indicate the direction of transcription. Other symbols are as described in the legend to Fig. 2.

TABLE 3. Level of 2,3-dihydroxybiphenyl dioxygenase in cell extracts[a]

Cell extract	*bph* genes	2,3-Dihydroxybiphenyl dioxygenase activity (U/g of protein)
PAO1161	None	<10
KF257(pMFB1)	*bphABC*	2,300
KF258(pMFB2)	*bphABC*	3,600
KF259(pMFB3)	*bphCBA*	2,350
KF260(pMFB4)	*bphA*	<10
KF271(pMFB5)	*bphC*	1,240

[a] The cells were grown in LB broth with streptomycin (200 μg/ml) except for PAO1161, which was grown without streptomycin.

dioxygenase in KF257(pMFB1). The transformant KF259 carrying pMFB3, in which *bphABC* is inserted in the opposite orientation, produced 65% of the 2,3-dihydroxybiphenyl dioxygenase activity found in pMFB2-carrying KF258 cells (Table 3), so it appears that there is another promoterlike sequence derived from the vector. To understand whether the cloned *bph* genes have their own promoter and how the *bph* genes are regulated, it will be necessary to subclone the same genes in a region where promoter sequences from the vector are lacking.

Reineke et al. (23, 24) and Chatterjee and Chakrabarty (6) have constructed chlorobenzoate-degrading *Pseudomonas* spp., including 4-chloro- and 3,5-dichlorobenzoate-utilizing strains, by introducing TOL genes into a 3-chlorobenzoate utilizer. It was suggested that the former acquired the ability to utilize 4-chlorobenzoate as a result of the recruitment of a toluate 1,2-dioxygenase gene from the TOL plasmid. Furukawa and Chakrabarty (10) previously have demonstrated that mixed cultures of a PCB-degrading *Acinetobacter* or *Arthrobacter* species (specifying degradation of PCB to chlorobenzoate) and a chlorobenzoate-utilizing *Pseudomonas* sp. (17) totally degraded some mono- and dichlorobiphenyls. If *bph* genes *bphABCD* were cloned into a broad-host-range vector such as an RSF1010-derived plasmid (2) and then introduced into a chlorobenzoate-utilizing *Pseudomonas* sp., some PCB congeners might be totally degraded by a single newly constructed strain. This kind of approach can be used to construct hybrid pathways for various environmental pollutants (24, 27).

Another aspect of *bph* gene cluster cloning is that *meta* cleavage compounds and dihydrodiol compounds can be produced and accumulated from biphenyls and chlorobiphenyls. Thus, by cloning of catabolism gene clusters and subsequent subcloning, a variety of catabolic intermediates can be produced from various starting materials. Such catabolic intermediates are often complicated in their molecular structure, making it advantageous to use microbial instead of chemical syntheses.

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