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Identification and Localization of 3-Phenylcatechol Dioxygenase and 2-Hydroxy-6-Oxo-6-Phenylhexa-2,4-Dienoate Hydrolase Genes of *Pseudomonas putida* and Expression in *Escherichia coli*

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The *bphC* and *bphD* genes of *Pseudomonas putida* involved in the catabolism of polychlorinated biphenyls or biphenyl were identified, localized, and studied for expression in *Escherichia coli*. This was achieved by cloning a 2.4-kilobase (kb) DNA fragment of recombinant cosmid pOH101 into *Hin*dIII site of pUC plasmids downstream of a *lacZ* promoter and measuring the enzyme activities of 3-phenylcatechol dioxygenase (3-PDase; a product of *bphC*) and the *meta*-cleavage product 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (a product of *bphD*). The amount of 3-PDase produced in *E. coli* was about 20 times higher than that of the enzyme produced by the parent, *P. putida*. Determination of expression of the *bphC* and *bphD* genes through their own promoter sequences or by using the *lacZ* promoter of pUC plasmids was done by cloning the DNA that encodes *bphC* and *bphD* genes in a *Hin*dIII site of a promoter selection vector (pKK232-8) upstream of the gene for chloramphenicol acetyltransferase (CAT). The recombinant plasmid (pAW787) constructed by inserting the 2.4-kb DNA in pKK232-8 expressed both 3-PDase and CAT activities. Another hybrid construct (pAW786) in which the DNA insert was cloned in the opposite orientation lacked CAT activity but produced normal amounts of 3-PDase activity. On the basis of these results, we suggest that the *bphC* and *bphD* genes were expressed by using promoter sequences that are independent of the promoter that expresses CAT activity in *E. coli*. The locations of the *bphC* and *bphD* genes were determined by insertional inactivation of the open reading frames of structural genes *bphC* and *bphD* by Tn*5* mutagenesis. On the basis of the results of analysis of the deletion mutants and restriction mapping analysis of Tn*5* on the Km^r mutant plasmids lacking 3-PDase activity, we found that the essential DNA regions required for the *bphC* and *bphD* genes were contained within a 2.1-kb *Hin*dIII-*Eco*RI fragment of pAW313.

Use of polychlorinated biphenyls (PCBs) as insulators, lubricating oils, conductors of heat, and solvents for paints has been prohibited because of toxicity to animals and humans (12, 20). Despite prohibition of their use, the environment is still burdened with these toxic chemicals. A variety of microorganisms have been found in chemically polluted soil environments which can degrade PCBs (1–3, 8–10, 13–15), and diverse bacterial metabolic pathways for degradation of PCBs have recently been reported (2, 13, 14, 19). Extensive studies on the bacterial degradation of PCBs have shown that most bacterial strains metabolize PCBs and biphenyl via common catabolic pathways by using biphenyl dioxygenase (a *bphA* gene product), dihydrodiol dehydrogenase (a *bphB* gene product), phenylcatechol dioxygenase (PDase; a *bphC* gene product), and a *meta*-cleavage product, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPDA) hydrolase (a *bphD* gene product) (9, 13–15, 19, 21, 25).

Bacterial genes (*bphABCD*) of *Pseudomonas putida* OU83 that specify degradation of PCBs to their corresponding chlorobenzoic acids have been cloned both in our laboratory (13, 14) and in others (19, 25). Furukawa and Miyazaki (9) previously reported cloning of a 7.6-kilobase (kb) DNA fragment containing *bphABC* genes from *P. pseudoalcaligenes*. Indirect evidence of an operon that encodes *bphC* and *bphD* has recently been obtained by DNA sequence analysis (15). Gas chromatographic-mass spectrophotometric (GC-MS) analyses of the metabolic products of 3-phenylcatechol (3-PC) have also shown formation of benzoic acid (15). DNA sequence analysis of the cloned DNA fragment revealed two reading frames (ORFI [882 base pairs] and ORFII [834 base pairs]). Further, unidirectional deletion mutants and Tn*5* insertional mutants suggested that ORFI codes for 3-PC dioxygenase (3-PDase). Nucleotide sequencing of the ORFII region and GC-MS analysis of metabolic products from bacteria containing the *bphC* and *bphD* genes indirectly suggested that ORFII contains HOPDA hydrolase activity (15).

In our laboratory, Khan et al. (13) have previously reported cloning of a 6.4-kb *Hin*dIII DNA fragment from *P. putida* that specifies broad substrate specificity 3-PDase (a gene product of *bphC*). In this report, we describe the identification and localization of *bphD* (a gene that specifies HOPDA hydrolase) and *bphC* (a gene that encodes 3-PDase) genes on a 2.4-kb *Hin*dIII DNA fragment subcloned from recombinant cosmid pOH101 (13). This *Hin*dIII DNA fragment, when cloned into pUC18 at a *Hin*dIII site downstream of the *lacZ* promoter, expressed both the *bphC* and *bphD* gene products in *Escherichia coli*. Further evidence of the expression of the genes by their own promoters was obtained by constructing a variety of recombinant plasmids by cloning the 2.4-kb DNA fragment of pOH101 into the *Hin*dIII site of promoter probe vector pKK232-8. Recombinant plasmids containing the 2.4-kb *Hin*dIII DNA insert conferred chloramphenicol resistance (Cm^r) and expressed the *bphC* and *bphD* gene products in *E. coli*. Further locations of DNA regions that specify the *bphC* and *bphD* genes on the subcloned DNA fragment were determined by transposon Tn*5* insertional mutagenesis.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain, plasmid, or phage	Characteristics and comments[a]	Source or reference
P. putida OU83	*cbpABCD*, isolated from PCB-contaminated soil	14
E. coli HB101	*pro leu recA hsdR hsdM*	26
pOH101	*cbpCD* Tc^r, 6.4-kb *Hin*dIII fragments cloned in pCP13	13
pAW313	*cbpCD* Am^r, 2.4-kb *Hin*dIII fragment cloned in pUC18	27
pAW3131	Am^r, 1.3-kb *Eco*RI-*Eco*RI fragment cloned in pUC18	This report
pAW3132	Am^r, 0.8-kb *Hin*dIII-*Eco*RI fragment cloned in pUC18	This report
pAW3133	Am^r, 0.3-kb *Eco*RI-*Hin*dIII fragment cloned in pUC18	This report
pAW413	*cbpCD* Am^r, 2.4-kb *Hin*dIII fragment cloned in pUC19	This report
pAW414	*cbpCD* Am^r, 2.4-kb *Hin*dIII fragment cloned in pUC19 in orientation opposite to that of pAW413	This report
pAW787	*cbpCD* Cm^r Am^r, 2.4-kb *Hin*dIII fragment cloned in promoter probe vector pKK232-8	This report
pAW786	*cbpCD* Am^r, 2.4-kb *Hin*dIII fragment cloned in promoter probe vector pKK232-8 in orientation opposite to that of pAW787	This report
pKK232-8	Am^r, *cat* cartridge just downstream of multiple cloning sites	4
λ 467	λ *b221 rex*::Tn*5* *c*I857 *O*am29 *pam-80* Km^r	K. T. Shanmugan; 5
pAW313-T2, pAW313-T4, pAW313-T8	*cbpD* Am^r Km^r pAW313::Tn*5*	This report

[a] The designations used for relevant genes are as follows: *cbp*, chlorobiphenyl; *bph*, biphenyl; *recA*, recombination deficient; *pro*, proline; *leu*, leucine; *hsdR*, host-specific restriction; *hsdM*, host-specific modification.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains, plasmids, and bacteriophage used in this study are listed in Table 1. Organisms were grown in previously described phosphate-buffered mineral salt medium (13) supplemented with 0.1% sodium succinate or 3-PC. Luria broth (L broth) consisted of 1% tryptone (Difco Laboratories, Detroit, Mich.), 0.5% yeast extract (Difco), 0.5% NaCl, and 0.1% D-glucose. *E. coli* strains were grown in M9 medium (17) amended with 20 μg of the appropriate amino acids per ml. Organisms containing plasmids were grown and maintained on L agar or L broth medium supplemented with appropriate antibiotics. For solid agar media, agar (Difco) was added at a concentration of 1.5%.

DNA isolation, restriction enzyme digestion, and ligations. Plasmid DNA was isolated by alkaline lysis (17) and purified by cesium chloride density gradient centrifugation. The enzymes *Eco*RI, *Hin*dIII, *Xho*I, *Bam*HI, *Sal*I, *Pst*I, and T4 DNA ligase were used for in vitro genetic manipulations and physical mapping of restriction sites of the DNA insert. These were obtained from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.); International Biotechnologies, Inc. (New Haven, Conn.); or Boehringer Mannheim Biochemicals (Indianapolis, Ind.).

Subcloning of DNA. Recombinant cosmid pOH101 DNA was digested to completion with *Hin*dIII, mixed with similarly digested plasmid vector pUC18 (1:1), and ligated with T4 DNA ligase at 12 to 16°C as described previously by Khan and Walia (14). The ligated DNA was transformed in *E. coli* JM83 by $CaCl_2$-heat shock treatment (16). The transformants were selected on L agar medium amended with 100 μg of ampicillin per ml and 40 μg each of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and isopropylthio-β-D-galactopyranoside per ml (18). Transformants lacking β-galactosidase activity were selected and tested for expression of 3-PDase activity by being sprayed with a 0.1% solution of 3-PC as described previously (13).

Transposon mutagenesis. Insertional inactivation of a gene by transposon mutagenesis was performed by transduction for kanamycin resistance (Km^r) by λ 467 as described by Bruijn and Lupski (5). Briefly, *E. coli* HB101 cells containing recombinant plasmid pAW313 were infected with λ::Tn*5* phage stock. Km^r transductants were selected on L agar plates containing 50 μg of kanamycin per ml and 100 μg of ampicillin per ml. Plasmid DNA was isolated from ampicillin-resistant (Am^r) Km^r transductants and used to transform *E. coli* HB101. Antibiotic-resistant transformants were selected and tested for expression of 3-PDase activity by being sprayed with a 0.1% solution of 3-PC. The position of Tn*5* within pAW313::Tn*5* plasmids was determined by restriction enzyme analysis of plasmid DNA isolated from Km^r *E. coli*. *Eco*RI, *Sal*I, and *Hin*dIII enzymes were used to localize the site of insertion of Tn*5*.

Enzyme assays. Activities of 3-PDase, HOPDA hydrolase, and chloramphenicol acetyltransferase (CAT) were assayed on supernatants (high speed) of extracts prepared by sonication of cells (Branson Sonic Power Co., Danburg, Conn.). The activity of 3-PDase was determined by measuring the formation of HOPDA at 434 nm after addition of 3-PC as the substrate (13). In a separate experiment, HOPDA hydrolase activity was determined by tracking the disappearance of HOPDA (as a substrate) at 434 nm (21) by using a dual-beam UV spectrophotometer (The Perkin-Elmer Corp., Norwalk, Conn.). CAT activity was determined by spectrophotometry as described by Shaw (23). Total protein was assayed by the dye-binding assay (24) as recommended by the supplier (Pierce Chemical Co., Rockford, Ill.).

Metabolite extraction, HPLC conditions, and GC-MS analysis. Chemical metabolites of 4′-chloro-3-phenylcatechol or 3-PC from the cell extracts, resting cell suspension, and growing cells were extracted with 2 volumes of ethyl acetate-diethyl ether (1:1). Extracted metabolites were passed through a prewashed (5 ml of methanol and 5 ml of deionized water) C18 column (J. T. Baker Chemical Co., Phillipsburg, N.J.). Samples were eluted with 5 ml of ethyl acetate, and metabolites were concentrated by evaporation of ethyl acetate to dryness. The metabolites were redissolved in appropriate amounts of solvent. A portion (20 μl) of each dissolved sample was analyzed by high-performance liquid chromatography (HPLC; Perkin-Elmer) with a C18 column (5-μm pore size; 250 by 4.6 mm; Alltech Associates, Inc., Applied Science Div., State College, Pa.) and a mobile phase consisting of methanol-water-acetic acid (60:35:5) at a flow rate of 1 ml/min. The UV A_{255} and A_{335} were monitored with

an LC235 diode array detector (Perkin-Elmer). For the HPLC elution profile and UV spectrum overlay of 3-PC and HOPDA, see Fig. 1A to D and E, respectively. Concentrations of individual components were determined from standard curves of concentrations versus peak areas by using the Omega II system (Perkin-Elmer). GC-MS analysis of the extracted metabolites was done by making *N,O*-bis(trimethylsilyl)trifluoroacetamide (Pierce) derivatives of extracted metabolites with a Shimadzu QP-1000 GC-MS system, and a fused-silica capillary column (15 m by 0.25 mm [internal diameter]; Quadrex, New Haven, Conn.) with a 1.0-μm-thick methyl silicone coating was used for the separations. The GC-MS conditions, temperature program, and gas flow rate used were as described previously (13).

Chemicals. The chemicals used in this study were as follows. 3-Dithiobisnitrobenzoic acid and dithiothreitol were purchased from Sigma Chemical Co., St. Louis, Mo. 3-PC and 4′-chloro-3-phenylcatechol were synthesized in the laboratory of G. Brieger (Department of Chemistry, Oakland University, Rochester, Mich.). HOPDA was synthesized by enzymatic conversion of 3-PC to HOPDA. Partially purified 3-PDase was prepared from a high-speed supernatant of *E. coli* cells containing recombinant plasmid pAW313 (27). Protein fractions containing 3-PDase activity were separated by DEAE-Sephadex chromatography and used to synthesize HOPDA.

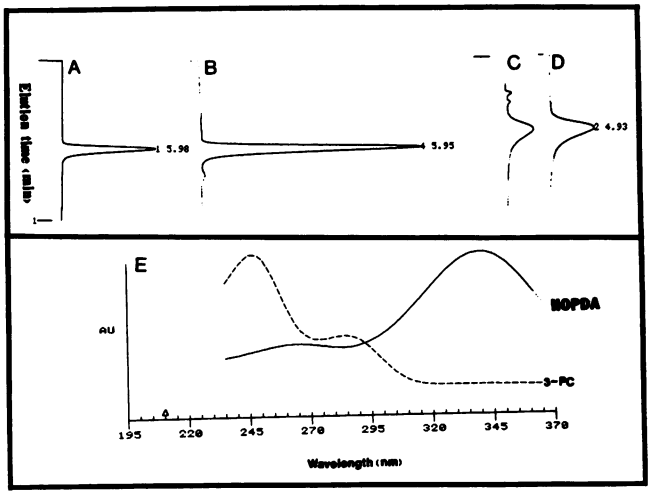


FIG. 1. HPLC elution profile and UV spectrum overlay. Panels: A, elution profile of 3-PC (retention time, 5.98 min; A_{255}); B, elution profile of 3-PC–HOPDA monitored at A_{255}; C, elution profile of 3-PC–HOPDA monitored at A_{335}; D, elution profile of HOPDA (retention time, 4.93; A_{335}); E, UV spectrum overlay of 3-PC and HOPDA. HPLC conditions: column, Econosphere C_{18} (250 mm by 4.6 mm, 5-μm pore size); mobile phase, methanol-water-acetic acid (60:40:5); flow rate, 1 ml min^{-1}; temperature, 25 ± 2°C; UV detector set at 255 and 335 nm.

RESULTS

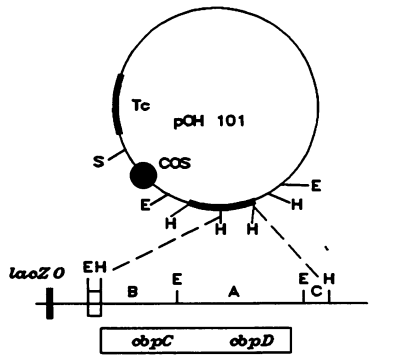
Metabolite analysis. Bacterial genes that specify the broad substrate specificity enzyme 3-PDase from *P. putida* were cloned in the *Hin*dIII site of cosmid pCP13 (22) and expressed in *E. coli* (13). *E. coli*(pOH101) cells were incubated with 4-chlorobiphenyl, and 3-PC in phosphate-buffered mineral salt medium, and metabolites were extracted and examined by GC-MS and HPLC. Both GC-MS and HPLC analyses of these metabolic products from 4-chlorobiphenyl extract showed no evidence of 4-chlorobiphenyl degradation. HPLC analysis of the 3-PC metabolites showed peaks with same retention time (4.93 min) and UV spectrum as standard HOPDA (Fig. 1). Further GC-MS analysis of 3-PC metabolites showed a metabolite peak with the same mass spectrum as standard benzoic acid.

Identification of genes for 3-PDase and HOPDA hydrolase. The regions on recombinant cosmid pOH101 that specify the *bphC* and *bphD* genes were determined by constructing a variety of derivative plasmids. Several recombinant plasmids were constructed by cloning *Hin*dIII fragments of pOH101 in the *Hin*dIII site of pUC18 and pUC19 plasmids downstream of the *lacZ* promoter. Bacterial colonies that expressed 3-PDase activity (a gene product of *bphC*) were detected by being sprayed with a 0.1% solution of 3-PC in 50 mM Tris hydrochloride–10% acetone buffer (pH 7.5) as described previously (13). Colonies that produced active 3-PDase turned yellow because of the formation of the *meta*-cleavage product of 3-PC (HOPDA). 3-PDase-producing bacterial colonies were analyzed for the DNA inserts in recombinant plasmids.

Restriction enzyme analysis of recombinant plasmids from 3-PDase-positive colonies showed a 2.4-kb *Hin*dIII fragment, suggesting that the *bphC* gene was contained in this fragment (Fig. 2). Recombinant plasmid derivatives of pUC18 and pUC19 containing the 2.4-kb *Hin*dIII fragment in the opposite orientation also showed expression of 3-PDase and HOPDA hydrolase activities. This suggests that expression of the *bphC* and *bphD* genes in *E. coli* is regulated by their own (*bph*) promoter sequences, independently of the pUC plasmid *lacZ* promoter (Fig. 2). In a recent study unlike ours, Kimbara et al. (15) have demonstrated that *Pseudomonas* genes that code for 3-PDase activity were not expressed from their own promoters in *E. coli*.

Confirmation of a native promoter on the cloned DNA was made by constructing hybrid plasmids of a promoter probe vector (pKK232-8). This was achieved by cloning the 2.4-kb *Hin*dIII DNA fragment that codes for 3-PDase activity in the *Hin*dIII site of the promoter probe vector and then measuring the expression of CAT activity. Promoter probe vector pKK232-8 contains a cartridge of genes for CAT which lacks its own promoter (4) and has multiple cloning sites for insertion of foreign DNA which is located upstream of the gene cartridge for CAT. A summary of enzyme activities, the orientation of the cloned *Hin*dIII DNA insert, and restriction analysis of the recombinant plasmids are shown in Fig. 3. Recombinant plasmid pAW787 expressed CAT, 3-PDase, and HOPDA hydrolase activities (Table 2). In contrast, a plasmid (pAW786) containing the *Hin*dIII fragment in the orientation opposite to that of pAW787 did not express CAT activity but expressed both 3-PDase and HOPDA hydrolase activities. Thus, the 2.4-kb *Hin*dIII fragment that encodes both 3-PDase and HOPDA hydrolase activities was expressed in *E. coli* from the promoter(s) contained in the cloned DNA fragment of *P. putida*.

Localization of the *bphC* gene by Tn*5* mutagenesis. The DNA regions on recombinant plasmid pAW313 that encodes the *bphC* and *bphD* genes were determined by insertional inactivation of the ORF by introduction of a Km^r marker by transposon mutagenesis. Precise localization of Tn*5* was determined by restriction enzyme analysis of plasmid DNA containing Tn*5* by *Eco*RI, *Hin*dIII, and *Sal*I. Figure 4 shows the agarose gel electrophoretic patterns of plasmid DNA digested with appropriate enzymes. Insertion of Tn*5* in cloned DNA was determined by the increase in the size of the 2.4-kb *Hin*dIII fragment (Fig. 4, lane 4, arrowhead). This



Recombinant Plasmids	*cbpC* (3-PDase)	*cbpD* (HOPDA Hydrolase)
pAW 313	+	+
pAW 3132	−	−
pAW 3131	−	−
pAW 3133	−	−
pAW 413	+	+
pAW 414	+	+
pAW 313-T2	−	+
pAW 313-T4	−	+
pAW 313-T8	−	+

FIG. 2. Plasmid constructions. The 2.4-kb *Hin*dIII fragment of pOH101 (solid bar) was inserted into pUC18 to produce pAW313 and into pUC19 to create pAW413 and pAW414. Fragments A (*Eco*RI-*Eco*RI, 1.3 kb), B (*Hin*dIII-*Eco*RI, 0.8 kb), and C (*Eco*RI-*Hin*dIII, 0.3 kb) of plasmid pAW313 were subcloned in pUC18 to construct pAW313, pAW3132, and pAW3133, respectively. ▼ Locations of Tn*5* (pAW313-T2, pAW313-T4, and pAW313-T8); Tc, tetracycline; H, *Hin*dIII; E, *Eco*RI; S, *Sal*I; □, multiple cloning sites; ■, *lacZ* promoters.

was further confirmed by analysis of an *Eco*RI digest of plasmid DNA. By using *Eco*RI (no site in Tn*5*), *Sal*I (one site in Tn*5*), and *Hin*dIII (two sites in Tn*5*), we were able to determine the location of Tn*5* on the DNA to within 100 base pairs. The locations of transposons and a summary of genes *bphC* and *bphD* are shown in Fig. 2. Essential DNA regions that code *bphC* genes were also determined by constructing a variety of hybrid plasmids (pAW3131, pAW3132, and pAW3133) containing fragments A, B, and C of pAW313 in pUC18. 3-PDase-coding sequences lie between the *Hin*dIII-*Eco*RI (800 base pairs) and *Eco*RI-*Eco*RI (1,300 base pairs) sites on the recombinant plasmid.

Expression of enzyme activities. Expression of 3-PDase, HOPDA hydrolase, and CAT activities was measured in appropriate *E. coli* cultures containing recombinant plasmids and *P. putida* OU83. The specific activities of the enzymes produced are shown in Table 2. *E. coli* containing recombinant plasmids showed expression of both 3-PDase and HOPDA hydrolase. High-level expression of 3-PDase was found in *E. coli*(pAW313) but not in the parent, *P. putida*. Expression of CAT was observed in pAW787, and no expression of CAT was found in pAW786, in which the DNA insert was in the opposite orientation. Previous reports (15) have suggested lack of expression of *P. putida* genes in *E. coli*.

DISCUSSION

Constitutive expression of genes that encode the broad substrate specificity enzyme 3-PDase was found on a 6.4-kb fragment of chlorobiphenyl-degrading *P. putida* DNA that was cloned in cosmid vector pCP13 (13, 22). In this study, we identified and localized two genes (*bphC* and *bphD*) involved in chlorobiphenyl catabolism on a 2.4-kb *Hin*dIII DNA fragment of recombinant cosmid pOH101. *E. coli* cultures carrying recombinant plasmid pOH101 showed no evidence of 4-chlorobiphenyl degradation. This suggests that pOH101 either does not specify an active gene product of *bphA* (biphenyl dioxygenase) or lacks a DNA region essential for the initial enzymes of the chlorobiphenyl or biphenyl degradation pathway. Analysis of metabolites obtained from *E. coli*(pOH101) incubated with 3-phenylcatechol revealed conversion of 3-phenylcatechol into the *meta*-cleavage compounds HOPDA and benzoic acid. These results indicated that the cloned DNA insert (6.4 kb) in pOH101 specifies genes for 3-PDase (a gene product of *bphC*) and *meta*-cleavage compound HOPDA hydrolase (a gene product of *bphD*). As in our studies, Kimbara et al. have reported similar results from cloning a 29-kb DNA insert in a broad-host-range cosmid (pKS13) which contained the *bphC* and *bphD* genes. The cloned DNA fragment in a 3.2-kb *Pst*I recombinant plasmid fragment (pKHI) expressed 3-PDase activity in *P. putida* constitutively and lacked the first two genes, *bphA* and *bphB*, of the biphenyl catabolic pathway (15).

The *bphC* gene described in this study specifies a broad substrate specificity 3-PDase and showed high-level expression in *E. coli* (Table 2). On the contrary, the pKHI cosmid of Kimbara et al. did not express 3-PDase activity in *E. coli* and showed narrow substrate specificity (15). Subcloning of *Hin*dIII DNA fragments of pOH101 into the *Hin*dIII site of

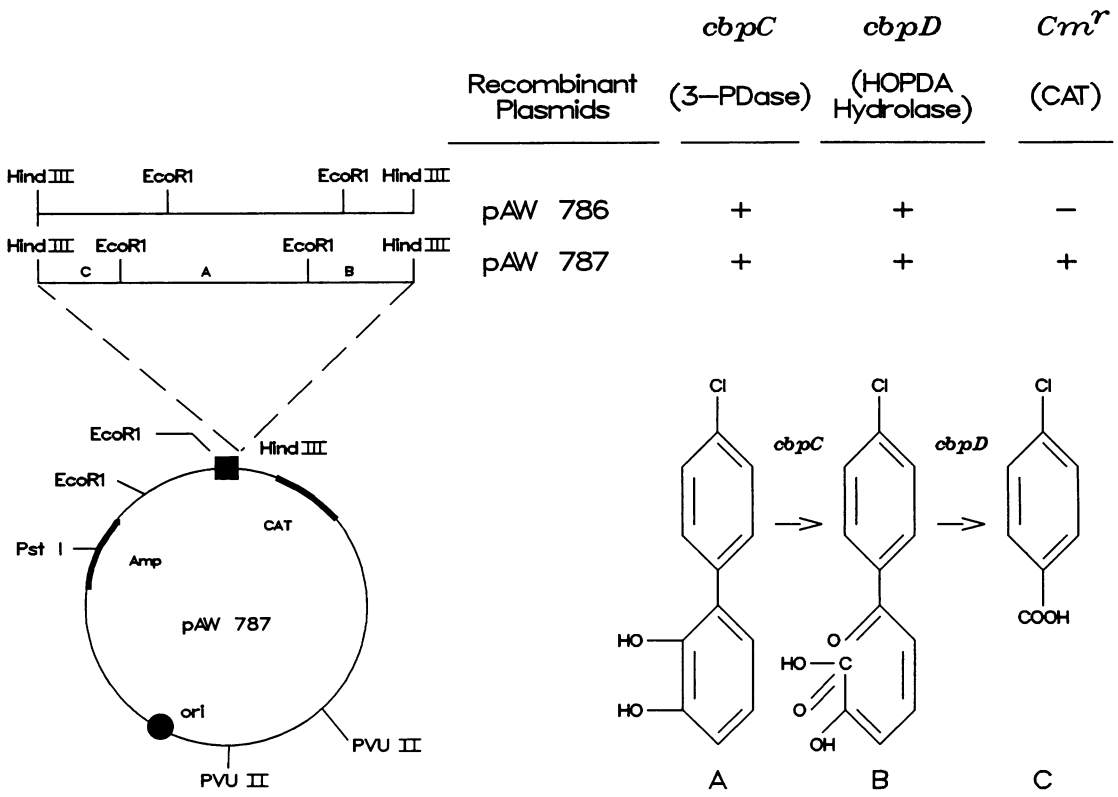


FIG. 3. Schematic of the cloning of a DNA insert (2.4-kb *Hin*dIII fragment) in promoter probe vector pKK232-8. Amp, Ampicillin; ■, multiple cloning sites; A, 4-chloro-3-phenylcatechol; B, 4-chloro derivative of HOPDA; C, 4-chlorobenzoic acid. ori, Origin of replication.

pUC18 and pUC19 downstream of the *lacZ* promoter showed expression of both 3-PDase and HOPDA hydrolase. These constructs allowed us to define essential regions of DNA that encode the *bphC* and *bphD* genes on a 2.4-kb DNA fragment and further strengthened our hypothesis that cloned DNAs might express their genes (*bphC* and *bphD*) through their own promoter. Further analysis was done by subcloning a 2.4-kb *Hin*dIII insert from pAW313 into a *Hin*dIII site of pKK232-8 upstream of the gene for CAT. CAT, 3-PDase, and HOPDA hydrolase activities were expressed in hybrid plasmid pAW787. Only CAT activity was lacking in hybrid plasmid pAW786 (Table 2). Since the cloned DNA was inserted into a promoter probe vector upstream of the Cm^r-encoding gene, the expression observed in *E. coli* must have been driven by a promoter contained in the cloned DNA sequences. Whether the same promoter functions in *P. putida* or not is not known. Expression of the *bphC* and *bphD* genes was found in either orientation (Fig. 3); therefore, the promoter responsible for CAT (Cm^r) expression appears to be independent of the promoter(s) used for expression of the *bphC* and *bphD* genes. This effect may be due to the opposite orientations of the cloned *Hin*dIII fragment in plasmid pAW786. On the basis of these results, we conclude that the cloned DNA insert in pAW313 expresses its genes with its own native promoter sequences. In contrast to our studies, Kimbara et al. (15) have observed expression of 3-PDase in *E. coli* and suggested that this expression is a readthrough product of the pUC *lacZ* promoter. Furthermore, it was also suggested that genes *bphC* and *bphD* were contained in an operon.

TABLE 2. Expression of 3-PDase, HOPDA hydrolase, and CAT activities

Bacterial strain or plasmid	Sp act of[a]:		
	3-PDase	HOPDA hydrolase	CAT
P. putida OU83	120.0	15.9	0.0
E. coli			
pAW313	2,285.0	3.8	0.0
pAW787	50.5	0.9	5.3
pAW786	77.4	0.9	0.0
pAW313-T2	0.0	0.9	ND
pAW313-T8	0.0	0.5	ND
pAW3131	0.0	0.0	ND
pAW3132	0.0	0.0	ND
pAW3133	0.0	0.0	ND
pAW413	1,780.0	ND	ND
pAW414	2,010.0	ND	ND

[a] One unit of CAT activity is equal to the chloramphenicol-dependent reduction of 1 μmol of dithiobisnitrobenzoic acid, which was measured as the rate of increase of the A_{412}. One unit of 3-PDase activity is equal to the formation of 1 μmol of the *meta*-cleavage product of the substrate per min at 25°C. One unit of HOPDA hydrolase activity is equal to the hydrolysis of 1 μmol of HOPDA, which was measured as the rate of decrease of the A_{434}. ND, Not determined.

With Tn*5* insertional mutations, it was found that a 2.1-kb region of the cloned DNA (indicated as A and B in Fig. 2) is essential for 3-PDase and HOPDA hydrolase activities. The locations of T2, T4, and T8 in 3-PDase-negative mutants were found in a *Hin*dIII-*Eco*RI fragment designated B (800 base pairs; Fig. 2). These 3-PDase-negative mutants expressed HOPDA hydrolase activity, suggesting that the *bphD* gene either is expressed through a Tn*5* promoter or has

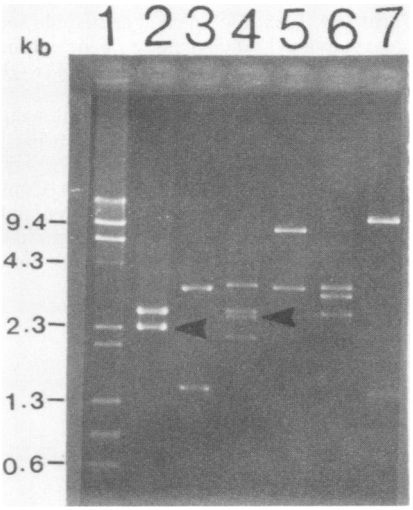


FIG. 4. Agarose gel electrophoresis of DNA restriction fragments of various plasmids. Lanes: 1, lambda *Hin*dIII and ϕX174 *Hae*III DNA markers; 2, pAW313 digested with *Hin*dIII; 3, pAW313 digested with *Eco*RI; 4, pAW313-T2 (pAW313::Tn*5*) digested with *Hin*dIII; 5, pAW313-T2 digested with *Eco*RI; 6, pAW313-T4 digested with *Hin*dIII; 7, pAW313-T4 digested with *Eco*RI. The arrowhead in lane 2 indicates the 2.4-kb *Hin*dIII fragment cloned in pUC18, and the arrowhead in lane 4 indicates the increase in the size of the 2.4-kb *Hin*dIII fragment caused by introduction of Tn*5*.

its own promoter. Further, construction of a hybrid plasmid containing the *Hin*dIII-*Eco*RI fragment (fragment B, 800 base pairs) showed lack of 3-PDase and HOPDA hydrolase activities (data not shown). On the basis of these results, we suggest that both fragments A (*Eco*RI-*Eco*RI, 1.3 kb) and B (*Hin*dIII-*Eco*RI, 800 base pairs) are needed for *bphC* and *bphD* expression of functional enzyme activities. Further subcloning of DNA and complementation studies will determine the exact boundaries of the *bphC* and *bphD* genes. This report provides, for the first time, direct biochemical evidence of the expression of HOPDA hydrolase genes contained in cloned DNA of recombinant plasmids. Previous reports have shown indirect evidence of HOPDA hydrolase function by either DNA sequence analysis or formation of benzoic acid from biphenyl (14, 19) or 3-PC (15). Extensive DNA homologies have been reported in catabolic genes contained in TOL (7), NAH, (6, 11), and *bph* (8, 15) DNAs. To the contrary, Southern hybridization analysis of pOH101 with ^{32}P-labeled DNA of pAW6194 (*bphC*) as a probe did not show positive DNA hybridization (13, 27). These results indicate lack of DNA sequence homologies and suggest divergence of catabolic genes in chlorobiphenyl-degrading bacteria.

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