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Expression, Localization, and Functional Analysis of Polychlorinated Biphenyl Degradation Genes *cbpABCD* of *Pseudomonas putida*

ASHRAF A. KHAN AND SATISH K. WALIA*

Department of Biological Sciences, Oakland University, Rochester, Michigan 48309-4401

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Genes of *Pseudomonas putida* strains that are capable of degrading polychlorinated biphenyls were cloned in the plasmid vector pUC19. The resultant hybrid plasmid, pAW6194, contained *cbpABCD* genes on a 9.0-kb DNA fragment that was necessary for the catabolism of polychlorinated biphenyls. These genes were further subcloned on an 8.0-kb *Hin*dIII fragment of pAW540. Degradation of 3-chlorobiphenyl, 2,4-dichlorobiphenyl, and 2,4,5-trichlorobiphenyl into a chloro derivative of benzoic acid was found in *Escherichia coli* harboring chimeric plasmid pAW540. Expression of *cbpA* (biphenyl dioxygenase, 6.2 U/mg of protein) and *cbpC* (3-phenylcatechol dioxygenase, 611.00 U/mg of protein) genes was also found in *E. coli* containing the hybrid plasmid pAW540. These enzyme activities were up to 10-fold higher than those found in *P. putida* OU83. These results led us to conclude that *cbpABCD* genes of *P. putida* OU83 were encoded on cloned DNA and expressed in *E. coli*. Whether the expression of *cbpABCD* genes of *P. putida* OU83 was driven by its own promoters located on the cloned DNA or by the *lacZ* promoter of pUC19 was examined by subcloning a 8.0-kb DNA fragment encoding the *cbpABCD* genes, in both orientations, in the *Hin*dIII site of the promoter probe vector pKK232-8. The resulting recombinant plasmids, pAW560 and pAW561, expressed *cbpABCD* genes and conferred chloramphenicol resistance only in *E. coli* harboring pAW560, indicating that the expression of chloramphenicol acetyltransferase is independent of *cbpABCD* gene expression. Physical mapping, subcloning, and deletion mutant plasmids allowed us to identify DNA regions encoding the *cbpBCD* genes on the 2.3-kb *Sal*I-*Hin*dIII fragment and the *cbpA* gene on the 2.8-kb *Sal*I fragment of pAW540. The locations of *cbpA* and *cbpBCD* genes were found to be 3.0 kb apart on the cloned DNA. The structural organization of the *cbpABCD* genes was also determined by Tn*5* insertional inactivation of the genes.

Bacterial degradation of polychlorinated biphenyls (PCBs) has been shown to occur by oxidative (1–4, 10–16, 21, 26) and reductive (23) metabolic mechanisms. The oxidative catabolic pathway of PCBs includes four enzymes: biphenyl dioxygenase (BPDase), dihydrodiol dehydrogenase (DHgenase), 3-phenylcatechol dioxygenase (3-PDase), and 2-hydroxy-6-phenylhexa-2,4-dienoate (HOPDA) hydrolase, encoded by the *cbpA*, *B*, *C*, and *D* genes, respectively (1, 13, 15, 21).

The cloning of the *cbpABCD* genes encoding enzymes that degrade PCBs has revealed considerable heterogeneity in their organization (1, 13, 21). In general, the *cbpABCD* genes were found to be clustered and organized in an operon (13, 21). However, the restriction enzyme fragment profiles of the cloned DNAs specifying PCB degradation differed in *Pseudomonas* spp. and *Alcaligenes* spp. (12, 13, 15, 21). In two reports the PCB degradation genes were found to be organized into more than one operon, since *cbpCD* genes alone formed one operon (16, 17). Whereas most PCB degradation genes from different bacteria have similar substrate specificities, the *cbpC* gene has been shown to be of two types; one specifies the enzyme with a broad substrate range (14), and the second encodes the enzyme with a narrow substrate specificity (15). In spite of these variations, the PCB degradation genes have been reported to be conserved in *Pseudomonas* spp. and *Alcaligenes eutrophus* (28). However, diversity of catabolic genes in soil microbial population has also been reported (27). Further studies on characterization of the PCB degradation genes should be helpful in understanding the organization and expression of these genes in various organisms.

In this report, we describe the subcloning, identification, localization, and expression of *cbp* genes of *Pseudomonas putida* OU83. Furthermore, we provide evidence for the PCB degradation genes being organized in an operon and precise localization of *cbpC* gene encoding narrow-substrate-specific 3-PDase.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophage. The bacterial strains, plasmids, and bacteriophage used in this study are listed in Table 1.

Bacterial growth medium and conditions. The organisms were grown in Luria broth containing 10% tryptone (Difco Laboratories, Detroit, Mich.), 0.5% yeast extract (Difco) 0.5% NaCl, and 0.1% glucose supplemented with appropriate antibiotics. For Luria agar plates, 1.5% agar (Difco) was added to Luria broth. Phosphate-buffered basal synthetic medium (BSM) supplemented with 1% KNO_3 was prepared as described by Bedard et al. (4). Appropriate substrate 4-chlorobiphenyl (4-CBP) (0.5 mM) or 0.1% D-glucose was also used as a carbon source. Organisms were incubated at 30°C (*P. putida*) and 35 ± 2°C (*Escherichia coli*). Long-term storage of bacteria was done in L broth containing 25% glycerol and appropriate antibiotics at −70°C.

DNA manipulation. Plasmid DNA was isolated by the alkaline lysis procedure (19) and further purified by cesium chloride-ethidium bromide density gradients. Plasmid DNA

* Corresponding author.

TABLE 1. Bacterial strains and plasmids used in this study

Organism, plasmid, or phage	Relevant characteristics and comments[a]	Source or reference
P. putida OU83	*cbpABCD*, isolated from PCB-contaminated soil	14
P. putida AC812	*trpB615*, *recA801*	9
pOH88	*cbpABCD*, Tc^r, 21-kb *Hin*dIII fragments of *P. putida* OU83 cloned in cosmid pCP13	15
E. coli HB101	*pro leu recA hsdR hsdM*	5
pAW6194	*cpbABCD*, Am^r, 9.0-kb *Eco*RI fragment of pOH88 cloned in pUC19	27
pAW540	*cbpABCD*, Am^r, 8.0-kb *Hin*dIII fragment of pAW6194 cloned in pUC19	This report
pAW520	*cbpC*, Am^r, 6.1-kb *Sal*I fragment of pAW6194 self-ligated, 3-PDase positive	This report
pAW583	*cbpC*, Am^r, 3.0 kb-*Sal*I fragment of pAW540 cloned in pUC19, 3-PDase negative	This report
pAW530	Am^r, 900-bp *Hin*dIII-*Eco*RI fragment of pAW6194 cloned in pUC19	This report
pAW550	Am^r, 900-bp *Bam*HI-*Hin*dIII fragment of pAW6194 cloned in pUC19	This report
pAW541	Am^r, 300-bp *Bam*HI fragment of pAW540 cloned in pUC19	This report
pAW542	Am^r, 900-bp *Bam*HI fragment of pAW540 cloned in pUC19	This report
pAW584	*cbpC*, Am^r, 2.3-kb *Sal*I fragment of pAW540 cloned in pUC19, 3-PDase positive	This report
pAW588	*cbpC*, Am^r, 2.3-kb *Sal*I fragment of pAW540 cloned in pUC19 in the opposite orientation to pAW584, 3-PDase positive	This report
pAW589	*cbpC*, Am^r, 2.3-kb *Sal*I fragment of pAW540 cloned in pBluescript SK$^+$, 3-PDase positive	This report
pAW590	*cbpC*, Am^r, 2.3-kb *Sal*I fragment of pAW540 cloned in pBluescript SK$^+$ in the orientation opposite to that of pAW589, 3-PDase positive	This report
pBluescript SK$^+$	Am^r, contains *lacZ* promoter for fusion protein induction	Stratagene Ltd.
pKK232-8	Am^r, CAT gene just downstream of cloning site	6
pAW560	*cbpABCD*, Am^r Cm^r, 8.0 kb *Hin*dIII fragment cloned in promoter probe vector pKK232-8	This report
pAW561	*cbpABCD*, Am^r Cm^r, 8.0-kb *Hin*dIII fragment cloned in pKK232-8 in the orientation opposite to that of pAW560	This report
pAW581	Am^r, 1.9-kb *Sal*I fragment of pAW540 cloned in pUC19, 3-PDase negative	This report
pAW585	Am^r, 1.1-kb *Sal*I fragment of pAW540 cloned in pUC19, 3-PDase negative	This report
pAW587	Am^r, 2.6-kb *Sal*I fragment of pAW540 cloned in pUC19, 3-PDase negative	This report
pAW582	Am^r, 3.7-kb *Sal*I fragment of pAW540 cloned in pUC19, 3-PDase negative	This report
pAW6194-T8	pAW6194::Tn*5*, Am^r Km^r, 3-PDase negative, no degradation of biphenyl	This report
pAW6194-T1 and T10	pAW6194::Tn*5*, Am^r Km^r, 3-PDase negative, formation of 3-phenylphenol from biphenyl	This report
pAW6194-T4, T5, T17, T26, T32, and T33	pAW6194::Tn*5*, Am^r Km^r, 3-PDase negative, formation of 3-phenylcatechol from biphenyl	This report
pAW6194-T7, T41, T43, T35, T38, T40, T36, and T42	pAW6194::Tn*5*, Am^r Km^r, 3-PDase positive, formation of 4-CHOPDA from 4-CBP	This report
λ467	λb221 *rex*::Tn*5* *c*I857 *Oam*29 *pam*-80 Km^r	7

[a] *cbp*, Chlorobiphenyl; *recA*, recombination deficient; *pro*, proline; *leu*, leucine; *trp*, tryptophan; *hsdR*, host-specific restriction; *hsdM*, host-specific modification; 4-CHOPDA, 4-chloro derivative of HOPDA.

restricted with appropriate enzymes was fractionated by agarose gel electrophoresis. The enzymes *Eco*RI, *Hin*dIII, *Xho*I, *Bam*HI, *Sal*I, and T4 DNA ligase were purchased from Bethesda Research Laboratories (Gaithersburg, Md.) and used as recommended by the vendor. Transformation of cells was performed by the $CaCl_2$-heat shock method (20). Transformants lacking β-galactosidase activity were selected on appropriate antibiotic plates as described previously (16). The ability of the transformants to convert 4-CBP or 3-phenylcatechol to the yellow *meta* cleavage product (HOPDA) by 3-PDase was tested by spraying a 0.1% solution of 3-phenylcatechol on transformant bacterial colonies as described previously (27).

Enzyme assay. 3-PDase activity was assayed by using high-speed supernatants of extracts prepared by sonication of the cells (sonifier from Branson Ultrasonic Corp., Danbury, Conn.) and measuring the formation of HOPDA or its chloro derivative (yellow *meta* cleavage product) at 434 nm at 30°C. BPDase activity was measured in whole cells by monitoring oxygen uptake with a YSI 53 monitor equipped with polarographic oxygen probe (11). Chloramphenicol acetyltransferase (CAT) activity was determined in the cell extract by spectrometry (24). Total protein was determined by the dye-binding assay (25) as recommended by the supplier of reagents for the protein assay (Pierce Chemical Co., Rockford, Ill.).

Transposon mutagenesis. Plasmid derivatives with transposon insertion mutations were isolated by transduction of Tn*5* with λ467 as described by Bruijin and Lupski (7). The location of Tn*5* was determined by restriction enzyme analysis with *Sal*I, *Hin*dIII, *Bam*HI, and *Eco*RI (16).

HPLC and GC-MS analyses. The degradation products of 4-CBP generated by the resting and growing cells were extracted by passing the samples through a prewashed (5 ml of methanol and 5 ml of deionized water) C_{18} column and eluting with either 3 ml of acetonitrile or hexane-ethyl acetate (1:1). An appropriate portion (2 to 20 μl) of each sample was analyzed by gas chromatography (GC) (16) or

high-pressure liquid chromatography (HPLC) with a C_{18} column (5-μm pore size, 250 by 4.6 mm; Alltech Associates, Inc.) and a mobile phase consisting of 100% acetonitrile and water-phosphoric acid (99:1) and an isocratic gradient of 50% acetonitrile and 50% water-phosphoric acid to 100% acetonitrile in 15 min at a flow rate of 1 ml/min. The A_{255} and A_{280} of metabolites were monitored with LC235 diode array detector (The Perkin Elmer Corp., Norwalk, Conn.). The identification and quantitation of the substrate and metabolite peaks were determined by comparing the HPLC cochromatography results, UV spectra, and peak areas with those of known standards. To determine the PCBs and metabolites by GC and GC-mass spectrometry (MS), the samples were extracted with hexane-ethyl acetate (1:1) and derivatized with trifluoroacetamide (Pierce). The samples were then analyzed on a QP-1000 (Shimadzu) GC-MS system. The GC-MS conditions, temperature program, and gas flow rate were as described previously (15).

Chemicals. 4-CBP was purchased from Lancaster Synthesis Ltd., Windham, N.H. 4-Chlorobenzoic acid (4-CBA), biphenyl, 5-bromo-4-chloro-3-indolyl-β-D-galactopyronaside, isopropylthio-β-D-galactopyronaside, kanamycin, ampicillin, and tetracycline were purchased from Sigma Chemical Co. (St. Louis, Mo.). 4-Chloro-3-phenylcatechol (4-CPC) and 3-phenylcatechol were synthesized in the laboratory of G. Brieger, Department of Chemistry, Oakland University, Rochester, Mich.

RESULTS

Degradation of PCBs by *E. coli* harboring pAW6194. Previously cloned DNA (pAW6194) of *P. putida* OU83 containing 3-PDase activity (15) was examined for the activity of other PCB-degradative enzymes by using 4-CBP, 3-CBP, 2,4-dichlorobiphenyl, and 2,4,5-trichlorobiphenyl in BSM (pH 7.5) as substrates. After the incubation of these compounds with the recombinant *E. coli*, the medium turned yellow within 15 min because of the formation of a *meta* cleavage product of PCBs. Analysis of the samples collected from incubation mixture containing only 4-CBP as the substrate (after extraction with ethyl acetate) revealed that 4-CBP was degraded to 4-CBA, suggesting that the *cbpABCD* genes of *P. putida* were expressed in *E. coli*. The kinetic analysis of degradation of 4-CBP in *E. coli* showed that the rate of degradation of 4-CBP was faster in the first hour of incubation at 30°C (Fig. 1). The elution profiles of 4-CBP (retention time, 15.23 min), 4-CPC (retention time, 7.47 min), and 4-CBA (retention time, 4.85 min) and the UV spectrum overlays of 4-CBP, 4-CPC, and 4-CBA standards are shown in Fig. 2. The retention times and UV spectra of two metabolites of 4-CBP shown in Fig. 2 were the same as those of 4-CPC and 4-CBA, respectively.

Physical mapping and analysis of transposon Tn*5* mutant plasmids. To determine the location and organization of the cloned *cbp* genes on plasmid pAW6194, a restriction map of cloned DNA fragment was constructed by using *Eco*RI, *Sal*I, *Hin*dIII, *Xho*I, and *Bam*HI (Fig. 3). Alignment of the DNA fragments on the restriction map was carried out by localizing the Tn*5* insertions in mutagenized derivatives of pAW6194.

The Tn*5* mutants were generated by transposition by using lambda::Tn*5*. Strains of *E. coli* containing pAW6194::Tn*5* were screened for kanamycin resistance and insertional inactivation of 3-PDase activity. Randomly selected kanamycin-resistant transductants (with or without 3-PDase activity) were further analyzed for the accumulation of

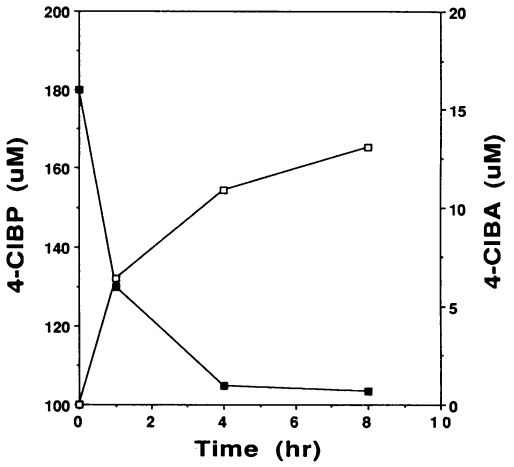

FIG. 1. Degradation of 4-CBP by cell suspension (optical density of 1.0 at 550 nm) of *E. coli* HB101 containing hybrid plasmid pAW6194. Shown are 4-CBP (■) and 4-CBA (□).

metabolites and site of insertion of Tn*5*. Six mutant transductants (T4, T5, T17, T26, T32, and T33) accumulated 4-CPC, two (T1 and T10) formed dihydrodiol from biphenyl (which in acidic conditions changed to 3-phenylphenol), one (T8) degraded neither 4-CBP nor biphenyl, seven (T35, T36, T38, T40, T41, T42, and T43) converted 4-CBP to 4-CBA, and one (T7) yielded HOPDA from biphenyl. The results showing formation of metabolites from 4-CBP are presented in Table 2.

The sites of insertion of Tn*5* in mutant derivatives of plasmid pAW6194::Tn*5* are shown in Fig. 3. The gel electrophoresis patterns of Tn*5* insertions in mutant plasmids digested with *Sal*I are represented in Fig. 4; Tn*5* has a unique *Sal*I site. The 1.0- and 6.1-kb *Sal*I fragments increased in size, and two new fragments appeared (Fig. 4, lanes 5 and 12). The 6.1- and 1.0-kb *Sal*I fragments of plasmid pAW6194 are indicated in lanes 2 and 13, respectively. Analysis of restriction enzyme data generated with other enzymes (*Eco*RI, no site in Tn*5*; *Bam*HI, one site in Tn*5*; *Hin*dIII, two sites in Tn*5*) helped in the precise localization of Tn*5* (data not shown).

Subcloning of *cbp* genes and analysis of deletion mutant plasmids. To locate the individual *cbp* genes on plasmid pAW6194, a number of deletion derivative plasmids were constructed by subcloning DNA. *Sal*I, *Hin*dIII, and *Bam*HI were used to construct deletion derivatives of the plasmid pAW6194. Plasmid pAW540 was constructed by cloning a 8.0-kb *Hin*dIII fragment of pAW6194 into plasmid pUC19 downstream of *lacZ* promoter, which expressed 3-PDase activity and formed 4-CBA from 4-CBP. To examine whether the subcloned DNA in plasmid pAW540 expressed *cbp* genes by using a promoter of pUC19 or of *P. putida*, the 8.0-kb *Hin*dIII fragment of pAW540 was cloned into a promoter probe vector, pKK232-8 (6). The promoter probe vector contains CAT genes downstream of the multiple cloning sites and lacks its own promoter. The resultant plasmids pAW560 and pAW561, in which the DNA fragment was cloned in opposite orientations, expressed 3-PDase activity in *E. coli* and degraded 4-CBP into 4-CBA (Fig. 5). The, chloramphenicol resistance was expressed only in plasmid pAW560 (Table 3). The 3-PDase gene was further subcloned on a 2.3-kb *Sal*I fragment into plasmid pBluescript SK^+ (Fig. 6). The cloned DNA in plasmid pAW589

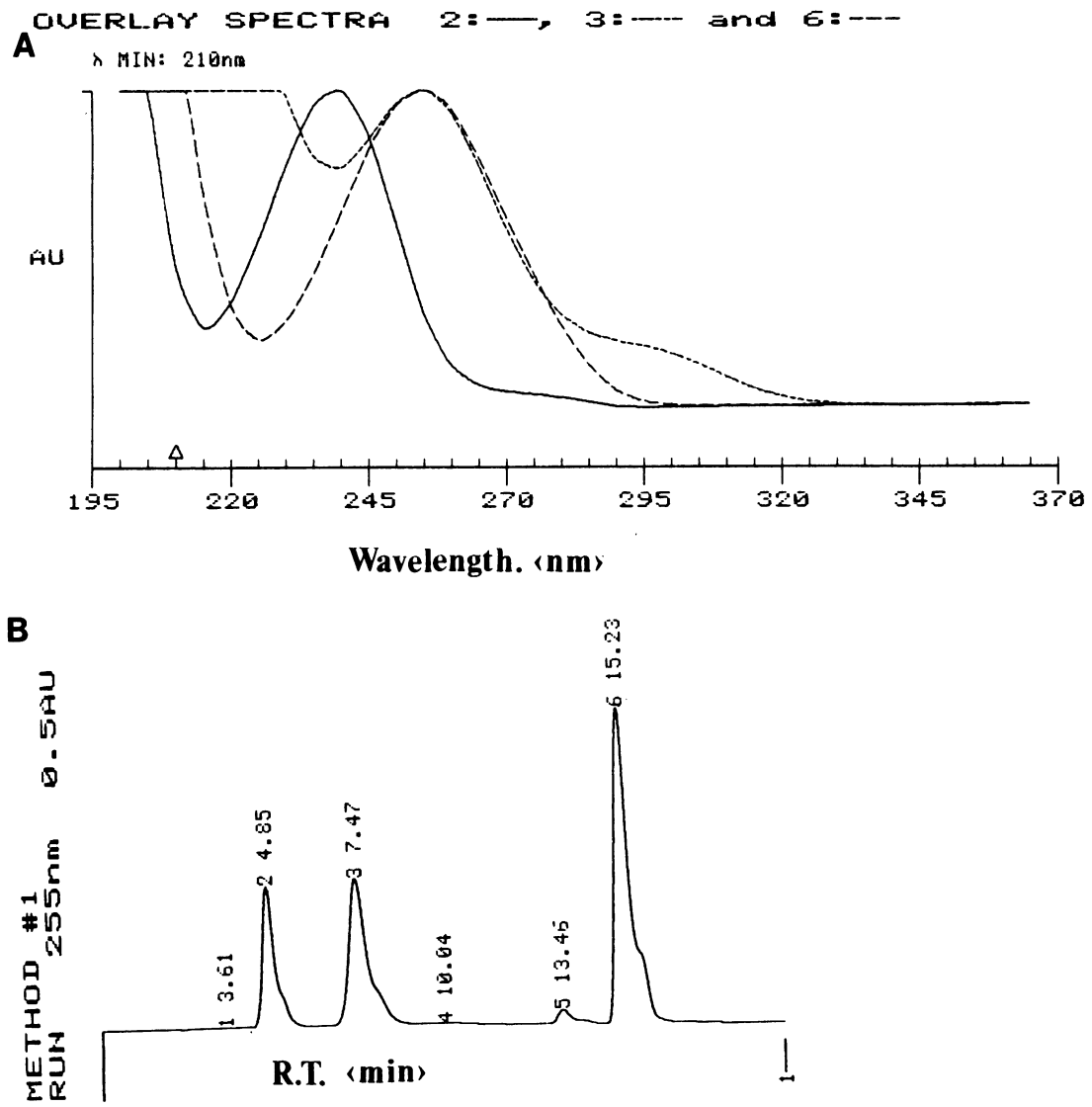

FIG. 2. UV spectrum overlay and high-pressure liquid chromatography elution profile. (A) UV spectrum overlay of 4-CBP (peak 6), 4-CPC (peak 3), and 4-CBA (peak 2). (B) Elution profiles of 4-CBP (retention time, 15 to 23 min; A_{255}), 4-CPC (retention time, 7.47 min; A_{255}), and 4-CBA (retention time, 4.85 min; A_{255}).

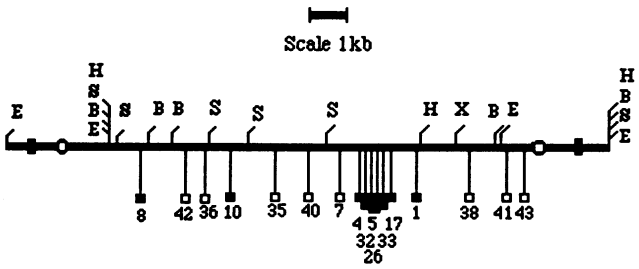

FIG. 3. Physical map of plasmid pAW6194 and site of insertion of Tn*5*. Shown are the site of Tn*5* insertion represented by numerical numbers that do (□) and do not (■) produce active 3-PDase enzyme. B, *Bam*HI; E, *Eco*RI; S, *Sal*I; H, *Hin*dIII; X, *Xho*I; ■, *lac*ZO, ○, origin of replication.

was in the orientation opposite to that of plasmid pAW590. The amount of 3-PDase produced by the cells containing pAW589 was approximately 10-fold greater than that of the cells harboring pAW590 (Table 3). Similar results were observed in *E. coli* harboring plasmids pAW584 and pAW588 containing the 2.3-kb *Sal*I fragment cloned in the orientation opposite to that of pUC19. Plasmids pAW581, pAW582, pAW585 and pAW587 with deletions in pAW540 neither produced 3-PDase nor degraded 4-CBP. Plasmid pAW520, constructed by deleting the internal *Sal*I fragment of pAW6194, failed to degrade 4-CBP but allowed conversion of 4-CPC into 4-CBA. Plasmids pAW550 and pAW530, generated by cloning the 900-bp *Hin*dIII-*Bam*HI and *Hin*dIII-*Eco*RI fragments of pAW6194 into pUC19, were negative for 3-PDase activity.

Enzymatic analysis. Regulation and expression of PCB catabolism genes was examined by assaying the PCB catabolism enzyme activities and utilization of various substrates.

TABLE 2. Metabolites of 4-CBP and biphenyl formed in *P. putida* and *E. coli* containing Tn*5* insertion mutant plasmid pAW6194::Tn*5*[a]

Organism	Formation of:			
	3-PP	4-CPC	4-CHOPDA	4-CBA
P. putida OU83	+	+	+	+
P. putida AC812	−	−	−	−
pOH88	+	+	+	+
E. coli HB101	−	−	−	−
pAW6194	+	+	+	+
pAW6194-T1	+	−	−	−
pAW6194-T4	+	+	−	−
pAW6194-T5	+	+	−	−
pAW6194-T7	+	+	+	ND
pAW6194-T8	−	−	−	−
pAW6194-T10	+	−	−	−
pAW6194-T17	+	+	−	−
pAW6194-T26	+	+	−	−
pAW6194-T32	+	+	−	−
pAW6194-T33	+	+	−	−
pAW6194-T35	+	+	+	+
pAW6194-T36	+	+	+	+
pAW6194-T38	+	+	+	+
pAW6194-T40	+	+	+	+
pAW6194-T41	+	+	+	+
pAW6194-T42	+	+	+	+
pAW6194-T43	+	+	+	+

[a] 3-PP, 3-Phenylphenol; 4-CHOPDA, 4-chloro derivative of HOPDA; +, formation of metabolites; −, no formation of metabolites; ND, not done.

E. coli strains containing pAW6194 and pAW540 were able to metabolize 4-CBP, glucose, and succinate (Table 4). However, *P. putida* was unable to oxidize succinate. Strains of *E. coli* harboring plasmid pAW520 had 3-PDase activity (Table 3) but were not able to degrade 4-CBP and 4-CBA. Strains of *E. coli* containing pAW540 expressed both biphenyl dioxygenase and 3-PDase. However, high-level expression of biphenyl dioxygenase was found in *P. putida* OU83. In contrast, the expression of 3-PDase was higher in strains of *E. coli* containing plasmids pAW540, pAW520, and pAW589 than in the parent strains, *P. putida* OU83. To determine the strength of the promoter on cloned DNA, CAT activity was assayed in *E. coli* containing pAW560 and pAW561. Expression of CAT was found in pAW560, and no expression of CAT was observed in pAW561, in which the DNA fragment was cloned in the orientation opposite to that of pAW560 (Table 3).

DISCUSSION

Genes specifying degradation of PCBs in *P. putida* OU83 (15, 16) were studied for the purpose of understanding their organization in this bacterium. This goal was achieved by subcloning the *cbp* genes that specify degradation of PCBs from the previously reported recombinant cosmid pOH88 (15). The relative positions of *cbp* genes expressed in *E. coli* were mapped by restriction enzyme analysis, and the boundaries of DNA encoding the *cbpABCD* genes were established on 8.0-kb DNA (Fig. 3). Others (1, 13, 21) reported similar findings of *cbpABCD* genes cloned from *Pseudomonas* spp. and expressed in *E. coli*. However, the size and restriction map of pAW6194 differed from the cloned DNA of *Pseudomonas* spp. encoding genes with similar functions. These observations suggest the divergence of *cbp* genes in bacteria during the course of evolution.

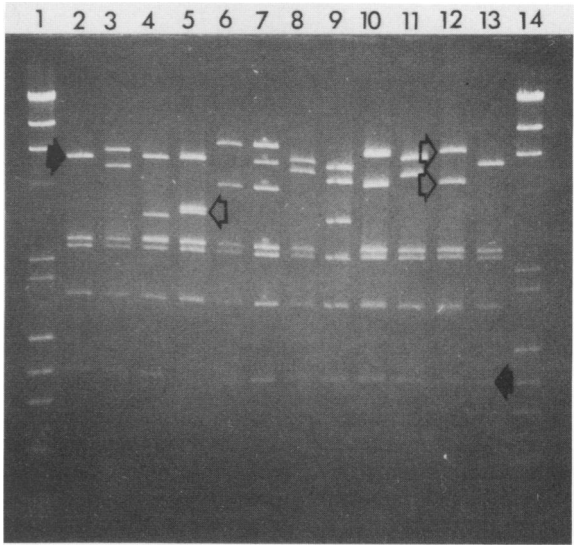

FIG. 4. Gel electrophoresis pattern of the plasmid pAW6194 and pAW6194::Tn*5*. Lanes: 1 and 14, lambda *Hin*dIII and ϕ174 *Hae*III markers; 2 and 13, plasmid pAW6194 DNA digested with *Sal*I; 2, pAW6194; 3, pAW6194-T1; 4, pAW6194-T8; 5, pAW6194-T10; 6, pAW6194-T34; 7, pAW6194-T37; 8, pAW6194-T7; 9, pAW6194-T17; 10, pAW6194-T17; 11, pAW6194-T5; 12, pAW6194-T33. The arrows in lanes 2 and 13 indicate 6.1- and 1.0-kb *Sal*I fragments of pAW6194, and the arrows in lanes 12 and 5 indicate the increases in size of the 6.1- and 1.0-kb *Sal*I fragments and the two new fragments. Tn*5* has a unique *Sal*I site.

Often catabolic genes are clustered and organized in an operon (12, 13, 18, 22). Transposons have been used for the genetic analysis of catabolic plasmids TOL (10, 30), SAL (29), and NAH (8). In this study, two types of transposon insertion mutant pAW6194::Tn*5* plasmids were found; one that converted 3-phenylcatechol into a yellow compound (HOPDA), indicating 3-PDase activity, and another that showed no accumulation of yellow compound, suggesting the lack of production of active 3-PDase. This seems to be due to the disruption of the open reading frame of the *cbp* genes by insertion of transposon Tn*5*. Further studies with 3-PDase-negative, Tn*5*-containing mutants showed either no degradation of 4-CBP or accumulation of 4-chloro-3-phenylphenol and 4-CPC from the degradation of 4-CBP. Based on the formation of metabolites (Table 2), three groups of transposon insertion mutant plasmids were identified; one did not degrade biphenyl, the second produced 3-phenylphenol, and the third accumulated 3-phenylcatechol from biphenyl. Inactivation of *cbp* genes in the former group indicates insertional mutations in the regulatory region or in the *cbpA* gene upstream of *cbpC*, suggesting a polar effect of Tn*5*. Mutations in second and third groups of the mutant plasmids are likely to be in the *cbpB* and *cbpC* genes. Mapping of sites of insertions of Tn*5* on plasmid pAW6194 showed the locations of the *cbp* genes (Fig. 3). Based on the relationship of the positions of insertions of Tn*5* on the restriction map, production of catabolic enzymes, and formation of metabolites, we propose that the *cbpA* gene is located on 2.8-kb *Sal*I fragment, which is 3.0 kb apart from the *cbpDCB* genes cluster contained on the 2.3-kb *Sal*I-*Hin*dIII fragment of pAW6194.

The results from this study further suggest that the order of *cbp* genes (*cbpA*, *cbpD*, *cbpC*, and *cbpB*) in pAW6194

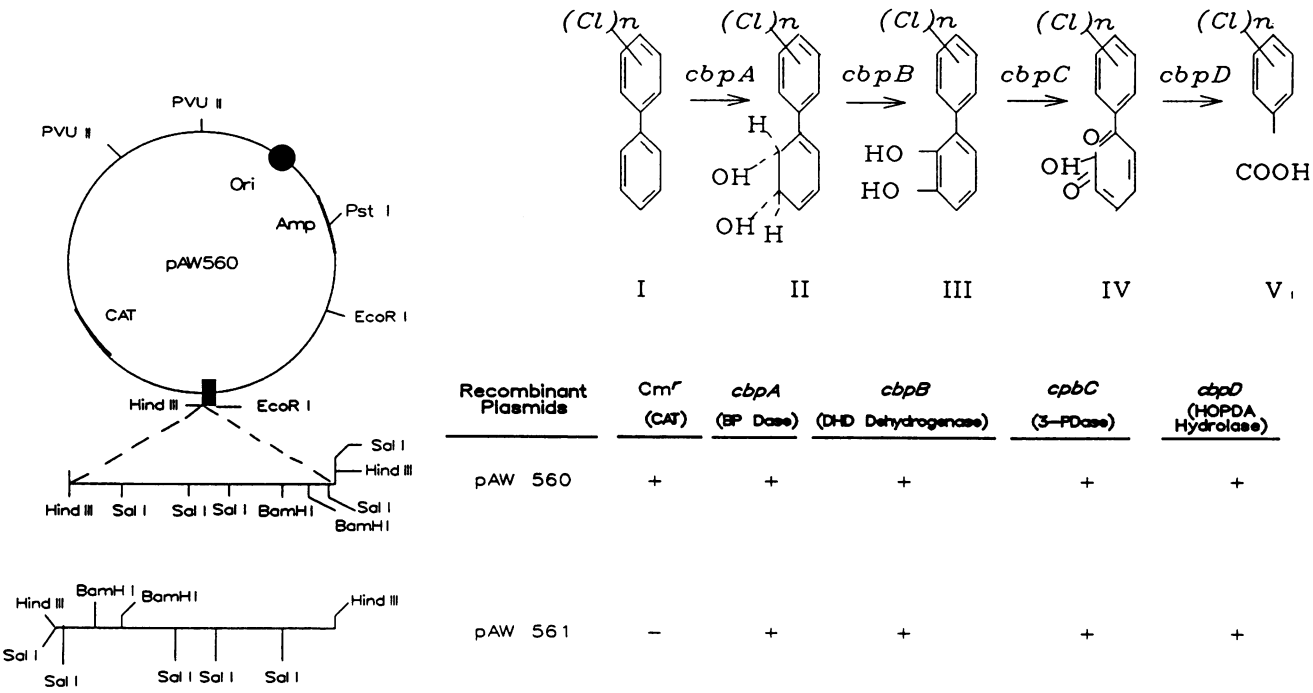

FIG. 5. Catabolic pathway of PCB degradation encoded on an 8.0-kb *Hin*dIII insert subcloned in the *Hin*dIII site of promoter probe vector pKK232-8. The DNA inserts in pAW560 and pAW561 are cloned in the opposite orientations. Genes: *cbpA* (gene encoding BPDase; *cbpB* (gene encoding dihydrodiol [DHD] dehydrogenase), *cbpC* (gene encoding 3-PDase), and *cbpD* (gene encoding HOPDA hydrolase). Chloro-derivative compounds: I, biphenyl; II, dihydrodiol compound; III, 3-phenylcatechol; IV, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate; V benzoic acid. Amp, ampicillin; +, formed; −, not formed; Ori, origin of replication.

differed from that of the previously cloned *cbp* genes from *Pseudomonas* spp. (1, 13, 21). The organization of *cbp* genes in pAW6194 appeared to be similar to that in plasmid pGEM456 constructed by Mondello (21) from a *Pseudomonas* sp., in which *cbpA* and *cbpBC* genes were also found 3.0 kb apart. The gene order of pAW6194 also differed from that of pKF715 created from a *Pseudomonas* sp. (13), in which the *cbpABC* genes were clustered and the *cbpD* gene was separated from the gene cluster on the cloned DNA. An operon from *P. putida* containing *cbpCD* genes specifying the broad-substrate-specific 3-PDase and HOPDA hydrolase have been described (14, 16). However, the *cbpB* gene function was not reported in this operon. It is likely that *cbpB* gene was also part of cloned DNA encoding the *Pseudomonas cbpCD* operon.

TABLE 3. Expression of BPDase, 3-PDase, and CAT activities

Bacterial strain or plasmid	Sp act of:		
	CAT[a]	BPDase[b]	3-PDase[c]
P. putida OU83	ND[d]	45.64	120.00
E. coli	0.00	0.00	0.00
pAW560	8.80	ND	27.53
pAW561	0.00	ND	59.43
pAW6194	0.00	6.28	205.00
pAW550	ND	0.00	0.00
pAW530	ND	ND	0.00
pAW520	ND	ND	1,690.82
pAW540	ND	ND	2,038.19
pAW583	ND	ND	0.00
pAW584	ND	ND	858.58
pAW588	ND	ND	44.04
pAW589	ND	ND	2,101.53
pAW590	ND	ND	256.86

[a] 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 A412.

[b] BPDase activity is equal to 1 nmol of oxygen consumed per min at 30°C.

[c] 3-PDase activity is equal to the formation of 1 μmol of *meta* cleavage product of 3-phenylcatechol per min at 25°C.

[d] ND, Not done.

The deletion of the 120-bp *Sal*I fragment in plasmids pAW582 and pAW587 caused loss of the BPDase (gene product of *cbpA*) activity, indicating an essential role of these DNA sequences in the expression of initial genes of PCB catabolic pathway. The DNA regions encoding *cbpC* and *cbpD* genes were localized on the 2.3-kb *Sal*I-*Hin*dIII fragments of plasmids pAW520 and pAW584. However, biochemical and genetic analyses of transposon Tn*5* mutant (pAW6194-T1) strongly suggest that *cbpB* gene is also contained in the 2.3-kb DNA fragment (Fig. 3). The amount of 3-PDase (with narrow substrate specificity) produced in *E. coli* containing recombinant plasmid pAW540 was higher than that of the parent, *P. putida* OU83. Previously, we have shown that the cloned *cbpC* gene on plasmid pAW313 encoding broad substrate specificity was expressed at high levels in *E. coli* (16). This may be attributed to the high copy number of hybrid plasmids, deletion of nonessential regions of DNA, or preferential use of promoter sequences by *E. coli* enzymes. Poor expression of *cbpCD* genes of *Pseudomonas* spp. have also been reported (17). Evidence of the expression of *cbp* genes in *E. coli* either driven by the promoters contained in the cloned DNA or through the *lacZ* promoter of plasmid vector pUC19 was obtained by cloning the 8.0-kb *Hin*dIII fragment of plasmid

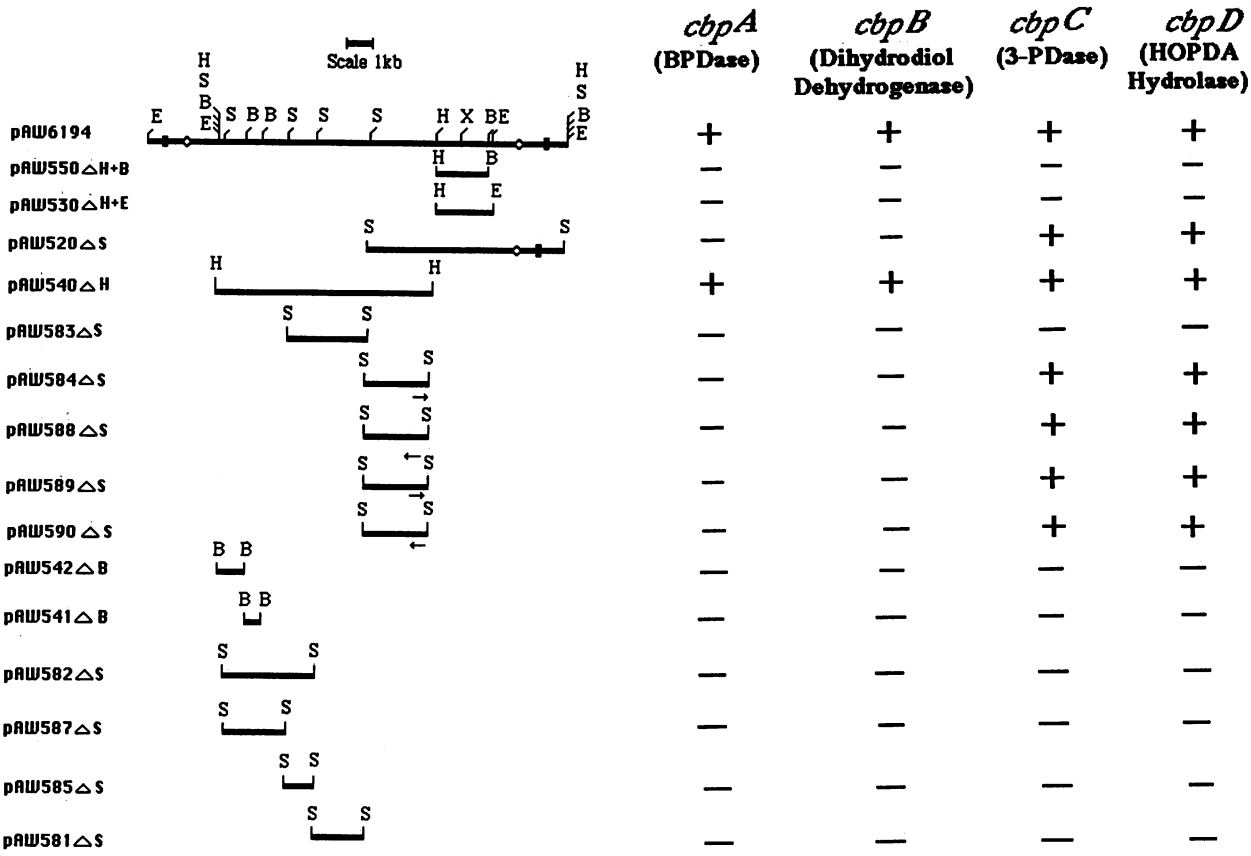

FIG. 6. Schematic of construction of deletion mutant plasmids by subcloning. Restriction sites: E, *Eco*RI, B, *Bam*HI; S, *Sal*I, H, *Hin*dIII; X, *Xho*I. Only the restriction sites used in cloning are shown. The arrow indicates direction of transcription of the *lacZ* promoter on plasmid vector. +, produced; −, not produced.

pAW540 in the *Hin*dIII site of the promoter probe vector pKK232-8. The resulting recombinant plasmids, pAW560 and pAW561, expressed *cbpABCD* genes in either orientation, indicating that cloned DNA has promoterlike sequences that are recognized by *E. coli* RNA polymerase. Whether the same promoter sequence functions in *P. putida* is not known. Since CAT genes can only be expressed in one orientation of the cloned DNA in pAW560, promoters used for the expression of *cbpABCD* genes may be independent of the promoters used for CAT gene expression. The amount of the production of 3-PDase in recombinant clones containing *cbp* genes varied by 2- to 10-fold. Further identification of the regulatory regions of *cbp* genes and substrate specificity mutations by DNA sequencing and substrate binding studies will be useful in laying the ground work for the genetic engineering of microorganisms with relaxed substrate specificity.

TABLE 4. Rates of oxygen uptake by washed bacterial cell suspension[a]

Bacterial strain or plasmid	Oxygen uptake rate (nmol of O_2 consumed per min per optical density of 1.0 for bacterial cell suspension in the electrode cell) in metabolism of:			
	4-CBP	4-CBA	Succinate	Glucose
P. putida OU83	114.12	39.35	0.00	7.87
E. coli				
pAW6194	15.70	0.00	55.09	15.74
pAW540	15.74	0.00	31.48	23.61
pAW520	0.00	0.00	35.41	43.28
pAW530	0.00	0.00	31.48	23.61

[a] The substrate was used at an initial concentration of 40 μM.

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