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Cloning of Bacterial Genes Specifying Degradation of 4-Chlorobiphenyl from *Pseudomonas putida* OU83

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Genes capable of 4-chlorobiphenyl (4-CBP) degradation were cloned from 4-CBP-degrading *Pseudomonas putida* OU83 by using a genomic library which was constructed in the broad-host-range cosmid vector pCP13. *P. putida* AC812 containing chimeric cosmid-expressing enzymes involved in the 4-CBP degradation pathway were identified by detecting 3-phenylcatechol dioxygenase activity (3-PDA). Chimeric cosmid clones pOH83, pOH84, pOH85, pOH87, and pOH88 positive for 3-PDA grew in synthetic basal medium containing 4-CBP (5 mM) as a carbon source. Restriction digestion analysis of recombinant cosmids showed DNA inserts ranging from 6 to 30 kilobase pairs. Southern hybridization data revealed that the cloned DNA inserts originated from strain OU83. Gas chromatography-mass spectrometry analysis of the metabolites of *P. putida* AC812(pOH88) incubated with 4-CBP and 4′-chloro-3-phenylcatechol showed the formation of 4-chlorobenzoic acid and benzoic acid. These results demonstrate that the cloned DNA fragments contain genes encoding for chlorobiphenyl dioxygenase (*cbpA*), dihydrodiol dehydrogenase (*cbpB*), 4′-chloro-3-phenylcatechol dioxygenase (*cbpC*), a *meta*-cleavage compound (a chloro derivative of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate) hydrolase (*cbpD*), and a new dechlorinating activity (*dcpE*). The location of the *cbpC* gene specifying 3-PDA was determined by subcloning an *Eco*RI DNA fragment (9.8 kilobase pairs) of pOH88 in plasmid vector pUC19. The cloned gene encoding 3-PDA was expressed in *Escherichia coli* HB101 and had substrate specificity only for 3-phenylcatechol and 4′-chloro-3-phenylcatechol.

Although the industrial and agricultural revolutions have brought economic prosperity, the environment, as a consequence, has been burdened with potentially carcinogenic and (mutagenic) halogen-substituted aromatic compounds, including polychlorinated biphenyls (24, 31). The majority of these compounds are resistant to microbial degradation (2, 3, 20, 34). However, bacterial strains have recently been isolated which have evolved the capability to degrade chlorinated biphenyls (1, 2, 6, 7, 17–19, 29, 46). Diverse biochemical pathways for the metabolism of chlorinated biphenyls have been reported (1, 4, 6, 7, 19, 29, 46, 48). At best, four enzymes (chlorobiphenyl dioxygenase (*cbpA*), dihydrodiol dehydrogenase (*cbpB*), 4′-chloro-3-phenylcatechol dioxygenase (*cbpC*), and a *meta*-cleavage compound (a chloro derivative of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid [HOPDA]) hydrolase [*cbpD*]) are believed to be involved in the catabolism of 4-chlorobiphenyl (4-CBP) (19, 29) into 4-chlorobenzoic acid (4-CBA). More recently, Omori et al. (32, 33) have purified one hydrolase and three *meta*-cleavage compound HOPDA-reducing enzymes (I, II, and III) that are responsible for the conversion of HOPDA into benzoic acid (BA). Accumulation of chloroacetophenone has also been shown with *Pseudomonas* species (4) and *Alcaligenes eutrophus* H850 (6).

Furukawa and Miyazaki (19) have cloned a 7.9-kilobase (kb) fragment of chromosomal DNA from *Pseudomonas pseudoalcaligenes* which specifies three genes (*bphA*, *bphB*, and *bphC*). Plasmid-mediated degradation of 4-CBP has also been reported (18, 37), but the detailed genetic basis of degradation has not been studied for far.

In this report we describe the molecular cloning of the genes involved in catabolic pathway of 4-CBP and their expression in *Pseudomonas putida* and *Escherichia coli*.

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MATERIALS AND METHODS

Chemicals. The chemicals used in this study were as follows: 4-CBP (Lancaster Synthesis, Ltd., Windham, N.H.); 4-chlorocatechol (American Tokyo Kasel, Inc., Portland, Oreg.); 3-phenylcatechol (3-PC; Sunny Microbiology International, Rochester Hills, Mich.); and 4′-chloro-3-phenylcatechol (4-CPC), which was synthesized by T. McGuire in the laboratory of G. Brieger (Department of Chemistry, Oakland University, Rochester, Mich.). Biphenyl (BP), catechol, 4-CBA, 4-methylcatechol, BA, and radiolabeled 4-CBP (4-Cl-$^{14}C_6H_4$-C_6H_5; 7.2 mCi/mmol) were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Synthesis of 4-CPC. The synthesis of 4-CPC was carried out as follows. 2-Phenylphenol (Aldrich Chemical Co., Inc., Milwaukee, Wis.) was acetylated by standard procedures (23) and chlorinated in a manner similar to that reported previously for 4′-chloro-4-hydroxybiphenyl (36). The resulting chloroacetate was recrystallized from petroleum ether (mp 80 to 81°C) and analyzed by gas chromatography (GC; flame ionization detector; 2-m column; 5% OV-17/80-100 Chrom W-HP), which indicated a single product with 98% purity. A nuclear magnetic resonance (NMR) spectrum at 250 MHz indicated a singlet at 2.09 ppm, which was also indicative of isomeric purity. Oxidative degradation yielded only 4-CBA, indicating that the desired 4′-chloro-2-hydroxyphenol acetate was obtained. The chloroacetate was saponified to the corresponding phenol. The 4′-chloro-2-hydroxyphenol was formulated in the position *ortho* to the hydroxyl group by a general method reported for 2-hydroxyphenol (10). After purification by recrystallization, the resulting aldehyde (mp 96.8 to 98.3) was oxidized by the Dakin modification of the Baeyer-Villager reaction (22). The final product was purified by repeated recrystallization from hexane to give a white solid (mp 106 to 108°C). GC analysis of the bis-trimethylsilyl ether derivative showed a purity of 98%. The elemental analysis of this compound was done by

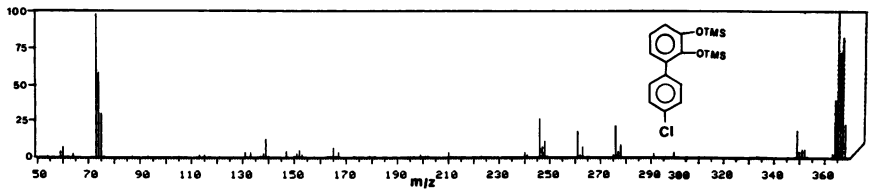

FIG. 1. Mass spectrum of the trimethylsilane (TMS) derivative of 4-CPC.

Galbraith Laboratories Inc. (Knoxville, Tenn.) and showed a composition of 65.45% C and 4.25% H. Oxidative degradation yielded only 4-CBA, which was indicative of isomeric purity. The proton NMR spectrum, obtained at 60 MHz in deuterochloroform (tetramethylsilane internal standard) was relatively simple. It consisted of a broad singlet at 5.25 ppm (2H), a sharp singlet at 6.70 ppm (3H), and another sharp singlet at 7.27 ppm (4H). The NMR spectrum closely resembled that of the previously reported 2,3-dihydroxybiphenyl, which showed the following NMR spectrum at 60 MHz in deuterochloroform (tetramethylsilane internal standard): a broad singlet at 5.57 ppm (2H), a sharp singlet at 6.68 (3H), and a sharp singlet at 7.25 (5H).

The mass spectrum of 4-CPC showed the following peaks: 365 (100, M^+), 349 (20, M-CH_3), 276 (23), 261 (20), 246 (27), 73 (98) (Fig. 1). The mass spectrum for the *N,O*-bis(trimethylsilyl)trifluoroacetamide derivative of 2,3-dihydroxybiphenyl, by way of comparison, showed the following peaks: 330 (49, M^+), 315 (29, M-CH_3), 242 (24), 227 (24), 212 (35), 73 (97).

Bacterial strains, growth conditions, and plasmids. The source and relevent characteristics of bacterial strains and plasmids used in this study are listed in Table 1. The organisms were grown in phosphate-buffered synthetic basal medium (SBM) containing the following, in grams per liter: K_2HPO_4, 5.6; KH_2PO_4, 2.1; NH_4Cl 2.7; $MgSO_4$, 0.19; $MnSO_4 \cdot H_2O$, 0.05; $FeSO_4 \cdot 7H_2O$, 0.001; $CaCl_2 \cdot 2H_2O$, 0.003; and BP or 4-CBP, 0.9. It was supplemented with 0.005% yeast extract as a carbon source. The medium was prepared by the instructions of Bedard et al. (7). Luria broth (L broth) was 1% tryptone (Difco Laboratories, Detroit, Mich.), 0.5% yeast extract (Difco), 0.5% NaCl, and 0.1% D-glucose. For Luria agar solid medium, 1.5 g of agar (Difco) was added to 100 ml of the broth.

DNA isolation, restriction enzyme digestion, and ligations. The chromosomal DNA from *P. putida* OU83 was isolated as described by Darzin and Chakrabarty (12). *P. putida* OU83 was grown overnight in SBM containing 5 mM BP and 0.5% sodium succinate. The cells were lysed in lysis solution (2.5 ml of 25% sucrose in 50 mM Tris hydrochloride [pH 8.0], 1 ml of lysozyme [10 mg/ml], 2.5 ml of 0.25 M EDTA, and 4 ml of 20% sodium dodecyl sulfate [SDS]). The suspension was then heated at 55°C for 10 minutes and extracted with phenol-chloroform (1:1), and the DNA was precipitated with isopropanol. DNA was further purified by cesium chloride gradient centrifugation (12).

The plasmid DNA was isolated by an alkaline lysis procedure (28) and purified on cesium chloride density gradients. The enzymes *Eco*RI, *Hin*dIII, *Xho*I, *Bam*HI, *Pst*I, and T4 DNA ligase were used and were purchased from Bethesda Research Laboratories (Gaithersburg, Md.), International Biotechnologies, Inc., or Boehringer Mannheim Biochemicals (Indianapolis, Ind.). Incubation and storage conditions were as recommended by the suppliers.

Genomic library construction. The chromosomal DNA of *P. putida* OU83 was partially digested with *Hin*dIII and mixed with cosmid pCP13 that was previously digested with *Hin*dIII at a ratio of 1:3. The ligations were carried out by adding T4 DNA ligase and incubating the mixture at 4°C for 16 h. The ligated DNA was packaged in vitro by using packaging extracts (Amersham Corp., Arlington Heights, Ill.) and transfected into *E. coli* AC80. The transfected cells were plated onto L-agar plates with tetracycline (30 μg/ml). The genomic library (recombinant cosmid clones) was stored in L broth containing 40% glycerol and 30 μg of tetracycline per ml.

Identification of genes specifying degradation of 4-CBP. Chimeric cosmid clones were transferred from *E. coli* AC80 to *P. putida* AC812 by triparental crossing as described by Carey et al. (9). Briefly, the donor strain AC80 (containing the cosmid genomic library), mobilizing factor (strain HB101 containing pRK2013), and recipient (*P. putida* AC812) were grown overnight in L broth and mated on nitrocellulose membrane filters (44). Transformants of *P. putida* containing recombinant cosmids were selected on *Pseudomonas* isolation agar containing 100 μg of tetracycline per ml. The transformed colonies containing recombinant plasmids and expressing 4-CBP degradation genes were identified by spraying the colonies with 3-PC (0.1% solution in 50 mM Tris hydrochloride [pH 7.5]–10% acetone). Positive clones quickly turned yellow by the formation of the *meta*-cleavage product HOPDA.

Growth of cosmid clones in synthetic basal medium. The organisms containing chimeric plasmids were grown in SBM containing 4-CBP (5 mM), BP (5 mM), or both as the sole carbon source. Growth was monitored at 540 nm, and after 7 days an increase in absorbance by more than 0.05 was considered as positive growth.

Transformation of 4-CBP and 4-CPC. The degradation of 4-CBP and 4-CPC was studied in SBM as described by Walia et al. (46). The medium for resting cells incubation was amended with 100 μg of chloramphenicol per ml (Sigma). Briefly, the cell suspension in SBM medium containing 500 μg of 4-CBP per ml and 2 μCi of ^{14}C-labeled 4-CBP (specific activity, 7.2 mCi/mmol) was incubated at 30°C on a rotary, shaking incubator (Labline Inc.) for 7 days. The degradation products were acidified, extracted with ethyl acetate, and then analyzed by thin-layer chromatography and GC-mass spectrometry (MS) (46). Abiotic controls used under the test conditions were heat-killed cell suspensions, which were indicators for the volatilization and nonbiological degradation of the test chemical compound.

Gel electrophoresis, Southern blotting, and DNA hybridization. The DNA was digested with appropriate restriction enzymes and separated on 1% agarose at 60 V for 2 h with a horizontal gel electrophoresis apparatus (Bio-Rad Laboratories, Richmond, Calif.) in TEA buffer (40 mM Tris acetate [pH 7.8], 2 mM EDTA). After denaturation and neutralization of the gel, the DNA fragments were then transferred onto nylon membrane (Gene Screen Plus; Dupont, NEN Research Products, Boston, Mass.) filters by the method of Southern (39), and the filter was prehybridized for 2 h at 65°C

in 20 ml of prehybridization solution containing 1 M NaCl, 1% SDS, and 10% dextran sulfate. The heat-denatured calf thymus DNA (100 μg/ml) and radioactive DNA probe containing 5×10^6 dpm/ml, which was labeled to a specific activity of 10^8 dpm/μg of DNA with the random primer extension labeling system (13), was added to the bag containing prehybridized membrane. The hybridization was carried out for 40 h at 65°C. The filter was washed twice with 2× SSC (0.3 M NaCl plus 0.03 M sodium citrate) at room temperature for 5 min with constant agitation, twice with 200 ml of a solution containing 2× SSC and 1% SDS at 65°C for 30 min with constant agitation, and twice with 0.1× SSC and 1% SDS at 65°C for 30 min. The filter was wrapped in plastic wrap and exposed to X-ray film with a single intensifying screen at −70°C.

Subcloning of the 3-phenylcatechol dioxygenase gene. The DNA fragment specifying 3-phenylcatechol dioxygenase in the chimeric cosmid pOH88 was subcloned into pUC19 in the following manner. A complete *Eco*RI digest of pOH88 and similarly digested pUC19 were mixed and ligated at 4°C as described by Walia and Duckworth (42). The ligation mixture was transformed into *E. coli* JM83 by $CaCl_2$-heat shock treatment (27). The transformants were selected on L-agar medium amended with 100 μg of ampicillin per ml and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside per ml (30). The positive clones specifying 3-phenylcatechol dioxygenase activity (3-PDA) were identified by spraying a 0.1% solution of 3-phenylcatechol. The positive clones turned yellow by forming the *meta*-cleavage product HOPDA from 3-PC.

Enzyme assay. Activities for 3-phenylcatechol dioxygenase and catechol 2,3-dioxygenase were assayed on supernatants of extracts prepared by disrupting the cells by sonication (Branson Utrasonic Corp., Danbury, Conn.), followed by centrifugation (28,000 × *g*) for 30 min at 4°C. The enzyme activity for 3-PC was determined by measuring the formation of the *meta*-cleavage compound at 434 nm after the addition of 4-CPC or 3-PC. Catechol 2,3-dioxygenase activity was measured by determining the rate of formation of 2-hydroxymuconic acid at 375 nm (35). The oxidation of 4-methylcatechol and 4-chlorocatechol was determined by following the increase of the A_{382} and A_{379}, respectively. A quantitative conversion of the catechols into ring fission products was obtained from the molar absorption coefficients (5). Protein content was determined by a dye-binding assay (38) by the instructions of the supplier (Pierce Chemical Co., Rockford, Ill.).

Analysis of chimeric plasmid-encoded gene products. The gene products encoded by the *Eco*RI fragment of pOH88 in pAW6194 were analyzed in crude cell extracts of *E. coli* HB101 containing chimeric plasmids, as described by Ghosal et al. (21). The proteins were separated in SDS-polyacrylamide gels and then stained with Coomassie blue (26, 45).

Thin-layer chromatography, autoradiography, and GC-MS. The degradation products from the resting cell suspension or growing cells in either 4-CBP or 4-CPC were acidified to pH 1.0 with 2 N H_2SO_4 and then extracted twice with equal volumes of ethyl acetate. The extracts were applied to precoated silica gel plates (Whatman Ltd., Maidstone, Kent, England) and then developed in a solvent system containing benzene-dioxane-acetic acid in a ratio of 20:4:1 (vol/vol/vol) (19). The developed plates were exposed to a UV lamp (Mineral light; UVP, Inc., San Gabriel, Calif.). Degradation of radiolabeled compounds was monitored by autoradiography of the thin-layer chromatographic plates. For GC-MS analysis the extracted metabolites were derivatized with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (Pierce Chemical Co.) (46). Analyses were carried out on a GC-MS system (QP-1000; Shimadzu). A fused-silica capillary column (15 m by 0.25 mm [inner diameter]; Quadrex, New Haven, Conn.) with a 1.0 μm methyl silicone coating was used for the separations. The carrier gas was helium, which was injected at 20 ml/min. Injections were made in the splitless mode with a 1.0-min delay time. The temperature program used was 2 min at 50°C, followed by a ramp rate of 20°C/min to 250°C. The MS was operated in the electron ionization mode (70 eV). The source and inlet transfer line were held at 250°C. Masses were scanned from 50 to 450 atomic mass units (amu) at 1.3 s per scan.

RESULTS

Cloning of 4-CBP-degrading genes. The purified genomic DNA of *P. putida* OU83 was partially digested with *Hin*dIII and ligated with cosmid pCP13 that was completely digested with *Hin*dIII (a unique *Hin*dIII site is located in the kanamycin resistance gene of pCP13 [12]). The ligated DNA was packaged in vitro into bacteriophage lambda heads (Amersham) and then transfected into *E. coli* AC80. The transfectants were selected on L-agar medium amended with tetracycline (30 μg/ml). The chimeric cosmids were mobilized from strain AC80 into *P. putida* AC812 by mobilizing plasmid pRK2013 (16). The frequency of transfer of the recombinant cosmid was 7.8×10^4. To determine the size of DNA inserts in the genomic library, the DNAs from 20 randomly chosen cosmid clones were isolated and digested with *Hin*dIII and analyzed on a 1% agarose gel. About 90% of the recombinant cosmids were found to contain an average of 23 kb of DNA insert of chromosomal DNA from *P. putida* OU83. This observation indicates that our genomic library gave a greater than 99% probability that any particular *P. putida* gene was cloned. Over 2,000 transconjugants of *P. putida* AC812 containing chimeric cosmids were tested for the production of 3-phenylcatechol dioxygenase. A total of 11 clones (containing chimeric cosmid) specifying 3-PDA were identified (Table 1).

Growth and degradation of 4-CBP by 3-phenylcatechol dioxygenase-positive clones. The clones positive for 3-phenylcatechol dioxygenase were grown in SBM amended with 4-CBP (5 mM), and the results of the growth experiments are summarized in Table 2. The growth yield of chimeric cosmid pOH88 was 235% compared with the growth of the parent *P. putida* OU83. The ability to degrade 4-CBP by the chimeric cosmid was also studied by the use of ^{14}C-radiolabeled 4-CBP as the substrate. The degradation products of ^{14}C-labeled 4-CBP were analyzed by thin-layer chromatography. A chromatogram of metabolites of 4-CBP and their corresponding autoradiogram are shown in Fig. 2. A common metabolite spot (R_f, 0.35) of 4-CBA and BA was detected in the ethyl acetate extract of 4-CBP degradation products from cosmid pOH85 (Fig. 2, lane A), pOH84 (Fig. 2, lane B), and pOH88 (Fig. 2, lane E). Only one radiolabeled metabolite spot was found in Fig. 2, lane C (strain OU83), which comigrated with 4-CBA and BA. No radiolabeled metabolite spot was found for strain 812(pOH810) (Fig. 2, lane F) or the negative control *P. putida* AC812 (Fig. 2, lane D). GC-MS analysis of the derivatized ethyl acetate extract of 4-CBP with pOH88 revealed peaks with retention times of 8.1 and 6.8 min and gave an identical mass spectrum to those of 4-CBA and BA, with peaks at 228 (m^+/z^+) and 194 (m^+/z^+), respectively. These results indicate that 4-CBP is converted into 4-CBA and BA. The degradation of 4-CPC by pOH88 in

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain	Plasmid	Phenotype or genotype[a]	Source or reference
P. putida			
OU83	ND[b]	4-CBP$^+$ BP$^+$ 2,3-DHBD$^+$	This paper
AC812		*trpB615 recA801*	A. M. Chakrabarty (11)
AC812	pOH81	*trpB615 recA801* 2,3-DHBD$^+$ Tc^r	This paper
AC812	pOH82	*trpB615 recA801* 2,3-DHBD$^+$ Tc^r	This paper
AC812	pOH83	*trpB615 recA801* 2,3-DHBD$^+$ Tc^r	This paper
AC812	pOH84	*trpB615 recA801* 2,3-DHBD$^+$ Tc^r	This paper
AC812	pOH85	*trpB615 recA801* 2,3-DHBD$^+$ Tc^r	This paper
AC812	pOH86	*trpB615 recA801* 2,3-DHBD$^+$ Tc^r	This paper
AC812	pOH87	*trpB615 recA801* 2,3-DHBD$^+$ Tc^r	This paper
AC812	pOH88	*trpB615 recA801* 2,3-DHBD$^+$ Tc^r	This paper
AC812	pOH89	*trpB615 recA801* 2,3-DHBD$^+$ Tc^r	This paper
AC812	pOH810	*trpB615 recA801* 2,3-DHBD$^+$ Tc^r	This paper
E. coli			
HB101		*pro leu recA hsdR hsdM*	43
HB101	pOH810	*pro leu recA hsdR hsdM* 2,3-DHBD$^+$ Tc^r	This paper
AC80	pOH101	*thr leu met hsdR hsdM* 2,3-DHBD$^+$ Tc^r	This paper
AC80		*thr leu met hsdR hsdM*	A. M. Chakrabarty (11)
HB101	pRK2013	*pro leu recA hsdR hsdM* Km^r	16
AC80	pCP13	*thr leu met hsdR hsdM*	A. M. Chakrabarty (12)
HB101	pOH101	*pro leu recA hsdA hsdM* 2,3-DHBD$^+$ Tc^r	This paper
HB101	pAW6194	*pro leu recA hsdM* 2,3-DHBD$^+$ Am^r	This paper
JM83	pAW6194	λ^- *ara* Δ(*pro-lac*) *thi* ϕ80d*lacZ* ΔM15 λ^- 2,3-DHBD$^+$ Am^r	This paper
JM83	pUC19	λ^- *ara* Δ(*pro-lac*) *thi* ϕ80d*lacZ* ΔM15 λ^- β-gal$^+$ Am^r	30
JM83		λ^- *ara* Δ(*pro-lac*) *thi* ϕ80d*lacZ* ΔM15 λ^-	30

[a] Designations used for the relevant genotype and phenotypes are as follows: BP, biphenyl; 4-CBP, 4-chlorobiphenyl; β-gal$^+$, β-galactosidase; 2,3-DHBD, 2,3-dihydroxybiphenyl dioxygenase; Am, ampicillin; Tc, tetracycline; Km, kanamycin; Sm, streptomycin; Rif, rifampin; *recA*, recombination deficient; *pro*, proline; *leu*, leucine; *trp*, tryptophan; *thr*, threonine; *met*, methionine; *hsdR*, host-specific restriction; *hsdM*, host-specific modification; *thi*, thiamine; *lac*, lactose; *ara*, arabinose.

[b] ND, Not detected.

a resting cell suspension is shown in Fig. 3, which revealed the formation of 4-CBA (retention time, 8.1 min [Fig. 3A], 228 [m^+/z^+] [Fig. 3C]), BA (retention time, 6.8 min [Fig. 3A], 194 [m^+/z^+] [Fig. 3B]), and parent 4-CPC (retention time, 11.9 min [Fig. 3A], 365 [m^+/z^+] [Fig. 1]). The accumulation of 4-CBA and BA was found in a ratio of 3:1 after incubation of 4-CPC for 4 h with a resting cell suspension of *P. putida* AC812(pOH88). No metabolite peak corresponding to 4-CBA or BA was detected in heat-killed cell suspensions of *P. putida* AC812(pOH88) when they were incubated either with 4-CBP or 4-CPC. These results demonstrate that chimeric cosmid pOH88 contains more than one gene for the conversion of 4-CBP, 4-CPC, or both into 4-CBA and BA.

Molecular analysis of chimeric cosmids. The electrophoretic analysis of *Hin*dIII-digested cosmid DNA indicated the

TABLE 2. Growth of *P. putida* AC812 containing chimeric cosmid DNA encoding genes for the degradation of 4-CBP

P. putida strain	Cosmid	A_{540} (% growth yield)[a]	
		BP	4-CBP
OU83		0.20 (100)	0.19 (100)
AC812		NG	NG
AC812	pOH81	0.48 (240)	ND
AC812	pOH86	0.66 (330)	ND
AC812	pOH87	0.44 (200)	0.42 (210)
AC812	pOH88	0.47 (235)	0.58 (290)
AC812	pOH810	NG	NG

[a] *P. putida* AC812 containing chimeric cosmid clones were grown on SBM amended with 5 mM BP or 4-CBP and 20 μM tryptophan. The A_{540} was followed for 7 days. NG, No growth. Growth yield was compared with that of parental *P. putida* OU83 over the same period of time. ND, Not done.

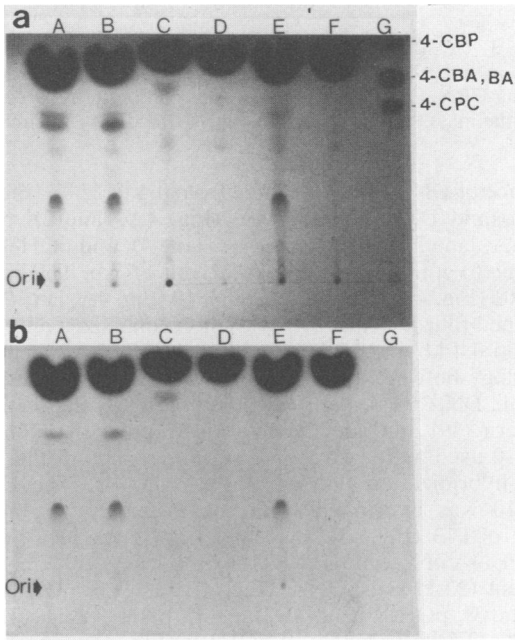

FIG. 2. Thin-layer chromatogram of the ^{14}C-radiolabeled metabolites of 4-CBP. (a) Lane A, *P. putida* AC812(pOH85); lane B, *P. putida* AC812(pOH84); lane C, *P. putida* OU83; lane D, *P. putida* AC812(pOH84); lane E, *P. putida* AC812(pOH88); lane F, *P. putida* AC812(pOH810); lane G, 4-CBP, 4-CBA, and 4-CPC. (b) Autoradiogram of panel a. Both 4-CBA and BA cochromatographed (R_f, 0.35) in the solvent system containing benzene-dioxane-acetic acid (20:4:1 [vol/vol/vol]).

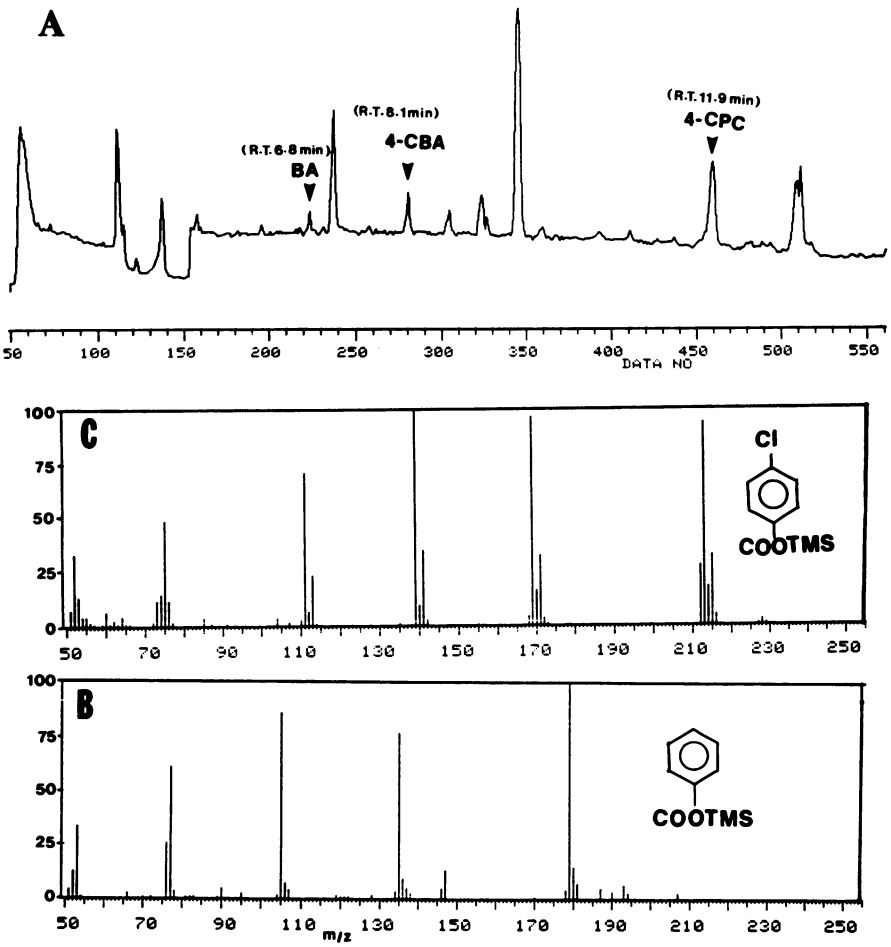

FIG. 3. GC-MS of trimethylsilane (TMS) derivatives of the metabolites of 4-CPC. (A) GC-MS profile of the metabolite peaks. R.T., Retention time. (B) Mass spectrum of the metabolite peak (retention time of BA, 6.8 min). (C) Mass spectrum of the metabolite peak (retention time of 4-CBA, 8.1 min). The mass spectrum of the compound peak (retention time of 4-CPC, 11.9 min) was the same as that given in Fig. 1, and the mass spectra shown in panels B and C were similar to the mass spectra of BA and 4-CBA.

presence of DNA inserts ranging from 0.4 to 22 kb (Fig. 4A). The cosmid DNA from pOH83 (Fig. 4A, lane 2), pOH84 (Fig. 4A, lane 3), pOH87 (Fig. 4A, lane 4), and pOH88 (Fig. 4A, lane 5) contained inserts of 22 and 1.7 kb. The cosmids pOH101 (Fig. 4A, lane 1) and pOH810 (Fig. 4A, lane 9) each contained four DNA inserts with molecular sizes of 2.6, 2.3, 0.8, and 0.4 kb and 18.5, 7.5, 2.3, and 2.0 kb, respectively. Homology between the cloned DNA and that of the parent genomic DNA was established by blotting by the method of Southern (39) and specific hybridization, with ^{32}P-labeled pOH810 used as the DNA probe. The results of the DNA-DNA hybridization are shown in Fig. 4B. The cosmid pOH810 was hybridized with the linearized fragment of vector pCP13 (Fig. 4B, lane 8), *Hin*dIII fragments of genomic DNA of *P. putida* OU83 (Fig. 4B, lane 10), a common fragment (22 kb) of pOH83 (Fig. 4B, lane 2), pOH84 (Fig. 4B, lane 3), pOH87 (Fig. 4B, lane 4), pOH88 (Fig. 4B, lane 5), and a 2.3-kb fragment of pOH101 (Fig. 4B, lane 1). The hybridization data established that the DNA inserts were from the parent strain OU83. To establish similarities or dissimilarities among the various clones, we compared them after they were digested with various restriction enzymes (*Hin*dIII, *Eco*RI, *Bam*HI, *Xho*I, and *Pst*I). Chimeric cosmids pOH83, pOH84, pOH87, and pOH88 all had identical fragment patterns. The data for only one chimeric clone (pOH88) is shown in Fig. 5, since all others (listed above) were identical and their *Hin*dIII restriction patterns are shown in Fig. 4. To determine the location of the *cbpC* gene on the cloned fragment, we subcloned a 9.8-kb *Eco*RI fragment from pOH88, and its expression was determined (see below). Plasmids containing the 9.8-kb *Eco*RI fragment were named pAW6194 (Fig. 5, lane 8).

Expression of cloned gene product in *E. coli*. The synthesis of cloned gene products was analyzed by preparing crude cell extract of 3-phenylcatechol dioxygenase-positive (pAW6194) and -negative (pUC19) clones. Subsequently, the cell extracts were then subjected to SDS-polyacrylamide gel electrophoresis. The proteins were produced so abundantly in *E. coli* HB101 that they could be readily visualized in conventional Coomassie blue-stained SDS-polyacrylamide gels. Figure 6 (lane 3) shows the protein profile of *E. coli* HB101 containing the plasmid vector pUC19 (negative control). The proteins synthesized by strain HB101 containing pAW6194 are shown in Fig. 6 (lane 2). The results shown in Fig. 6 (lane 2) indicate that the cloned DNA (pAW6194) expressed at least two peptides with M_rs of 55,000 and 22,000.

Level of 3-PDA and substrate profile. The expression of 3-PDA was measured in *E. coli* and *P. putida* containing recombinant plasmids. The relative activities of this enzyme

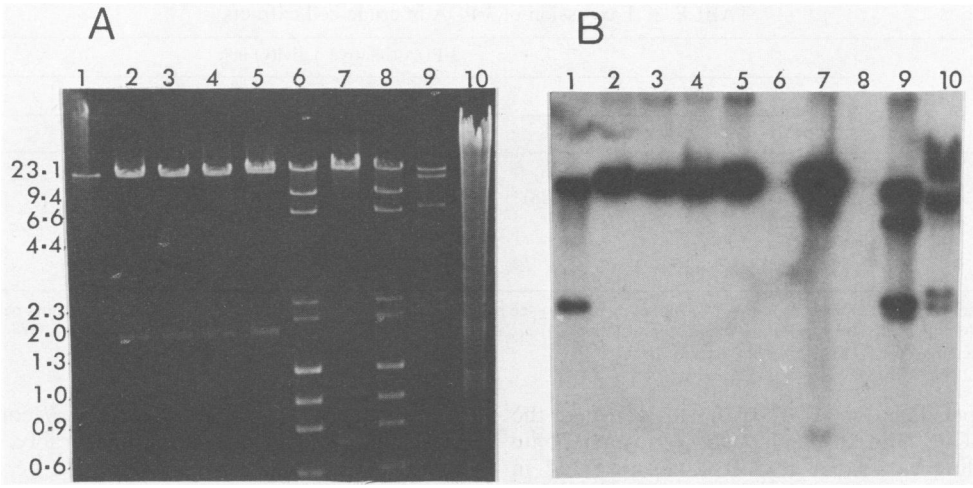

FIG. 4. Agarose gel electrophoresis of *Hin*dIII restriction fragments of chimeric cosmids and chromosomal DNA and the corresponding Southern blot hybridization. The sizes (in kilobases) of the DNA fragments are given on the left. (A) Lane 1, pOH101; lane 2, pOH83; lane 3, pOH84; lane 4, pOH87; lane 5, pOH88; lanes 6 and 8, lambda *Hin*dIII and φX174 *Hae*III DNA markers, respectively; lane 7, cosmid pCP13 (parent); lane 9, pOH810; lane 10, chromosomal DNA of *P. putida* OU83. (B) Autoradiogram of DNA-DNA hybridization of the corresponding lanes in panel A, with cosmid pOH810 used as the probe.

with various substrates are given in Table 3. The level and the substrate profile of 3-PDA varied in different clones. The 3-PDA specified by pOH88 and pAW6194 had a narrow substrate specificity only for 3-PC and 4-CPC. The enzyme specified by pOH810 had a broad substrate specificity, including catechol, 4-methylcatechol, 4-CPC, and 3-PC. Taira et al. (41) have also reported similar 3-PDAs in *Pseudomonas paucimobilis* and *P. pseudoalkaligenes*.

DISCUSSION

We described here the cloning of genes specifying the catabolism of 4-CBP. The metabolism of 4-CBP via the *meta*-cleavage pathway needs at least four different enzymes (chlorobiphenyl dioxygenase [*cbpA*], dihydrodiol dehydrogenase [*cbpB*], 3-phenylcatechol dioxygenase [*cbpC*], and a *meta*-cleavage compound hydrolase [*cbpD*]) (19). In this study, GC-MS analysis of the metabolites of 4-CBP revealed the conversion of 4-CBP into 4-CBA and BA by strain AC812(pOH88). This observation confirms the 4-CBP degradation pathway proposed by Furukawa and Miyazaki (19) and Ahmed and Focht (1) and further suggests that the genes (*cbpA*, *cbpB*, *cbpC*, and *cbpD*) encoding at least four enzymes that are necessary for the catabolism of 4-CBP into 4-CBA were cloned. The identification of BA among the degradation products of 4-CBP suggests that there is a new dechlorination activity or metabolism of trace amounts of biphenyl if it is present in 4-CBP (98% purity by GC analysis). GC-MS analysis of the metabolites of 4-CPC (98% purity with no evidence of the presence of biphenyl) with

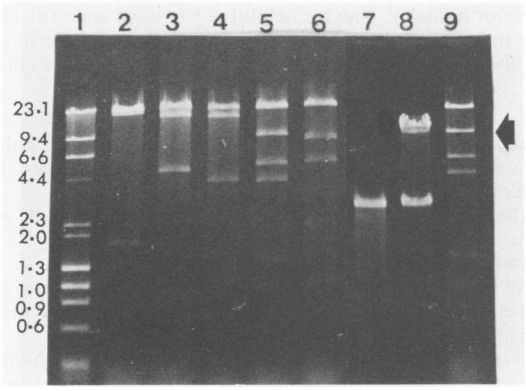

FIG. 5. Agarose gel electrophoresis of DNA restriction fragments of various plasmids. Lane 1, Lambda *Hin*dIII and φX174 *Hae*III DNA markers; lane 2, pOH88 digested with *Hin*dIII; lane 3, pOH88 digested with *Xho*I; lane 4, pOH88 digested with *Hin*dIII-*Xho*I; lane 5, pOH88 digested with *Eco*RI; lane 6, pOH88 digested with *Eco*RI-*Hin*dIII; lane 7, pUC19 digested with *Eco*RI; lane 8, pAW6194 digested with *Eco*RI; lane 9, pOH88 digested with *Eco*RI. The arrows indicate the 9.8-kb *Eco*RI fragment containing the 3-phenylcatechol dioxygenase gene. Numbers to the left of the gel are in kilobase pairs.

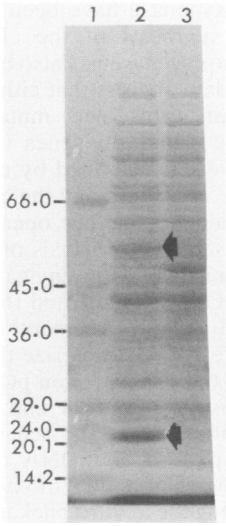

FIG. 6. SDS-polyacrylamide gel electrophoresis of crude cell extract. Lane 1, Molecular weight marker; lane 2, *E. coli* HB101 containing pAW6194; lane 3, *E. coli* HB101 containing pUC19. The arrows indicate the peptide bands encoded by chimeric plasmid. The sizes (in kilodaltons) of the peptides are given on the left.

TABLE 3. Expression of 3-PDA in crude cell extracts

Substrate	3-PDA (relative activity) in[a]:				
	P. putida AC812		*E. coli* HB101		
	pOH810	pOH88	pOH101	pOH810	pAW6194
3-PC	133 (72)	29.0 (15)	185 (100)	26.0 (14)	100 (54)
4-CPC	79.0 (188)	54.0 (128)	42.0 (100)	38.0 (90)	89.0 (211)
Catechol	710 (986)	NA	72.0 (100)	67.0 (93)	NA
4-Chlorocatechol	454 (926)	NA	49.0 (100)	40.0 (81)	NA
4-Methylcatechol	188 (606)	NA	31.0 (100)	42.0 (135)	NA

[a] One unit of enzyme was equal to the formation of 1 μmol of *meta*-cleavage product of substrate per min at 25°C. 3-PDA activity (units per milligram of protein) in crude cell extract. Numbers in parentheses are the percent relative activity relative to that of *E. coli* HB101(pOH101), which was 100%. *P. putida* AC812 and *E. coli* HB101 were negative for 3-PDA. NA, No activity.

resting cell suspensions of AC812(pOH88) revealed the conversion of 4-CPC into BA and 4-CBA. No metabolite peak corresponding to 4-CBA and BA was detected in abiotic controls [heat-killed suspensions of strain AC812 (pOH88) with 4-CPC]. This observation confirms the first possibility that there is dechlorinating activity of the cloned DNA insert in pOH88. The formation of BA from the degradation of 4-CBP has been shown in *P. putida* OU83 (46). More recently, dechlorination of pentachlorophenol has been demonstrated in *Flavobacterium* species (40). Previously, Furukawa and Miyazaki (19) cloned a 7.9-kb DNA fragment of *P. pseudoalcaligenes* that contained three clustered genes (*bphA*, *bphB*, and *bphC*) but that lacked the fourth gene *bphD*. The genes for the metabolism of the aromatic compounds toluene (15), naphthalene (8, 21), xylene (15, 21), and chlorobenzoate (11, 47) were also found to be clustered.

The enzymes involved in the catabolism of aromatic compounds are generally inducible (14, 18). In our studies, however, we found constitutive synthesis of 3-phenylcatechol dioxygenase from *P. putida* OU83 (46). The constitutive synthesis of 3-phenylcatechol dioxygenase may be due to the selection of a mutant in the regulatory region of the *cbp* operon. It appears that this mutant strain was selected during the isolation of chlorobiphenyl-degrading bacteria (46). The selection of mutants for the constitutive synthesis of catechol 2,3-dioxygenase have been reported previously (14). Constitutive synthesis of the cloned gene product 3-phenylcatechol dioxygenase has also been found in cosmid clones (25). These data suggest that either the complete *cbp* operon, including the (presumed) mutated regulator gene, were cloned or the structural genes (*cbpA*, *cbpB*, *cbpC*, *cbpD*, and *dcpE*) were transcribed by using the kanamycin promoter of pCP13. To understand the order of the gene and the regulatory elements of the *cbp* operon, further subcloning and nucleotide sequence analysis of catabolic genes are necessary. Subcloning of 3-phenylcatechol dioxygenase from pOH88 in pUC19 has indicated that the *cbpC* gene is located in a 9.8-kb *Eco*RI fragment of pOH88. Further studies are in progress to characterize the structural organization and order of the *cbp* genes on pOH88 and pAW6194.

The chimeric cosmids (pOH88 and pOH810) encoding 3-phenylcatechol dioxygenase showed variations in the restriction fragment pattern and DNA hybridization which suggest either two types of related dioxygenases, deletion of the *Hin*dIII site during the in vitro packaging of ligating DNA or rearrangement of DNA sequences. The multiple location of 2,3-dioxygenase genes, inversion, deletion, and rearrangement of DNA fragments have been reported in the haloaromatic-degrading operon (20, 25).

The relative activity of 3-phenylcatechol dioxygenase in crude extracts of *P. putida* or *E. coli* containing different recombinant cosmids varied considerably. This variation in the amount of enzyme activity could be attributed to the expression and regulation of *cbp* genes. The minilysate prepared from the *E. coli* HB101 containing pAW6194 showed differences in the molecular weight(s) of cloned gene-encoded peptides, indicating that a number of gene products were expressed. The enzyme specified by pAW6194 showed a narrow substrate specificity (3-PC and 4-CPC) similar to that of the enzyme characterized by Furukawa and Arimura (17). In contrast, the genes found in pOH810 encoded enzyme activities with a broad substrate profile (catechol, 4-methycatechol, 4-chlorocatechol, 3-PC, and 4-CPC). To understand the importance and the physiological differences among various types of dioxygenases, it will be necessary to purify and characterize these enzymes. Further information on the comparison of the nucleotide sequence will aid in the understanding of the mechanisms of gene expression and regulation of the catabolism of 4-CBP.

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