APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1988, p. 2664–2671
0099-2240/88/112664-08$02.00/0
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Vol. 54, No. 11

Molecular Cloning of 3-Phenylcatechol Dioxygenase Involved in the Catabolic Pathway of Chlorinated Biphenyl from *Pseudomonas putida* and Its Expression in *Escherichia coli*†

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Received 11 April 1988/Accepted 5 August 1988

Genes encoding 3-phenylcatechol dioxygenases were cloned from the chlorobiphenyl-degrading *Pseudomonas putida* strain OU83, using broad-host-range cosmid vector pCP13. Restriction enzyme analysis of DNA from 2,3-dioxygenase-positive chimeric cosmids showed DNA inserts ranging in size from 6.0 to 30 kilobases. The origin of the DNA insert in hybrid clones was established by using ^{32}P-labeled hybrid clones (pOH101 and pOH810). A 2.3-kilobase *Hin*dIII fragment was common to two clones. The 2,3-dioxygenase from the parent *P. putida* strain, OU83, and the recombinant clones (pOH101 and pOH8101) showed similar characteristics as determined by isoelectric focusing and polyacrylamide gel electrophoresis. The 2,3-dioxygenase from the *Escherichia coli* recombinant cosmid showed a pI of 5.0, a K_m of 14 μM, and broad substrate activity with catechol, 4-chlorocatechol, 4-methylcatechol, and 2,3-dihydroxybiphenyl.

The increased applications of halogen-substituted aromatic compounds in agricultural and industrial activities have burdened the environment with their excess release. The presence of these xenobiotic chemical compounds in the ecosystem poses a threat to the preservation of an ecological balance in this hemisphere. Numerous investigators have isolated pure cultures of bacteria capable of degrading halogenated aromatic compounds (1–3, 5–8, 16, 29). Diverse metabolic pathways for the catabolism of chlorinated biphenyls have been reported (1, 3, 5–7, 13–15, 20, 29). More recently, accumulation of chloroacetophenone has been shown with *Alcaligenes eutrophus* H850 (5) and *Pseudomonas* sp. (3). Extensive studies on the bacterial degradation of chlorinated biphenyls have shown that a majority of bacterial strains metabolize polychlorinated biphenyls via extradiol-type 2,3-dioxygenase (5, 15). In a majority of cases, accumulation of chlorobenzoic acid was found as a dead-end metabolite (1, 5, 15, 20, 29), which may be due to lack of an enzyme system in bacteria to metabolize the end product further. However, total mineralization of chlorinated biphenyls by mixed cultures has been reported (14).

The genes encoding enzymes that degrade chlorinated biphenyls were found on both chromosomes (15) and plasmids (14, 23). Furukawa and Miyazaki (15) have described cloning of a 7.9-kilobase (kb) fragment of chromosomal DNA from *Pseudomonas pseudoalcaligenes* which encodes genes specifying biphenyl dioxygenase, dihydrodiol dehydrogenase, and 2,3-dihydroxybiphenyl dioxygenase (2,3-DHBD) and further demonstrated that the purified 2,3-dioxygenase from both the parent and the cloned genes showed narrow substrate activity for only 2,3-dihydroxybiphenyl (2,3-DHBP) or 3-phenylcatechol (3-PC) (13). In this paper, we describe for the first time molecular cloning of genes specifying 2,3-dioxygenase with broad substrate specificity from the chlorinated biphenyl-degrading *P. putida* strain OU83 and expression of this gene in *Escherichia coli*.

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† Dedicated to the memory of Tom Pinkerton.

MATERIALS AND METHODS

Chemicals. The chemicals used in this study were as follows: biphenyl, 4-methylcatechol (Sigma Chemical Co., St. Louis, Mo.), 4-chlorocatechol (American Tokyo Kasel, Inc., Portland, Ore.), 4-chlorobiphenyl (Lancaster Synthesis Ltd., Windham, N.H.), and 3-PC (Sunny Microbiology International, Rochester Hills, Mich.). 3-PC was a colorless crystalline powder with a meniscus point of 104.5°C and a liquefaction point of 105.8°C (melting point apparatus with digital thermometer MFB-595; Gallenkamp, Leicestershire, U.K.). Gas chromatographic analysis (flame ionization detector, 2-m column, 5% OV-17/80–100 Chromosorb W-HP) of the corresponding bistrimethyl ether indicated a single product of >98% purity. The proton nuclear magnetic resonance spectrum, obtained at 60 MHz in deuterochloroform (tetramethylsilane internal standard) consisted of a broad singlet at 5.57 ppm (2H), a sharp singlet at 6.68 ppm (3H), and a sharp singlet at 7.25 ppm (5H). The mass spectrum of the *N*,*O*-bis(trimethyl silyl)trifluoroacetamide derivative of 2,3-DHBP showed the following peaks (Fig. 1):330 (49 M^+); 315 (29 M^+, M-CH_3); 242 (24 M^+); 227 (24 M^+); 212 (35 M^+); and 73 (97 M^+).

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

Media and cell growth. The organisms were grown in phosphate-buffered mineral salt medium containing the following (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 biphenyl or 4-chlorobiphenyl, 0.9, as carbon source. The medium was supplemented with 0.005% yeast extract and was prepared by using the instructions of Bedard et al. (6). 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. When maltose was needed in L broth, 0.2% maltose was substituted for glucose. For Luria agar solid medium, 1.5 g of agar (Difco) was added to 100 ml of the broth.

Transformation. The transformation of *E. coli* AC80 or HB101 was carried out as described previously (18), and transformants were selected on L-agar medium supple-

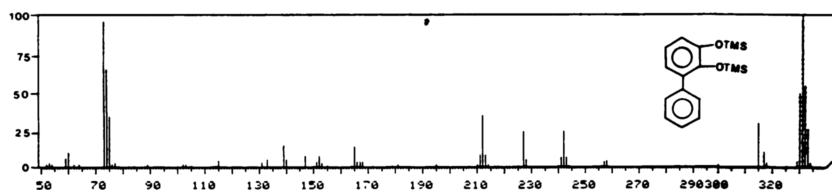


FIG. 1. Mass spectrum of trimethylsilane (TMS) derivative of 3-PC.

mented with tetracycline (30 μg/ml). *P. putida* AC812 containing recombinant cosmid and strain OU83 were selected on *Pseudomonas* isolation agar (Difco) with and without tetracycline (100 μg/ml). The DNA encoding genes for the biphenyl degradation pathway were tested by spraying a 0.1% solution of 2,3-DHBP. The clones expressing genes for 2,3-DHBD turned yellow within 1 to 2 min, and the colonies lacking this enzyme did not change their color.

Preparation of DNA. Plasmid DNA was isolated by the alkaline lysis procedure (19) and further purified by cesium chloride-ethidium bromide density gradient centrifugation (21). The purified DNA was dried under vacuum, dissolved in 10 mM Tris hydrochloride–1 mM EDTA, pH 8, and kept at −20°C until further use. The chromosomal DNA from *P. putida* OU83 (chlorobiphenyl-degrading soil bacteria [29]) was isolated as described before (9). Briefly, *P. putida* OU83 was grown overnight in 200 ml of minimal basal medium containing 5 mM biphenyl and 0.5% sodium succinate. The culture was passed through sterile glass wool to remove biphenyl particles. The cells were centrifuged at 5,000 × *g* (Sorval centrifuge RC2B) and washed with 10 mM Tris hydrochloride, pH 8. The washed cells were suspended in 10 ml of 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, 4 ml of 20% sodium dodecyl sulfate). The suspension was heated at 55°C for 10 min and extracted with phenol-chloroform (1:1), and the DNA was precipitated with 1 volume of isopropanol. DNA was recovered with a sterile glass rod. The DNA was dissolved in 10 mM Tris hydrochloride (pH 8)–1 mM EDTA (TE buffer) and was further purified on a cesium chloride-ethidium bromide gradient.

Construction and screening of *P. putida* OU83 gene library. The partially *Hin*dIII-digested chromosomal DNA (9) was mixed with *Hin*dIII-digested cosmid vector pCP13 DNA in a final concentration of 300 μg/ml (vector DNA, 3 μg; chromosomal DNA, 1 μg), and the ligations were carried out with T4 DNA ligase (International Biotechnologies Inc.). The ligation reaction was monitored on a 0.5% agarose gel (19). The ligated DNA was packaged in vitro by using packaging extracts (Amersham Corp., Arlington Heights, Ill.) according to the manufacturer's instructions. The recombinant cosmid pCP13 molecules packed in vitro in lambda heads were used to transfect *E. coli* AC80. Strain AC80 was grown in 10 ml of L broth with 0.2% maltose and 10 mM $MgCl_2$ to an optical density of 2.5 at 550 nm. A 200-μl amount of this culture and 10 μl of in vitro packaged recombinant cosmids were mixed and incubated for 30 min at room temperature; 2 ml of L broth was added, and the mixture was incubated at 37°C for 60 min. The mixture was then plated on L-agar plates with tetracycline (30 μg/ml). The hybrid clones were tested for tetracycline resistance and kanamycin sensitivity by the replica-plating method. The gene library was stored in L broth containing 40% glycerol and tetracycline (30 μg/ml).

The genomic library of *P. putida* OU83 was screened by mobilization of recombinant cosmid DNA into *P. putida* AC812 by a triparental cross (27). Triparental matings of *E.*

TABLE 1. Bacterial strains and plasmids

Strain	Bacterial species	Plasmid	Phenotype or genotype[a]	Reference
OU83	*P. putida*	ND[b]	4-CBP$^+$ BP$^+$ 2,3-DHBD$^+$	This paper
AC812	*P. putida*		*trpB615 recA801*	8
AC812	*P. putida*	pOH81	*trpB615 recA801* 2,3-DHBD$^+$ Tc^r	This paper
AC812	*P. putida*	pOH82	*trpB615 recA801* 2,3-DHBD$^+$ Tc^r	This paper
AC812	*P. putida*	pOH83	*trpB615 recA801* 2,3-DHBD$^+$ Tc^r	This paper
AC812	*P. putida*	pOH85	*trpB615 recA801* 2,3-DHBD$^+$ Tc^r	This paper
AC812	*P. putida*	pOH86	*trpB615 recA801* 2,3-DHBD$^+$ Tc^r	This paper
AC812	*P. putida*	pOH87	*trpB615 recA801* 2,3-DHBD$^+$ Tc^r	This paper
AC812	*P. putida*	pOH88	*trpB615 recA801* 2,3-DHBD$^+$ Tc^r	This paper
AC812	*P. putida*	pOH89	*trpB615 recA801* 2,3-DHBD$^+$ Tc^r	This paper
AC812	*P. putida*	pOH810	*trpB615 recA801* 2,3-DHBD$^+$ Tc^r	This paper
MMB 2442	*P. putida*	pOH21	*trp hsdR* 2,3-DHBD$^+$ Tc^r Sm^r Rif^r	This paper
MMB 2442	*P. putida*	pOH22	*trp hsdR* 2,3-DHBD$^+$ Tc^r Sm^r Rif^r	This paper
MMB 2442	*P. putida*		*trp hsdR* Sm^r Rif^r	M. Bagdasarian
HB101	*E. coli*		*pro leu recA hsdR hsdM*	26
HB101	*E. coli*	pOH810	*pro leu recA hsdR hsdM* 2,3-DHBD$^+$ Tc^r	This paper
AC80	*E. coli*	pOH101	*thr leu met hsdR hsdM* 2,3-DHBD$^+$ Tc^r	This paper
AC80	*E. coli*		*thr leu met hsdR hsdM*	8
HB101	*E. coli*	pRK2013	pro *leu recA hsdR hsdM* Km^r	12
AC80	*E. coli*	pCP13	*thr leu met hsdR hsdM*	9

[a] Designations used for relevant genotype and phenotypes are as follows: BP, biphenyl; 4-CBP, 4-chlorobiphenyl; 2,3-DHBD, 2,3-dihydroxybiphenyl dioxygenase; 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.

[b] ND, Not detected.

coli AC80 (containing cosmid genomic library), *E. coli* HB101 (pRK2013, mobilizing plasmid [12]), and *P. putida* AC812 (recipient) were done on nitrocellulose membrane filters (26). The transconjugants of strain AC812 were selected on *Pseudomonas* isolation agar containing tetracycline (100 μg/ml). Screening for the positive clones capable of degrading chlorobiphenyl was identified by spraying a 0.1% solution of 2,3-DHBP. The positive clones turned yellow within 1 to 2 min as a result of the conversion of 2,3-DHBP into 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HOPDA; yellow-colored *meta*-cleavage compound).

Enzyme assay. *P. putida* and *E. coli* strains containing hybrid cosmid DNA were grown in L broth with and without tetracycline. The cells were pelleted and washed with 50 mM Tris hydrochloride (pH 7.5)–10% acetone. The cells were disrupted by sonication (Branson Sonic Power Corp., Danbury, Conn.), and lysate was centrifuged at 28,000 × *g* for 30 min at 4°C. The supernatant was used as a cell extract for enzyme assays. 2,3-DHBD was assayed by measuring the formation of HOPDA at 434 nm after the addition of 2,3-DHBP. The molar extinction coefficient at 434 nm for HOPDA was 22,000 (15). Catechol 2,3-dioxygenase activity was measured by determining the rate of formation of 2-hydroxymuconic acid at 375 nm (22). The oxidation of 4-methylcatechol and 4-chlorocatechol was determined by following the increase of A_{382} and A_{379}. Quantitative conversion of the catechols into ring fission products was obtained from the molar absorption coefficients (4, 22). Protein content was determined by a dye binding assay (25) according to the instructions of the supplier (Pierce Chemical Co., Rockford, Ill.).

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis of the crude enzyme extract was carried out in nondenaturing conditions on a 10% polyacrylamide slab gel as described before (28). The protein band containing the enzyme activity was localized in the gel by equilibrating the gel in 50 mM Tris hydrochloride–10% acetone buffer, pH 7.5 (Tris-acetone buffer), for 15 min and then overlaying a Whatman no. 1 filter paper soaked with a 0.1% solution of 2,3-DHBP in Tris-acetone buffer. The band containing 2,3-dioxygenase turned yellow, indicating the presence of functional enzyme. The molecular weight of the native enzyme was determined with the help of standard protein markers: thyroglobulin (bovine), 670,000; gamma globulin (bovine), 158,000; ovalbumin (chicken), 44,000; myoglobulin (horse), 17,000; and vitamin B_{12}, 1,350.

Isoelectric focusing. Electrofocusing was carried out in agarose by using Isogel (pH 3 to 10; FMC Corp., Philadelphia, Pa.). Electrofocusing was performed at 750 V for 1 h at 5°C (Hoeffer). pI markers (FMC Corp.) were used as marker protein. The enzyme activity was localized as described above. The pI of the protein band containing enzyme activity was determined by comparing its mobility with that of standard pI markers. The electrophoresed gels were stained with Coomassie brilliant blue R-200 according to the supplier's (FMC Corp.) instructions to locate the protein band.

Agarose gel electrophoresis of DNA and hybridization analysis. Chromosomal DNA and cosmid clones containing the gene for 2,3-dioxygenase were digested with *Hin*dIII, *Bam*HI, *Sal*I, and *Eco*RI-*Hin*dIII restriction enzymes and were separated on 1% agarose. The DNA fragments were then transferred onto charged nylon membrane filter Gene Screen-plus (Dupont Co., NEN Research Products, Boston, Mass.) by the method of Southern (24); after denaturation and neutralization of the gel, the filter was prehybridized for 2 h at 65°C in 20 ml of prehybridization solution containing 1 M NaCl, 10% dextran sulfate, and 1% sodium dodecyl 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 of DNA per μg by a random primer extension labeling system (10), was added to the bag containing prehybridized membrane. Hybridization was carried out overnight at 65°C. The filter was washed twice with 2× SSC (0.3 M sodium chloride plus 0.03 M sodium citrate) at room temperature for 5 min with constant agitation, twice with 200 ml of solution containing 2× SSC and 1% sodium dodecyl sulfate at 65°C for 30 min with constant agitation, and twice with 0.1× SSC–1% sodium dodecyl sulfate 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.

RESULTS

Construction of a genomic library of *P. putida* OU83 in cosmid pCP13. A broad-host-range cosmid vector, pCP13, was used to clone DNA fragments containing genes involved in the catabolism of chlorobiphenyl. This cosmid vector (size, 23 kb) is derived from cosmid pLAFRI (12), has unique restriction sites (*Hin*dIII, *Eco*RI, etc.), is non-self-transmissible but mobilizable, and contains antibiotic resistance determinants for kanamycin and tetracycline. *E. coli* AC80 was transformed to *tet*^{r} by transfecting with hybrid cosmid pCP13 DNA, which was first packaged with the ligated DNA into lambda phage head. Transfectants were further tested for the loss of kanamycin resistance. In all cases, 90% of transfectants were *tet*^{r} and *kan*^{s}. The efficiency of transfection was 2 × 10^4 clones per μg of ligated DNA. A random sample of 20 *tet*^{r} *kan*^{s} clones were tested to determine the average size of the cloned DNA fragments in cosmid vector pCP13. The recombinant clones were found to contain DNA inserts ranging from 6 to 30 kb in size. This indicates that our genomic library of *P. putida* OU83 would be representing the entire chromosomal DNA in about 500 to 750 randomly selected clones and the DNA sequences encoding chlorobiphenyl degradation should be present in our cosmid library. The cosmid library, containing about 5,000 *tet*^{r} clones, was suspended in L broth containing 30 μg of tetracycline per ml and 40% glycerol and then frozen at −70°C.

Screening of hybrid cosmid clone containing genes encoding 2,3-dioxygenase activity. Previous studies have shown that genes from *Pseudomonas* species are poorly expressed in *E. coli* (11). To overcome this problem, the hybrid cosmid gene bank was mobilized in *P. putida* AC812 with the help of plasmid pRK2013 (ColE1 replicon, *tra*$^+$ function, *kan*^{r} [12]). The results of the mobilization of the cosmid gene bank from *E. coli* to *P. putida* with plasmid pRK2013 are given in Table 2. These data showed a high frequency of transfer of cosmid DNA. Some 2,000 transconjugants of *P. putida* were screened for their ability to express 2,3-dioxygenase by spraying a 0.1% solution of 2,3-DHBP. The positive clones turned yellow as a result of the formation of a *meta*-cleavage product (HOPDA) from 2,3-DHBP. Several *tet*^{r} *P. putida* clones exhibiting their ability to convert 2,3-DHBP into a yellow-colored *meta*-cleavage compound were identified. *P. putida* AC812 without recombinant cosmid DNA lacked this activity. The hybrid cosmid DNA from 2,3-DHBD$^+$ clones were isolated and transferred into *E. coli* HB101 and AC80 by transformation. The tetracycline-resistant transformants of *E. coli* were further tested for 2,3-DHBD$^+$ activity. Surprisingly, the genes for *P. putida* specifying 2,3-DHBD$^+$ activity were expressed in both *E. coli* HB101 and AC80.

TABLE 2. Frequency of transfer of hybrid cosmid DNA by triparental mating[a]

Mobilizing plasmid	Recipient *P. putida* strain	Transfer of hybrid cosmid DNA[b]	
		Total no. of clones	Clones with 2,3-DHBD$^+$ activity
pRK2013	AC812	7.8×10^4	10
pRK2013	MMB2442	5.3×10^3	2

[a] Donor *E. coli* was from an OU83 cosmid gene bank in strain AC80.
[b] Number of hybrid cosmid clones per milliliter of mating mixture selected on *Pseudomonas* isolation agar.

Growth characteristics and 2,3-DHBD activity. The time course of growth and 2,3-DHBD$^+$ activity in parent *P. putida* OU83, *P. putida* AC812, and *E. coli* HB101 with and without hybrid cosmid DNA are shown in Fig. 2. The organisms were grown in L broth and tested for synthesis of 2,3-DHBD$^+$ activity. *P. putida* OU83 had slow growth compared with *P. putida* AC812 and *E. coli* strain HB101, but the level of enzyme activity (2,3-DHBD$^+$) in strain OU83 was higher than in strains AC812 and HB101 containing recombinant plasmid pOH810 (Fig. 2). The expression of genes encoding enzyme (2,3-DHBD$^+$) activity in strains HB101(pOH810) and AC812(pOH810) was about the same, as seen by the level of enzyme produced when strains were grown in L broth containing tetracycline (Fig. 2B and C).

Restriction enzyme analysis and DNA-DNA homology studies. The hybrid cosmid DNA was isolated, purified, and digested with *Hin*dIII and then electrophoresed on agarose gel. Table 3 shows the number and molecular size of *Hin*dIII fragments. The sizes of the DNA inserts among 2,3-dioxygenases-positive clones varied from 0.4 to 22 kb. Figure 3A shows the DNA fragment patterns of recombinant cosmid clones containing 2,3-dioxygenase activity in comparison with chromosomal DNA of strain OU83. DNA homology between the cloned DNA inserts from strain OU83 and other 2,3-DHBD$^+$ clones was estimated by Southern blotting (24). The restricted DNA was electrophoresed, transferred to a Gene Screen-plus membrane, and then probed with ^{32}P-labeled DNA of recombinant cosmids pOH101 and pOH810. Figure 3B shows the results of the specific hybridization with ^{32}P-labeled pOH101 as DNA probe. For example, plasmid pOH101 was found to hybridize to a linearized fragment of vector DNA (lanes 2 to 4 and 6 to 10), DNA inserts in pOH101 (lane 9), *Hin*dIII digest of chromosomal DNA (lane 1), and HD fragment of pOH810 (lane 10). The results of insert size and specific DNA hybridization are summarized in Table 3. The hybridization data suggest that genes encod-

TABLE 3. *Hin*dIII fragment pattern and hybridization of various fragments in hybrid cosmid clones specifying 2,3-dioxygenase activity

Chimeric clone and its size (kb)	*Hin*dIII fragments and their size (kb)[a]	Fragments that hybridized with ^{32}P-labeled probe	
		pOH101	pOH810
pOH101 (27.9)	HA (22.0), HB (2.6), HC (2.3), HD (0.8), HE (0.4)	HA, HB, HC, HD, HE	HA, HC
pOH810 (51.9)	HA (22.0), HB (18.5), HC (7.5), HD (2.3), HE (2.0)	HA, HD	HA, HB, HC, HD, HE
pOH81 (45.7)	HA (22.0), HB (22.0), HC (1.7)	HA, HB	HA, HB
pOH82 (45.7)	HA (22.0), HB (22.0), HC (1.7)	HA, HB	HA, HB
pOH83 (45.7)	HA (22.0), HB (22.0), HC (1.7)	HA, HB	HA, HB
pOH84 (45.7)	HA (22.0), HB (22.0), HC (1.7)	HA, HB	HA, HB
pOH85 (44.0)	HA (22.0), HB (22.0)	HA, HB	HA, HB

[a] Fragments of recombinant clones were named alphabetically, using the first letter of enzyme followed by alphabetical size notation, with A being the largest.

ing 2,3-dioxygenase activity may be present on a common 2.3-kb *Hin*dIII fragment in pOH810, pOH101, and strain OU83. The common 2.3-kb *Hin*dIII fragment of DNA was lacking in several 2,3-DHBD$^+$ clones, pOH81, pOH82, pOH83, pOH84, pOH85, pOH86, pOH87, pOH88, and pOH89, but ^{32}P-labeled pOH101 hybridized to *Hin*dIII HA and HB fragments and two common *Hin*dIII-*Eco*RI fragments of 1.3 and 0.6 kb (Fig. 3B). A preliminary restriction map of pOH101 indicating the regions of DNA homology with pOH810 is shown in Fig. 4.

Level of 2,3-DHBD. The level of 2,3-DHBD was measured in crude cell extract from strains OU83, AC812, AC80, and HB101 with and without recombinant cosmid DNA. The level of enzyme activity varied in different clones even in the same host background (Table 4). The enzyme activity was highest in strain AC80 containing cosmid pOH101.

In situ localization and characterization of 2,3-DHBD.

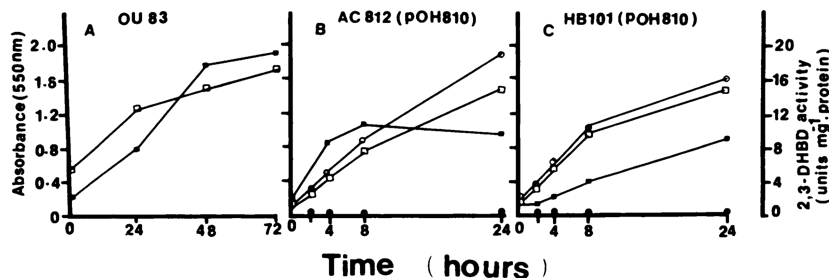


FIG. 2. Time course of growth and 2,3-DHBD enzyme activity. (A) *P. putida* OU83 growth (□), 2,3-DHBD (■); (B) *P. putida* AC812 growth (○), 2,3-DHBD (●), (*P. putida* 812(pOH810) growth (□), 2,3-DHBD (■); (C) *E. coli* HB101 growth (○), 2,3-DHBD (●), *E. coli* HB101(pOH810) growth (□), 2,3-DHBD (■). One unit of enzyme is equal to the formation of 1 μmol of the *meta*-cleavage compound (HOPDA) of 2,3-DHBP at 25°C.

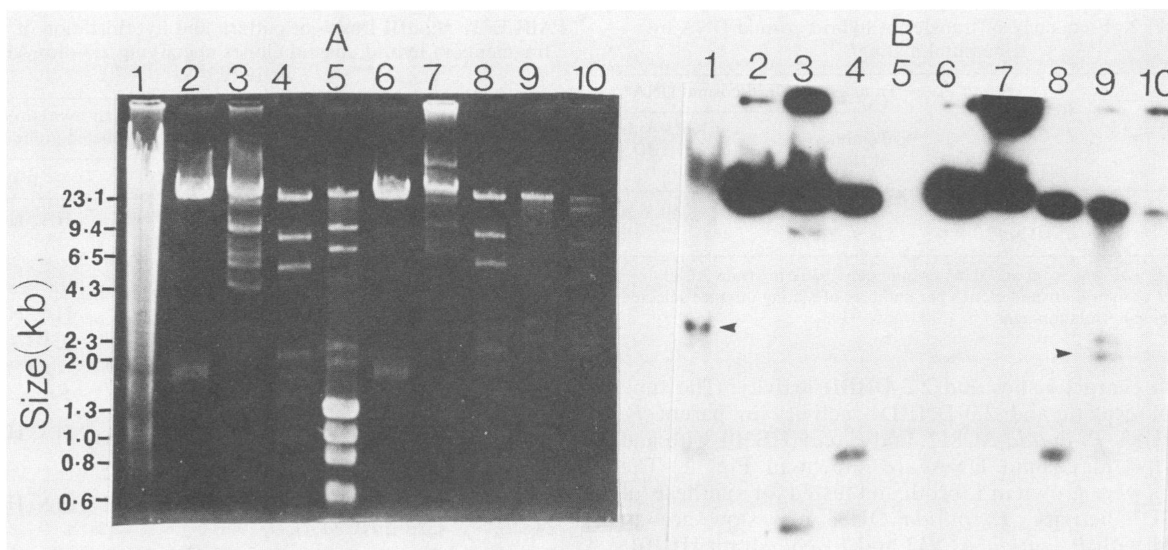


FIG. 3. Agarose gel electrophoresis of recombinant cosmids encoding genes specifying 2,3-dioxygenase activity. (A) Lane 1, Chromosomal DNA of strain OU83 digested with *Hin*dIII; lane 2, cosmid pOH83 digested with *Hin*dIII; lane 3, cosmid pOH83 digested with *Eco*RI; lane 4, cosmid pOH83 digested with *Hin*dIII-*Eco*RI; lane 5, Molecular weight marker DNA, lambda phage DNA digested with *Hin*dIII, and φX174 DNA digested with *Hae*III; lane 6, Cosmid pOH84 digested with *Hin*dIII; lane 7, cosmid pOH84 digested with *Eco*RI; lane 8, pOH84 digested with *Hin*dIII-*Eco*RI; lane 9, pOH101 digested with *Hin*dIII; lane 10, pOH810 digested with *Hin*dIII. (B) Autoradiogram of corresponding Southern blot of panel A after hybridization with ^{32}P-labeled cosmid pOH101 as probe. Arrowheads indicate positions of the common DNA fragment (2.3 kb) hybridized to ^{32}P-labeled probe (pOH101) with genomic DNA of *P. putida* OU83.

2,3-DHBD$^+$ activity was localized in situ on polyacrylamide and agarose gels. The electrophoresed gel was equilibrated with 4× Tris-acetone buffer (50 mM Tris–10% acetone buffer, pH 7.5) for 15 min. Whatman no. 1 filter paper soaked with a 0.1% solution of 2,3-DHBP in 1× Tris-acetone buffer, pH 7.5, was overlaid on the gel. Within 5 min of the application of soaked filter paper, a specific yellow band appeared on the gel exhibiting 2,3-DHBD$^+$ activity. Figure 5A (lanes 1 and 5, arrowhead) shows the development of a specific single band containing 2,3-DHBD$^+$ activity. The position of these bands in a corresponding Coomassie blue-stained gel is shown in Fig. 5B (lanes 1 and 5, arrowheads). There was no difference found in the mobility of the protein band containing 2,3-DHBD$^+$ activity from the crude extracts of strain OU83 and strain AC812, AC80, or HB101 containing recombinant cosmid DNA (pOH101 and pOH810). The molecular weight of this enzyme in nondenaturing conditions appeared to be over 200,000 (Fig. 5). The pI of the enzyme (2,3-DHBD$^+$) in the crude extracts of strain OU83, AC812(pOH101), AC80(pOH101), and HB101(pOH810) appeared to be 5.0 (Fig. 6B). The arrowhead in the Coomassie blue-stained gel (Fig. 6A) shows the location of the corresponding protein band exhibiting enzyme activity in the same gel before staining.

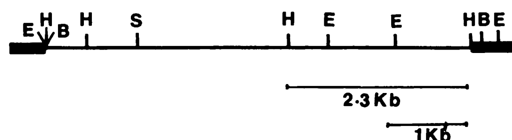


FIG. 4. Preliminary restriction map of the pOH101 containing 6.0-kb *Hin*dIII fragments of *P. putida* OU83. The heavy line represents the cosmid vector DNA, and the thin line represents the cloned *Hin*dIII DNA insert. The length of fragment HC of pOH101 has DNA sequence homology with pOH810 and is marked 2.3 kb. E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI; S, *Sal*I.

Substrate profile and K_m of 3-PC dioxygenase. The K_m (14.0 μM) of 3-PC dioxygenase was calculated from the Lineweaver-Burk plot (Fig. 7A). The kinetic parameters of the crude enzyme extract are shown in Fig. 7B. The results of the substrate profile are summarized in Table 5. The rate of oxidation of different substrates related to the maximal rate of oxidation of 3-PC was in the following order: 3-PC, 100%; catechol, 39%; 4-chlorocatechol, 26%; and 4-methylcatechol, 17%.

DISCUSSION

The expression of genes specifying 2,3-DHBD from *P. putida* to *E. coli* has been shown for the first time in this study. Previously, Furukawa and Miyazaki (15) demonstrated expression of cloned gene encoding 2,3-dioxygenase in *P. aeruginosa*. Numerous reports have indicated poor expression of *Pseudomonas* genes specifying catabolic enzymes involved in the metabolism of chlorobenzoate (16, 30), toluene (11, 17), and naphthalene (7) in *E. coli*. The level

TABLE 4. Expression of 2,3-DHBD in *P. putida* and *E. coli*

Bacterial strain (plasmid)	2,3-DHBD activity (U/mg of protein)[a]
P. putida OU83	120
P. putida AC812	ND
P. putida AC812(pOH810)	81
P. putida AC812(pOH83)	17
P. putida AC812(pOH84)	31
P. putida AC812(pOH85)	76
E. coli HB101	ND
E. coli HB101(pOH810)	92
E. coli AC80	ND
E. coli AC80(pOH101)	260

[a] One unit of enzyme activity is equal to the formation of 1 μmol of *meta*-cleavage product of 2,3-DHBP per min at 25°C. ND, Not detected.

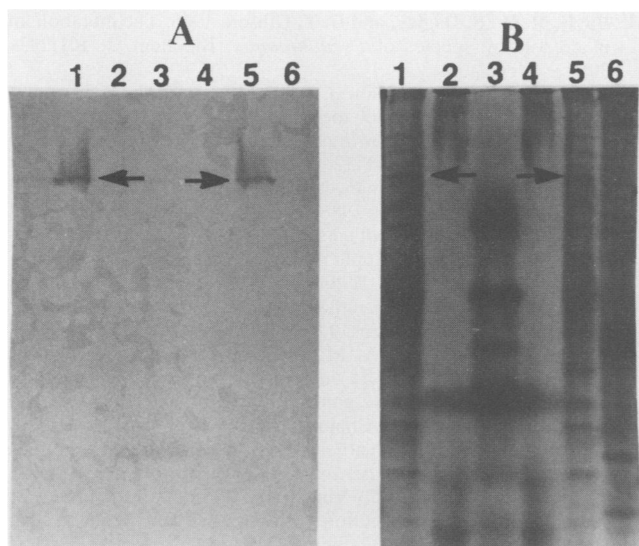


FIG. 5. Polyacrylamide gel electrophoresis of 2,3-DHBD. (A) Electrophoresed gel treated with a 0.1% solution of 2,3-DHBP in 50 mM Tris hydrochloride–10% acetone, pH 7.5. Lane 1, Crude extract of *P. putida* OU83; lane 2, proteins without enzyme activity; lane 3, molecular weight marker proteins; lane 4, proteins without enzyme activity; lane 5, crude extract of *P. putida* AC812 containing cosmid pOH810; lane 6, *P. putida* AC812 without cosmid pOH810, lacking 2,3-DHBD activity. (B) Coomassie blue-stained gel corresponding to panel A. The arrows in lanes 1 and 5 indicate the positions of protein bands containing 2,3-DHBD activity.

of enzyme activity varied in different hybrid clones. The hybrid clone (pOH101) containing the smallest insert, 6.0-kb, was found to have the highest level of enzyme activity in *E. coli*. This variation in enzyme activity may be because of the size of the cloned DNA insert.

The evidence for hybrid cosmid DNA specifying 2,3-dioxygenase activity was obtained through the transfer of recombinant clones by conjugation and transformation into *E. coli* and *P. putida*. The specific hybridization of ^{32}P-labeled hybrid clones carrying the 2,3-dioxygenase gene to chromosomal DNA of strain OU83 indicates that the cloned genes were originated from strain OU83 DNA. A common *Hin*dIII fragment (size, 2.3 kb) in recombinant cosmids pOH101 and pOH810 and in chromosomal DNA of strain OU83 was found to have DNA homology with ^{32}P-labeled probe (pOH101). These hybridization studies provide indirect evidence that the genes for 2,3-dioxygenase may be present on fragment HC of pOH101 and fragment HD of pOH810. Further evidence for the exact location of the 2,3-dioxygenase gene in pOH101 and pOH810 will be obtained by subcloning of the cloned DNA inserts. Restriction enzyme analysis of pOH81, pOH82, pOH83, pOH84, pOH86, and pOH87 showed two fragments (1.7 and 22 kb). None of these clones showed the 2.3-kb DNA fragment that was found to be common to pOH101 and pOH810.

DNA sequence homology with one *Hin*dIII and two *Eco*RI-*Hin*dIII fragments suggests the following possibilities. There may be multiple copies of the 2,3-dioxygenase genes scattered on the parent chromosomal DNA, or there may have been rearrangement of DNA sequences with the loss of the *Hin*dIII site by deletion, inversion, or insertion of DNA sequences during DNA packaging or propagation of the recombinant DNA. The multiple locations of 2,3-dioxygenase genes have been previously demonstrated in plasmids degrading toluene (7, 17). Inversions, deletions, and

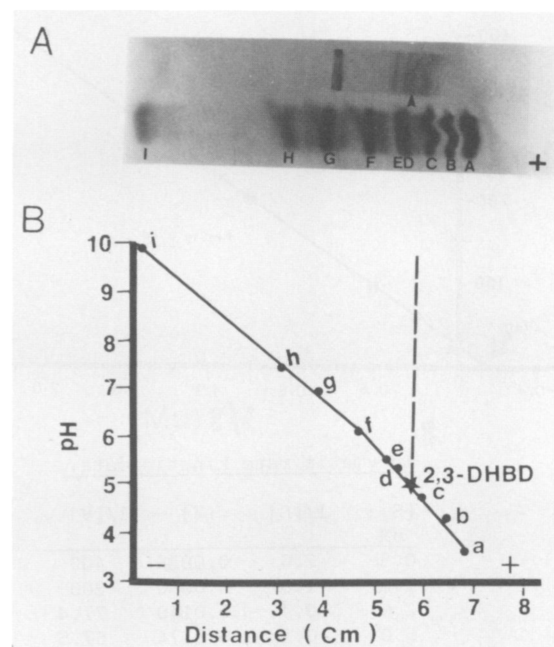


FIG. 6. Isoelectric focusing of 2,3-DHBD from crude extract. (A) Electrofocused and Coomassie blue-stained isogel (FMC Corp.). Lane 1, Crude enzyme extract of *P. putida* OU83; arrow indicates position of 2,3-DHBD. Lane 2, pI standard markers: A, amyloglucosidase; B, glucose oxidase; C, ovalbumin; D, β-lactoglobulin B; E, β-lactoglobulin A; F, carbonic anhydrase; G, myoglobin (minor band); H, myoglobin (major band); I, cytochrome *c*. (B) Graph showing relationship between pH of marker proteins and distance of migration on isogel.

rearrangement of DNA fragments have also been shown in 3-chlorobenzoate-degrading bacteria (8, 16).

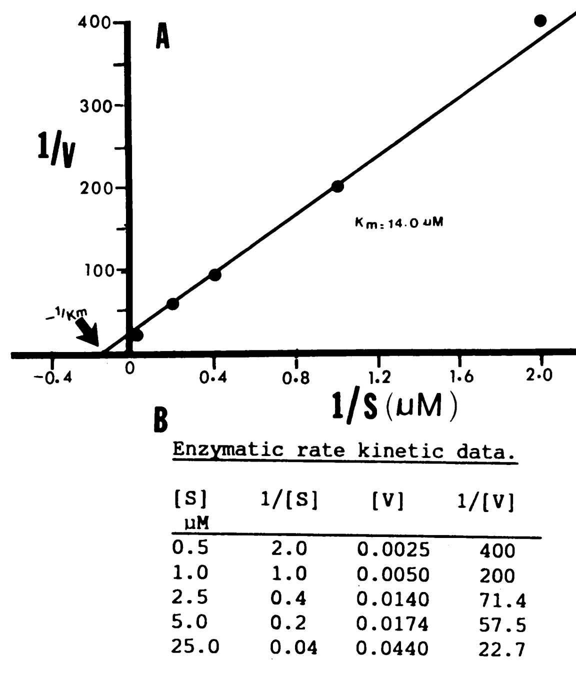
That the enzyme was not pure prevents a definitive description of the properties of the 2,3-dioxygenase; however, it was necessary to determine whether the gene product reflects the same characteristics as that observed in original *P. putida* OU83. The electrophoretic mobility of the enzyme in nondenaturing conditions, pI, K_m values, and the substrate specificity of cloned gene products from pOH810 and parent *P. putida* OU83 were similar. The data show that the cloned enzyme has common properties with parent strain OU83. The physical properties of the protein did not appear to be altered when the gene was expressed in *E. coli*.

Furukawa and Miyazaki (15) have cloned and characterized DNA fragments specifying 2,3-dioxygenase involved in the metabolism of biphenyls from *P. pseudoalcaligenes* and further established a narrow substrate activity only for 2,3-DHBP. In contrast, we have found that our cloned

TABLE 5. Substrate profile of 3-PC dioxygenase from crude extract of *E. coli* HB101(pOH101)

Substrate	3-PC dioxygenase activity (U/mg of protein)[a]	Relative activity (%)
Catechol	72	39
3-PC	185	100
4-Methylcatechol	31	17
4-Chlorocatechol	49	26

[a] One unit of enzyme is equal to the formation of 1 μmol of *meta*-cleavage product of substrate per min at 25°C. 3-PC dioxygenase activity was not detected in *E. coli* HB101.



Enzymatic rate kinetic data.

[S] uM	1/[S]	[V]	1/[V]
0.5	2.0	0.0025	400
1.0	1.0	0.0050	200
2.5	0.4	0.0140	71.4
5.0	0.2	0.0174	57.5
25.0	0.04	0.0440	22.7

FIG. 7. Determination of K_m for 3-PC from crude cell extract of *E. coli* HB101(pOH101). (A) Lineweaver-Burk plot: graph of substrate (1/S) versus initial velocity (1/V) of reaction. (B) Enzymatic rate kinetic data.

2,3-dioxygenase gene produces an enzyme with broad specificity including activities for catechol, 4-chlorocatechol, 4-methylcatechol, and 2,3-DHBP. Further comparisons of the structural organization, sequence homology, and nucleotide sequences of dioxygenase genes from different bacteria capable of degrading chlorinated biphenyls will be useful in elucidating mechanisms of regulation of the metabolic pathways of aromatic compounds. Furthermore, this may also indicate whether they share a common ancestor and, if so, may provide evidence about the mechanism underlying their evolutionary divergence.

ACKNOWLEDGMENTS

We thank A. M. Chakrabarty, M. Sylvestre, M. Bagdasarian, and K. T. Shanmugam for providing bacterial strains and plasmids; T. Rao, L. Bhatnagar, T. Grudzien, and P. Ketchum for useful suggestions; and G. Brieger for analysis of mass and nuclear magnetic resonance spectra of 3-PC.

This work was supported in part by funds from Public Health Service grant 507RR713 from BRSG, National Institutes of Health; Providence Hospital Research Foundation, Southfield, Mich.; and Sunny Microbiology International, Rochester, Mich.; and by Environmental Protection Agency grant R-812827 to S.W.

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