Cloning and Sequencing of Two Tandem Genes Involved in Degradation of 2,3-Dihydroxybiphenyl to Benzoic Acid in the Polychlorinated Biphenyl-Degrading Soil Bacterium *Pseudomonas* sp. Strain KKS102

KAZUHIDE KIMBARA,¹ TOSHIYUKI HASHIMOTO,¹ MASAO FUKUDA,¹ TAKAO KOANA,² MASAMICHI TAKAGI,¹* MICHIO OISHI,³ and KEIJI YANO¹

Department of Agricultural Chemistry¹ and Institute of Applied Microbiology,³ The University of Tokyo, Bunkyo-ku, Tokyo 113, and Biotechnology Department, Industrial Research Institute Japan, 1201 Takada, Kashiwa, Chiba 227,² Japan

Received 15 August 1988/Accepted 22 February 1989

Two genes involved in the degradation of biphenyl were isolated from a gene library of a polychlorinated biphenyl-degrading soil bacterium, *Pseudomonas* sp. strain KKS102, by using a broad-host-range cosmid vector, pKS13. When a 3.2-kilobase (kb) *PstI* fragment of a 29-kb cosmid DNA insert was subcloned into pUC18 at the *PstI* site downstream of the *lacZ* promoter, *Escherichia coli* cells carrying this recombinant plasmid expressed 2,3-dihydroxybiphenyl dioxygenase activity. Nucleotide sequencing of the 3.2-kb *PstI* fragment revealed that there were two open reading frames (ORFI [882 base pairs] and ORFII [834 base pairs], in this gene order). Results of analysis of Tn5 insertion mutants and unidirectional deletion mutants suggested that the ORFI coded for 2,3-dihydroxybiphenyl dioxygenase. When the sequence of ORFI was compared with that of *bphC* of *Pseudomonas pseudoalcaligenes* KF707 (K. Furukawa, N. Arima, and T. Miyazaki, J. Bacteriol. 169:427–429, 1987), the homology was 68%, with both strains having the same Shine-Dalgarno sequence. The result of gas chromatography-mass spectrometry analysis of the metabolic product suggested that the ORFII had *meta* cleavage compound hydrolase activity to produce benzoic acid. DNA sequencing suggested that these two genes were contained in one operon.

Polychlorinated biphenyls (PCBs) are environmental pollutants which are distributed widely in the world. Because of their chemical stability, incombustibility, high insulation property, high fat solubility, and low volatility, PCBs have been used widely as insulators, conductors of heat, lubricating oils, solvents for paints, etc. But production and use of PCBs has been prohibited since their toxicity and contamination to environment were discovered. The first report of biodegradation of PCBs, by Ahmed and Focht (1), appeared in 1973. Since then, several studies have reported the biodegradation and catabolic pathways of PCBs (3-5, 13, 15, 16). As it is generally accepted that the major catabolic pathway is common for PCB and biphenyl, many experiments have been performed with biphenyl as a substrate. The major catabolic pathway of PCB is as follows. (i) Molecular oxygen is introduced at the 2 and 3 positions of one of the two rings by biphenyl dioxygenase (a gene product of bphA in the case of Pseudomonas pseudoalcaligenes [17]). (ii) A resulting dihydrodiol derivative is then dehydrogenated by dihydrodiol dehydrogenase (a gene product of bphB [17]). (iii) A 2,3-dihydroxybiphenyl derivative thus formed is cleaved at the 1 and 2 positions by 2,3dihydroxybiphenyl dioxygenase (23DBDO) (a gene product of bphC [17]), and (iv) a resulting meta cleavage compound, a chlorinated derivative of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate (HOPD), is hydrolyzed by hydrolase (a gene product of a putative gene bphD [17], which has not been isolated yet).

Furukawa and Miyazaki (17) previously reported the

A PCB-degrading bacterium, *Pseudomonas* sp. strain KKS102, was recently isolated in our laboratory from soil as one of the components of a mixed culture which was composed of two *Pseudomonas* strains. This mixed culture exhibited a capability to degrade a mixture of highly chlorinated PCBs (PCB48, a mixture of mainly tetrachlorobiphenyls) which is rather resistant to degradation by bacteria so far isolated. It was shown that the two strains in the mixed culture, *Pseudomonas fluorescens* KKL101 and *Pseudomonas* strain KKS102, had a symbiotic relationship with each other and that KKS102 played a major role in degradation of PCBs (23).

In this report, we describe cloning, sequencing, and expression of two tandem genes from KKS102 corresponding to *bphC* and *bphD*, which were proved to be involved in biphenyl catabolism. These genes may also be involved in PCB catabolism.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Pseudomonas* strain KKS102 was isolated from soil near an oil refinery in Tokyo by repeated enrichment cultures with biphenyl and PCBs as the carbon sources (23). Host cells transconjugated with recombinant DNA were grown in a mineral salts medium composed of KH₂PO₄ (1.7 g/liter), Na₂HPO₄ (9.8 g/liter), (NH₄)₂SO₄ (1.0 g/liter), MgSO₄ · 7H₂O (0.1 g/liter), FeSO₄ · 7H₂O (0.95 mg/liter), MgO (10.75 mg/liter), CaCO₃ (2.0 mg/liter), ZnSO₄ · 7H₂O (1.44 mg/liter), CuSO₄ · 5H₂O (0.25 mg/liter), CoSO₄ · 7H₂O

cloning of an operon, bphABC, from P. pseudoalcaligenes, but bphD was not contained in this operon.

^{*} Corresponding author.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference	
Pseudomonas sp. KKS102		23	
P. fluorescens KKL101		23	
P. putida PpY101 E. coli	met nal	12	
HB101	F ⁺ pro leu thi lacY str hsdR hsdM endA recA	24	
JA221	leu trp thr thi lacI recA hsdR hsdM	7	
LE392(λ467)	F ⁻ met trp lacY galK galT supE supF hsdR (λ467")	9	
MV1184	ara Δ(lac-pro) str thi φ80 lacIZΔM15 Δ(srl-recA):: Tn10 (F' traD proAB lacI ^Q ZΔM15)	32	
Plasmids			
pRK2013	Km ^r Tra ⁺ ColE1 replicon	28	
pUC18	Apr lacZ' pMB9 replicon	32	
pMFY40	Apr Tcr Mob+ RSF1010 replicon	12	
pKTY320 ^b	Apr Cmr Mob+ p15A replicon	This labora- tory	
pKS13	Tcr cos Mob+ RK2 replicon	This study	
pKH1	bphCD in pKS13	This study	
pKH10	11-kb BamHI fragment in pUC18	This study	
pKH20	bphCD in pUC18	This study	
pKH30	bphCD in pMFY40	This study	
pKH101	bphCD in pUC18	This study	
pKH131	bphCD in pMFY40	This study	
pKH132	bphC in pMFY40	This study	

^a λ467 (λ::Tn5) phage has the following genotype: λ b221 rex::Tn5 cI857 Oam29 Pam80.

(0.28 mg/liter), H₃BO₃ (0.06 mg/liter), and concentrated HCl (51.3 μl/liter). Biphenyl or succinate was added at a concentration of 1 mg/ml. KKS102 was grown in DL broth, which was composed of bacto-tryptone (3.3 g/liter), yeast extract (1.7 g/liter), and NaCl (5 g/liter). The other *Pseudomonas* strains and *Escherichia coli* strains were grown in L broth. L broth was composed of Bacto-tryptone (10 g/liter), yeast extract (5 g/liter), and NaCl (5 g/liter). For solid media, agar was added at a concentration of 1.5%.

DNA cloning and related experiments. Total DNA from the strain KKS102 was isolated by using a modification of the procedure of Marmur (25). Plasmid DNA was isolated by a modification of the procedure described by Maniatis et al. (24).

For cloning of the genes involved in the degradation of biphenyl, a gene library of DNA of KKS102 was constructed in *E. coli*. Total DNA was partially digested with *Sau*3AI and ligated with a broad-host-range cosmid vector, pKS13. This vector derived from the cosmid vector pCP13 (8) is 21.7 kilobase pairs (kb) in size, confers tetracycline resistance, contains a *Bam*HI site for cloning a foreign gene, and is mobilizable but not self-transmissible (Mob⁺ Tra⁻). The ligated DNA was packaged in vitro into lambda phage particles, which were then infected into *E. coli* HB101. The genomic library was amplified by growing the cells in L broth supplemented with tetracycline. Amplified genomic libraries were preserved in 20% glycerol at -80°C. *E. coli* cells

containing the cosmid gene library thus prepared were mated with *Pseudomonas putida* PpY101 with the help of the plasmid pRK2013 (28). pRK2013 contains the RK2 *tra* functions and kanamycin resistance gene ligated to a ColE1 replicon and can mobilize the recombinant pKS13 derivatives into various gram-negative hosts. Triparental matings, in which *E. coli* HB101(pRK2013) was used as a source of the mobilizing plasmid pRK2013, were performed as described by Ruvkun and Ausubel (28).

After mating, transconjugants were selected on basal salts medium containing 1 g of succinate per liter and 10 µg of tetracycline per ml. Clones expressing 23DBDO activity were identified by spraying 2,3-dihydroxybiphenyl solution (1 g/liter) over the colonies. Positive clones turned yellow quickly by forming the *meta* cleavage compound, HOPD (17). Quantitative analysis of 23DBDO activity was carried out as described previously (18).

Southern blot analysis was performed by transferring DNA from agarose gel to a Hybond nylon membrane filter (Amersham Corp.). Hybridization with ³²P-labeled, nick-translated DNA was performed as described by Southern (31).

Tn5 mutagenesis was performed as described by de Bruijn and Lupski (9). The 3.2-kb subcloned fragment was inserted into pKTY320, and the λ::Tn5 phage stock prepared from E. coli LE392 (λ467) was used to infect E. coli JA221 carrying this plasmid. The transductants resistant to kanamycin (20 μg/ml) were selected. The plasmid DNA mixture was isolated from the transductants and used to transform E. coli JA221. Km^r transformants were selected as the Tn5 insertion mutants, and their 23DBDO activity was assayed. The sites of transposon insertions in the recombinant plasmids were mapped accurately with errors of less than 0.1 kb by digesting the plasmids with appropriate restriction enzymes.

DNA sequencing was carried out by the dideoxy-chain termination method of Sanger et al. (29) with plasmid pUC119 and *E. coli* MV1184 as described by Vieira and Messing (32).

Gas chromatography-mass spectrometry analysis. Authentic benzoic acid and the major metabolite of 2,3-dihydroxy-biphenyl were treated with N-methyl-N-trimethylsilyltrifluoroacetoamide to make trimethylsilyl derivatives. These derivatives were analyzed by gas chromatography-mass spectrometry (model JMS DX303, JEOL Ltd.). The column used in the analysis was an OV-1 capillary column (25 m by 0.25 mm inner diameter). Column operating conditions were as follows. The column temperature during gas chromatography was increased from 120 to 250°C at a rate of 16°C/min. The electron impact mass spectrometry was measured at a 70-eV ionization potential, 300-μA trap current, and 200°C ion source temperature.

RESULTS

Isolation from a gene library of a clone which showed 23DBDO activity. By spraying the 2,3-dihydroxybiphenyl solution, a single yellow colony was obtained after screening of about 3×10^3 colonies of transconjugants. A recombinant cosmid (designated pKH1) isolated from this yellow colony was transformed into $E.\ coli$ HB101. pKH1 was then isolated from $E.\ coli$ and reintroduced into $P.\ putida$ PpY101 to confirm that the cloned sequence contained in pKH1 was responsible for degradation of 2,3-dihydroxybiphenyl to HOPD. The result indicated that pKH1 had a gene coding for 23DBDO. This gene on pKH1 was not expressed when the cosmid was in $E.\ coli$ HB101. pKH1 was 50.7 kb in size and

^b pKTY320 (4.8 kb) was constructed from pACYC177 by replacing the Km^r-coding region with the *mob* region of RK2 and the Cm^r gene of pBR328.

2742 KIMBARA ET AL. J. BACTERIOL.

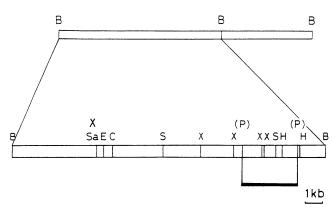


FIG. 1. Cloning of a 29-kb fragment from the genome of *Pseudomonas* strain KKS102 by using a cosmid vector (pKS13) and the restriction map of the 18-kb *Bam*HI fragment in it. The fragment consisted of the two *Bam*HI fragments (11 and 18 kb). The heavy line between the two Ps represents a 3.2-kb *Pst*I fragment subcloned in the later experiments from one (18 kb) of the two *Bam*HI fragments. Abbreviations: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sma*I; Sa, *Sac*I; X, *Xho*I.

contained a 29-kb DNA insert, which gave 11- and 18-kb fragments when digested with BamHI (Fig. 1).

Subcloning of the genes. A variety of derivative plasmids were constructed with the restriction fragments from the 29-kb DNA inserted in pKH1 by using plasmid vectors for E. coli and P. putida. None of the plasmids with 11- or 18-kb BamHI fragments conferred 23DBDO activity on E. coli. However, 23DBDO activity was observed when pKH101, constructed by inserting a 3.2-kb PstI fragment into a vector (pUC18) at the *PstI* site downstream of the *lacZ* promoter, was transformed into E. coli HB101. The restriction enzyme analysis indicated that this 3.2-kb fragment came from the middle of the 29-kb insert (Fig. 1 and 2), suggesting that a gene for 23DBDO could be expressed from its own promoter in *Pseudomonas* strains. pKH131, constructed by inserting the 3.2-kb fragment into a broad-host-range plasmid (pMFY40 [12]) at the PstI site, conferred high 23DBDO activity to P. putida but low activity to E. coli HB101. The results of these experiments are summarized in Fig. 2.

Southern blot analysis. Total DNA isolated from *Pseudomonas* strain KKS102, *Pseudomonas* strain KKL101, or *P. putida* PpY101 was digested with *Pst*I. DNA fragments thus obtained were run by agarose gel electrophoresis, transferred to a nylon membrane filter, and probed with a

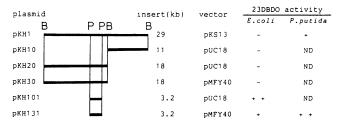


FIG. 2. Subcloning of a gene for 23DBDO. A variety of plasmids were constructed by using the restriction fragments obtained from the original 29-kb insert in pKH1. The names of the constructed plasmids and the structures and the sizes of the inserted DNA fragments are shown at the left. The 23DBDO activity in the cells of E. coli or P. putida carrying one of the constructed plasmids is shown at the right. ++, High activity; +, low activity; -, no activity; ND, not determined; B, BamHI; P, Pst1.

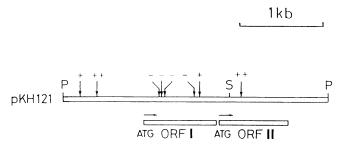


FIG. 3. Positions of Tn5 insertion in the subcloned 3.2-kb fragment and the effect of insertion on 23DBDO activity. Vertical arrows indicate the Tn5 insertion sites, and + and - represent the presence and the absence, respectively, of 23DBDO activity in each strain carrying a plasmid with Tn5 insertion at the specific site. The positions of ORFI and ORFII determined in the following sequencing analysis are indicated; directions of translation are shown by horizontal arrows. Abbreviations: P. PstI; S. SmaI.

nick-translated ³²P-labeled 3.2-kb *PstI* fragment. Southern blot experiments showed that the cloned fragment was derived from KKS102 and not present in the genomes of the other two strains (data not shown).

Tn5 mutagenesis. Tn5 mutagenesis was carried out to determine the region essential for 23DBDO activity in the 3.2-kb subcloned fragment. The results are summarized in Fig. 3. It was suggested that the gene coding for 23DBDO was located in the middle of the 3.2-kb fragment.

Nucleotide sequencing of the cloned DNA fragment. The 3.2-kb PstI fragment was inserted into pUC119, which contains the intergenic sequence of phage M13. A series of mutants which were deleted unidirectionally with exonuclease III and mung bean nuclease were constructed. By using the dideoxy-chain termination method, about 2,100 base pairs (bp) in the middle of the 3.2-kb fragment, including the 23DBDO-coding region suggested by the Tn5 insertion experiment described above, were sequenced; the result is shown in Fig. 4. Analysis of this sequence by a computerized analytical system revealed that there were two open reading frames, ORFI (882 bp) and ORFII (834 bp), which were linked tandemly and translatable to the same direction. The deduced amino acid sequences of these two open reading frames are also shown in Fig. 4.

From the Tn5 insertion analysis (Fig. 3), it was suggested that ORFI coded for 23DBDO. Furukawa et al. previously reported the nucleotide sequence of bphC, which codes for 23DBDO of P. pseudoalcaligenes KK707 (14). The nucleotide sequence of ORFI of KKS102 was compared with the sequence of bphC of KK707. The homology in the coding region was 68%, although the 3'-terminal region of about 30 bp found in KF707 was lacking in KKS102 (Fig. 5). Both genes had the same putative Shine-Dalgarno sequence. Figure 6 shows the comparison of the amino acid sequences deduced from the nucleotide sequences of these two corresponding genes. Although an amino acid is present at the 256th position in KF707 but not in KKS102, the homology was 66%. On the other hand, when the deduced amino acid sequence of ORFI was compared with that of the catechol 2,3-dioxygenase gene (27), the homology was less than 20%.

Function of ORFII. Furukawa determined the N-terminal 33-amino-acid sequence of purified *meta* cleavage compound hydrolase from *P. pseudoalcaligenes* KF707 (personal communication). Figure 7 shows the comparison of the amino acid sequence deduced from the DNA sequence of ORFII of KKS102 and the N-terminal amino acid sequence of KF707. The homology in this region was 67%. Thus, it was strongly

100. AM. AGT. 600. CGT. CGT. CGG. CGG. CGG. CGG. CGG. CGG	4. 2103 AAA.AAA.CGC.GAG.GAG.ACT.CCC.ATG. d amino acid sequences of ORFI and ORFII are shown. Possible Shine-Dalgarno (SD)	* 1023 1023 1028* GTG. GAT. TCC. TCC. TCG. ACG. GCG. CGA. CAC. AGG. CGC. ACG. GCC. ATG. TCG. GGT. CAC. AAG. TCG Yal-Asp-Ser-Ser-Trp-Thr-Yal-Ala-Arg-His-Ser-Arg-Thr-Ala-Met-Trp-Gly-His-Lys-Ser FIG. 4. Nucleotide sequence of the middle part of the 3.2-kb fragment. The deduced
100.000.000.000.000.000.000.000.000.000	. GAG. GCG. CGG. CCC. T	3CC.GAC.ACG.CCG.TCG.CCC.ATG.ATG.GAG.GTC.GAG.TTC.GGC.TGG.GGG.CCG N1a-Asp-Thr-Pro-Ser-Pro-Met-[le-Glu-Val-Glu-Phe-Gly-Trp-Gly-Pro
MI. GGT. GGC. CTG. CGT. GGC. GGC. GGC. GGC	ACG.GCT.GAG.CGC.CGT.T	962* GAC.GCC.GCC.GGG.CGC.ATC.ACC.TCC.CTG.CTC.GGG.CGC.CAC.ACC.AAC.GAC.CAG.ACG.CTC.TCC Asp-Ala-Ala-Gly-Arg-fle-Thr-Ser-Leu-Leu-Gly-Arg-His-Thr-Asn-Asp-Gln-Thr-Leu-Ser
MI. GGT. GAC. CTC. TGT. GGT. TGC. CGT. GGG. GGG. CAT. GGT. GGG. AGA. AGA. TGC. WEINER-Gly-Glin-Arg-ene. **AC. GTG. TTC. TTT. GGC. AGG. GGC. GGC. GAC. AGC. GGC. AGA. AGC. GGG. GGC. AGA. AGC. TGA. AGC. GGC. AGC. AGC. AGC. AGC. AGC. A	.ATT.GGG.CGC.AGT.GGG.Av -[le-Gly-Arg-Ser-Gly-S	902* CAC.CAC.TTC.ATG.CTG.CAA.GCC.AAC.ACC.ATC.GAC.GAT.GTG.GGC.TAC.GCC.TTC.GAT.CGG.CTG His-His-Phe-Net-Leu-Gin-Ala-Asn-Thr-lie-Asp-Asp-Vai-Gly-Tyr-Ala-Phe-Asp-Arg-Leu
MT. GGT. GAC. CTC. TGT. GGT. TGC. CGT. GGG. GGG. CAT. GGT. GGG. CAT. GGT. GGG. AAA. TAC. ### AF GGT. AAC. CTC. TGT. GGT. TGC. GGG. GGG. CAT. GGT. GGG. AAA. GGG. GGG. AAA. ### AF GGT. AAC. CAG. CGC. GGC. GGC. GGC. AAC. AGC. TTC. CGG. GGG. ACA. AGC. TGG. AGC. AGC. AGC. AGC. AGC. AGC. AGC. A	AAG.CTG.ATC.GC Lys-Leu-!le-Al	#42* CAC.TGC.AAT.GGC.CGT.CAC.CAC.ACG.ATC.GCC.CTG.GCT.GCT.TTC.CCG.ATC.CCG.AAG.CGC.ATC HIS-Cys-Asn-Gly-Arg-His-His-Thr-lie-Ala-Leu-Ala-Ala-Phe-Pro-lie-Pro-Lys-Arg-lie
MI. GGT. GAC. CTC. TGT. GGT. TGC. GGT. GGG. GGG. CAT. GGT. GGT. GGT. GGT. GGT. GGT. GGT. G	AAG.ACC Lys-Thr	782* • 723 CTG.TGG.GAC.ATC.ATT.GAC.ATC.CAG.ATG.GGG.GCG.GAA.ACG.AGC.GTG.CCC.GCG.CAC.TTT.CTG Leu-Ser-Asp-Ile-Ile-Asp-Ile-Gln-Met-Gly-Pro-Glu-Thr-Ser-Yal-Pro-Ala-His-Phe-Leu
1083 1083 1084 1085	. 1743 AAC.TTC.ATC.CTG.AGC.GCA.CAA.AAA.GTG.CCG.CTC.TCG.GCT.TCG.GAC.GTG.TCG.GCG.CGT.CTG ASN-Phe-Ile-Leu-Ser-Ala-Gln-Lys-Val-Pro-Leu-Ser-Ala-Trp-Asp-Val-Ser-Ala-Arg-Leu	722* • 663 CGC.CGC.GTG.CCC.GAC.ACG.GCG.AAG.GCG.ATG.GCT.TTC.TAC.ACC.GAG.GTG.CTT.GGC.TTT.GTG Arg-Cys-Val-Pro-Asp-Thr-Ala-Lys-Ala-Net-Ala-Phe-Tyr-Thr-Glu-Val-Leu-Gly-Phe-Val
**************************************	AA.GGA	662* * 603 CTG.CCT.AGC.GCC.GCC.GTG.TCG.GGC.TTC.GTC.ACG.GGC.GAC.CAG.GGC.ATC.GGG.CAC.TTC.GTG Leu-Pro-Ser-Ala-Pro-Yal-Ser-Gly-Phe-Yal-Thr-Gly-Asp-Gln-Gly-Ile-Gly-His-Phe-Yal
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### 1083 62* 674.00G.00G.00G.00G.CAG.00G.TGA.00G.TGA.00G.GAA.TAG. 62* 674.00G.00G.00G.CAG.00G.TGA.00G.00G.CTG.00G.00G.TGA.00G.00G.CTG.00G.00G.TGA.00G.00G.CTG.00G.00G.TGA.00G.00G.00G.CTG.00G.00G.TGA.00G.00G.00G.00G.TGA.00G.00G.00G.00G.TGA.00G.00G.00G.00G.00G.00G.00G.00G.00G.0	* 1562 AGC:TTG:TTC:ACC:GCC:ATG:CCC:ATG:GAA:GGC:ATC:AAG:CTG:CTG:TTC:AAG:CTT:TAC:GCC:GAG Ser-Leu-Phe-Thr-Ala-Het-Pro-Het-Glu-Gly-lle-Lys-Leu-Phe-Lys-Leu-Tyr-Ala-Glu	542* CGC.GGT.GAC.GAA.GCG.CTC.ATG.CAG.CAG.GGC.AAG.GTC.ATG.GGC.CTG.CTG.TGT.CTG.CAA.GAT Arg-Gly-Asp-Glu-Ala-Leu-Met-Gln-Gln-Arg-Lys-Yal-Met-Gly-Leu-Leu-Cys-Leu-Gln-Asp
1083	1582* CTG.GAA.TAC.CCT.GAG.CGC.ACC.GGC.AAA.CTC.ATC.CTC.ATG.GGG.CCG.GGC.GGA.TTG.GGC.AAC Leu-Glu-Tyr-Pro-Glu-Arg-Thr-Gly-Lys-Leu-[le-Leu-Met-Gly-Pro-Gly-Gly-Leu-Gly-Asn	482* ### 423 GAC. GCC. GCC. GCG. GTC. GAG. GCC. ATG. GCC. GAC. AAG. GTG. GGC. GAG. GCA. GGG. GTG. GCC. TTC. ACC ##################################
AT. GCT. GAC. CTC. TGT. GCT. TCC. CGT. GGG. GCG. CAT. GCT. GTG. GCA. GCG. GAA. TAC ATA. GCC. GCC. GCC. GCC. GCG. GCG. GCG. CAT. GCT. GTG. GCA. GCG. GAA. TAC ATA. GCC. GCC. GCC. GCC. GCC. GCC. ACC. AC	;CG.CAC.CTC.GTG.GC ,Ia-His-Leu-Yai-Gl	422* CGC.ATC.GCC.GTG.CAG.CCG.GGC.GAA.CTC.GAC.GAC.CTG.GCC.TAC.GCA.GGC.TTG.GAA.GTG.GAT Arg-lle-Ala-Yal-Gln-Pro-Gly-Glu-Leu-Asp-Asp-Leu-Ala-Tyr-Ala-Gly-Leu-Glu-Yal-Asp
AT. GGT. GAG. CTC. TGT. GGT. TCC. CGT. GGG. GCG. CAT. GCT. GTG. GCA. GGG. GAA. TAC ***********************************	# 1383 GAC.GAA.CAG.CGC.GGC.CTG.GTC.AAT.GCG.CGC.TCG.GTC.AAG.GGC.ATG.ATG.ATG.GAC.GTG.CTC.GGC Asp-Glu-Gln-Arg-Gly-Leu-Yal-Asn-Ala-Arg-Ser-Yal-Lys-Gly-Met-Met-Asp-Yal-Leu-Gly	362* TTG.ATG.GCT.GCG.GGT.TCG.GCT.GGC.GAC.GCT.GCG.CTG.TAC.CGG.GCC.GAT.CAG.CGT.GCC.TGG Leu-Net-Ala-Ala-Gly-Ser-Ala-Gly-Asp-Ala-Ala-Leu-Tyr-Arg-Ala-Asp-Gln-Arg-Ala-Trp
AT. GGT. GAG. GTG. TGT. GGT. TGC. GGT. GGG. GCG. CAT. GCT. GTG. GCA. GGG. GAA. TAC ATA. GGC. GGC. GGC. GGC. GGG. GCG. CAT. GCT. GTG. GCA. GGG. GAA. TAC ATA. GGC. GGC. GGC. GGC. GGC. GCG. GCG. TTC. CCC. ACG. ACA. GGC. GCG. GCG. TTC AT. GGT. GGC. ATG. GGC. ACG. GGC. GGC. GAC. ACC. TTC. CCC. ACG. ACA. AGG. GCG. GCG. TTC BY 0 0 0 1 82* TTC. GGT. TTC. ATG. GGC. GGG. GAG. AAG. ATG. AGT. ATC. GAA. CGT. TTG. GGC. ACG. GCG. GCG. GGC. GGC. GGG. GGC. GAT. AGC. AGC. AGC. GAT. AGC BY 0 0 0 1 82* TTC. GGT. TTC. ATG. AGC. AAG. AAG. AAG. AAG. ATG. AGT. ATC. GAA. CGT. TTG. GGC. TTG. GGC. GGC. GGG. GGC. GGG. GGC. GGG. GGC. TGG. AGC TTC. GGT. TTC. ATG. ACC. AAG. GAG. AAG. AAG. ATG. AGT. ATC. GAA. CGT. TTG. GGC. TGG. AGC TTC. GGT. TTC. ATG. ACC. AAG. GAG. AAG. AAG. AAG. ATG. AGT. ATC. GAA. CGT. TTG. GGC. TGG. AGC TTC. GGT. TTC. ATG. ACC. AAG. GAG. AAG. AAG. ATG. AGT. ATC. GAA. CGT. TTG. GGC. TGG. AGC TTC. GGT. TTC. ATG. ACC. AAG. GAG. AAG. AAG. ATG. AGT. ATC. GAA. CGT. TTG. GGC. TGG. AGC TTC. GGT. TTC. ATG. ACC. AAG. GAG. AAG. AAG. ATG. AGT. ATC. GAA. CGT. TTG. GGC. TGG. AGC TTC. GGT. TTC. ATG. ACC. AAG. GAG. AAG. AAG. ATG. AGT. ATC. GAA. CGT. TTG. GGC. TGG. AGC TTC. GGT. TTC. ATG. ACC. AAG. AAG. AAG. AAG. ATG. AGT. ATC. GAA. CGT. TTG. GGC. TGG. AGC TTC. GGT. TTC. ATG. ACC. AAG. AAG. AAG. ATG. AGT. ATC. GAA. CGT. TTG. GGC. TTG. GGC. GGC. GGC. GGC	IGC.GTG.CTC.CTC.CCG.GA Nrg-Yal-Leu-Leu-Pro-As	GAT.GTA.CCC.GCC. Asp-Yal-Pro-Ala-
AT. GGT. GAC.CTC.TGT.GCT.TCC.CGT.GGG.GCG.CAT.GCT.GTG.GCA.GCG.GAA.TAC	* 1263 GGC.CCG.GGC.GCG.GGG.GGC.TGG.AGC.AAC.TAT.TAC.CGC.AAC.ATG.GGG.CCC.TTG.GTC.GAG.GCT Gly-Pro-Gly-Ala-Gly-Gly-Trp-Ser-Asn-Tyr-Tyr-Arg-Asn-Ile-Gly-Pro-Phe-Val-Glu-Ala	GCT.TTC.ATA.ACC.AAG.GAG.AAG.ACA.ATG.
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62* * 1083 ANT.GCT.GAC.CTC.TGT.GCT.TCC.CGT.GGG.GCG.CAT.GCT.GTG.GCA.GCG.GAA.TAC GTA.CGC.GGC.CAG.CGC.TGA.GCC.GC Val-Arg-Gly-Gln-Arg-***	C.AAC.GAA.AGC.TCG.ACG.AGC.AAA.TTT.GTC.ACC.ATC.AAC.GA u-Asn-Glu-Ser-Ser-Thr-Ser-Lys-Phe-Yel-Thr-lle-Asn-Gl	63 .GGA.GCC.TAC.GTG.TTC.TTT.GCC.ACG.CGC.GGC.GAC.ACC.TTC.CCC.ACG.ACA.GG
	ΤGA.GCC.GCG.TCA.TTT.ATT.TAA.CTT.TT <u>T</u> .C <u>AG.GA</u> C	NAT.GCT.GAC

FIG. 4. Nucleotide sequence of the middle part of the 3.2-kb fragment. The deduced amino acid sequences of ORFI and ORFII are shown. Possible Shine-Dalgarno (SD) sequences and a promoterlike sequence are underlined.

2744 KIMBARA ET AL. J. BACTERIOL.

			-0.00	05.550	00.500	04*40	00+00
540 TGCTTGGCTT **** ***** TGCTCGGCTT	600 CCGCGCACTT * * * * * * * * * * * * CTGTGTACTT	660 TCCCGAAGCG * * * * * * * * * * * * * * * * * * *	720 CCTTCGATCG * ** ***** CATTTGATCG 720	780 ACCAGACGCT ***	840 GGGGGCCGCG ** * ** GGAGTGCCCG	900 GGGGTCACAA **** ***** GGGCCACAA	961 CATTACCAT 1 1 1 AA <u>[FAG</u> DACCO
530 1ACACCGAGG ** **** * TATACCGACG	590 ACGAGCGTGC * * * * GTGACGGTTC 590	650 GCTTTCCCGA 1* ****** GCATTCCCGC	710 CTGGGCTACG ** **** ** OTCGGCTTTG	770 CACACCAACO ******* * CACACCAATO	830 GAGTTCGCCT **** **** GAGTATGGCT 830	890 ACGGCCATGT ** ***** CCGAGCATGT	950 CTTTTTCAGA * CAACAACCTG
1	\$60		680 690 720 TTCATGCTGC AAGCCAACAC CATCGACGAT GTGGGCTACG CCTTCGATCG	110 710 740 750 760 760 770	790 800 810 820 830 830	1	1 910 920 930 940 950 960 950 960 950 960 950 960 950
510 CGGCGAAGGC *** ***** CGGACAAGGC	560 570 GACATCATTG ACATCCAGAT *** ***	610 ACCACACGAT ****** * ACCACACCCT	690 AAGCCAACAC * * * * * AAGTCGCCTG	140	810 CGTCGCCCAT **** CGTCCGG	870 CGGTGGCGCG *** * * TTGTGGTGAQ	930 GAICCCCTC * * * * * * * TCCCCCAAC
1 490 500 CGTGCGTGC GTGCCCGACA ******* * * * * * * * * * * * * * * *		610 620 CTGCACTGC AATGGCCGTC *********	670 680 690 690 CATCCACCAC TTCATGCTGC AAGCCAACAC	740 GCCGGCCGCA * *** * GACGGCTTGA	800 GCCGACACGC CGTCC *** ** **** GCCTCGACCC CGTCC	860 TCCTCCTGGA ****** CGCTCCTGGG	920 GGCCAGCGC <u>I</u> * * * GACAAAGCAC
481 490 CGTCCGCTGC ********** CGTCCGCTGC	541 550 TGTGCTGTGG * **** CCAGTTGTGT 541 550	601 610 TCTGCACTGC ********* CCTGCACTGC	661 670 CATCCACCAC *** *** CATTCATCAC 661 670	721 730 GCTGGACGCC # * ***** GGTTGACGCG 721 730	781 790 CTCCTTCTAT 1: :::::: GTCGTTCTAT 781 790	841 850 CACAGTGGAT ** ** ** TACCGTTGAC 841 850	901 GTCGGTACGC *** ** *** GTCTGCGGG
60 AACGTTTGGG * ******* GAAGTTTGGG	120 CCAAGAGCGT * *** * CGCAGAAGT	180 ATCAGCGTGC 18 1 18 ATTCGAGAGC 180	240 GCTTGGAAGT *** ** ** GCTACGAGGT	300 GGGTGGCCTT * * * * * GTATCGCAGT	350 360 360 ATGGCCTCCA	420 TTCATGAGCC * * * * * TCGAAAACC	470 GACCAGGGGA TCGGGCACTT ** ** ** * * * * * * * * * * * * * * *
50 *TCAGTATCG ***** *** ATGAGCATCA 50	CACTTTCTGA ******* TCGTTTCTGA	170 180 TACCGGGCCG ATCACCTCC * **	210 210 GCCTACGCAG GCTTGGAAGT **** ** ** ** ** ** GCCTTGCCG GCTACGAGGT 210 210	290 CGCCAGGCAG **** * AAACAGGCCG	350 ATGGCCTGC * ** ** ACGGGATTGA	410 GCAGAAATCT * * * AGCGAGGTGT	460 0000 0ACCAGGGA TCGGGCACTT 11 11 11 11 11 11 11 11 11 11 11 11 11
10 GGAGAAGACA ***** GGAGACAGTA SD 0	100 cgccTgggAC ## ### TGCTTGGCGT	COCTGCGCTG 11	220 CGACGACCTG **** *** TGACGATCTG	280 CGACAAGCTG ******* TGACAAGCTC	340 GCGCAAGGTC *** *** CCGCGGGGTG	400 CTACGCCCC *** ** * CTATGCCCC	
TCATAACCAA ** ** CATCGACGAA	10 10 120 100 110 120	130	190 200 210 220 CTGGCGCATC GCCGTGCACCTG CTGGCGCATC GCCGTGCACCTG CTGGCGCATC GCCGTTCAGC AGGGCGAGGT TGACGATCTG 190 200 210 22	1	10 310 320 330 340 340 340 340 340 340 340 340 34	370	
20 AAACTCGCTT * * * CATCCCTAAG	80 TTCGCCGTCA 8	140 GCTGCGGGTT * **** GAAGCGGGCA	200 GCCGTGCAGC ***** **** GCCGTTCAGC	260 GCCGCGCTCG ** * ** * * * * * CCAGGGCTGG	320 GACGAAGCGC ** * * GATGCCTCAT	380 GGCCTGCCGC ******** GGCCTGCCGT	440 AGGGCCCCG * ** *** GGTGCGGCCG
KKS102 1 10 50 50 50 50 50 60 60 60 60 60 60 60 60 60 60 60 60 60	61 CTACCTCGGC 111 111 ATACATGGGG 61	121 GGGTTTGATG 111 111111 GGGCTTGATG 121 130	181 CTGGCGCATC ****** *** CTGGGGGATC 181	241 GGATGACGCC ** ** ** GGCCGATGCG 241	301 310 CACCGGGGT *** *** CACCACGGG	361 AGATCCOTTC ** ***** CGACCCOTTT 361	421 CTTCCTCCT ******** GTTCCTCCT 421 430
KKS1 KF70							

FIG. 5. Comparison of the nucleotide sequence of ORFI with that of the *bphC* gene of *P. pseudoalcaligenes* KF707. The nucleotide sequence of ORFI is shown from the downstream region of the putative promoter sequence indicated in Fig. 4 together with that of *bphC*. A possible Shine-Dalgarno (SD) sequence in each structure, the translation start codon ATG, and the stop codons TGA and TAC are boxed. *, Coincidence of nucleotides in both genes.

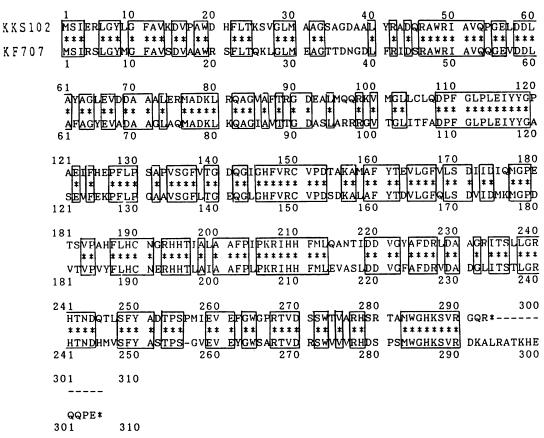


FIG. 6. Comparison of the deduced amino acid sequence coding for 23DBDO of *Pseudomonas* strain KKS102 with that of *P. pseudoalcaligenes* KF707 (the gene product of bphC). Homologous amino acids are boxed.

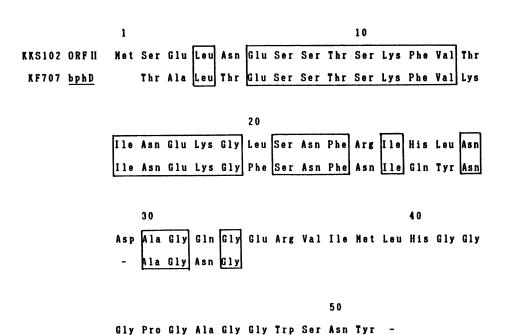


FIG. 7. Comparison of the N-terminal amino acid sequence of ORFII of *Pseudomonas* strain KKS102 (deduced from the nucleotide sequence) with the N-terminal amino acid sequence of *meta* cleavage compound hydrolase (a gene product of *bphD*) of *P. pseudoalcaligenes* KF707. Homologous amino acids are boxed.

2746 KIMBARA ET AL. J. BACTERIOL.

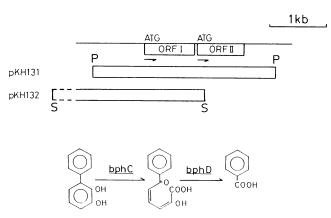


FIG. 8. Structures of DNA fragments in pKH131 and pKH132. pKH131 has the complete ORFI and ORFII in a broad-host-range plasmid. pKH132 has the complete ORFI and a part of ORFII in the same plasmid. The positions of ORFI and ORFII are indicated, with the initiation codon ATG in front of each ORF and the directions of translation shown by the horizontal arrows. The metabolic steps catalyzed by the gene products of bphC and bphD are shown in the lower part. As described in the text, it was concluded that ORFI and ORFII corresponded to bphC and bphD, respectively.

suggested that ORFII coded for *meta* cleavage compound hydrolase.

To confirm this, the following experiment was performed with two kinds of plasmids. The structures of the inserted DNA fragments in these plasmids are shown in Fig. 8. pKH131, consisting of the vector pMFY40 and the 3.2-kb DNA fragment as described above, contained the complete ORFI and ORFII. On the other hand, pKH132, consisting of the vector and the SmaI fragment derived from the middle of the 18-kb BamHI fragment (Fig. 1), contained the complete ORFI and only a part of ORFII. Each of these plasmids was introduced into P. putida PpY101 by using the triparental mating method, and catabolism of 2,3-dihydroxybiphenyl was examined. In the strain carrying pKH131, 2,3-dihydroxybiphenyl was quickly converted to the yellow meta cleavage compound, HOPD, and then slow accumulation of benzoic acid was observed. In contrast, in the strain carrying pKH132, 2,3-dihydroxybiphenyl was quickly converted to HOPD, but accumulation of benzoic acid was not observed. On the basis of these results, it was concluded that ORFII coded for meta cleavage compound hydrolase.

DISCUSSION

In the present study, we cloned two genes corresponding to bphC and bphD, which are involved in the degradation of biphenyls and possibly in the degradation of PCBs, from the total DNA of KKS102. The cloned DNA fragment (29 kb) in the cosmid pKH1 expressed 23DBDO activity in P. putida constitutively, even though the gene coding for it was located in the middle of the 29-kb DNA fragment (Fig. 2). pKH1 did not express 23DBDO activity in E. coli, but when the subcloned fragment (3.2 kb) was inserted downstream of the promoter of lacZ in the vector pUC18, 23DBDO activity was also detected in E. coli. These results indicated that the gene coding for 23DBDO was expressed from its own promoter only in P. putida and not in E. coli. Expression of low activity of 23DBDO in E. coli by a plasmid consisting of the 3.2-kb fragment and the vector pMFY40 might be due to readthrough of ORFI from the vector sequence. Much information is available about the structures of promoters and their regulation systems for degradative genes of *Pseudomonas* spp. (2, 10, 20–22, 26, 30). Upstream of the gene coding for 23DBDO there is a promoterlike sequence (Fig. 4), a GG-GC combinationlike sequence (10), and sequences similar to the proposed *P. putida* consensus promoter sequences, A-AGGC-T and GCAATA (2, 26). By using unidirectional deletion mutants, it was shown that the gene coding for 23DBDO with only 58 bp upstream from the initiation codon ATG was expressed in *P. putida* (data not shown), suggesting that the promoterlike sequence located between –42 to –56 from ATG may be functional as a promoter in *P. putida*. This promoter may also have read through ORFII to produce its gene product, which produced benzoic acid from HOPD in *P. putida*, since there is no promoterlike sequence in front of ORFII (Fig. 4).

P. putida carrying pKH1 had no activity to produce HOPD from biphenyl. This may be because the genes which code for enzymes involved in conversion of biphenyl to 2,3-dihydroxybiphenyl (corresponding to the genes bphA and bphB) did not exist in the cloned 29-kb fragment, or it may be because the expression of these genes was masked in P. putida. The latter possibility is more probable, since Southern blot analysis of the upstream region of the gene coding for 23DBDO in the 29-kb fragment, using the bphA gene of P. pseudoalcaligenes KF707 as a probe (17), gave a positive result; that is, there was a DNA sequence homologous to the bphA gene in the upstream region of this gene. Thus, it is strongly suggested that the genes corresponding to bphA and bphB were also contained in the cloned 29-kb DNA fragment. An expression of a positive regulatory gene like xylS (20) and nahR (30) might be required for active transcription of these genes, which was not apparently essential for the expression of ORFI and ORFII. Nucleotide sequencing of this upstream region is being done to determine whether there are any open reading frames corresponding to bphA and bphB and whether there are any specific sequences around their promoter regions for positive regulation of degradative genes.

It was reported that P. pseudoalcaligenes KF707 has one operon consisting of three genes, bphA, bphB, and bphC, for degradation of biphenyl to the meta cleavage compound HOPD, and a fourth gene, bphD (for conversion of the meta cleavage compound to benzoic acid), which was found neither in the same operon nor in the region just downstream of the operon (16). In contrast, two genes involved in degradation of 2,3-dihydroxybiphenyl to benzoic acid were tandemly and unidirectionally arranged, and were probably in one operon, in KKS102. Thus, the gene organizations in these two strains are similar but not identical. The absence or presence of the fourth gene in this gene cluster characterizes the operon structures in each strain, although at least two operons should be involved in both systems. In this context, we point out that the gene clusters of xyl (11) and nah (19) consist of two regulatory units or two operons.

The Tn5 insertion mutation analysis shown in Fig. 3 indicated that an insertion of Tn5 in the 3' region of ORFI did not abolish the activity of 23DBDO, suggesting that a part of the C-terminal region of 23DBDO may not be essential for its enzyme activity.

The N-terminal amino acid sequence deduced from ORFII in KKS102 showed high homology to that from the bphD gene product in KF707 (Fig. 7) determined by Edman degradation of the purified enzyme protein (K. Furukawa, personal communication). These results indicated that the structures of the genes involved in the two specific steps of biphenyl degradation in these strains are similar to each

other in nucleotide sequence. It is suggested that these two biphenyl-degrading gene systems might have evolved from the same origin but diverged from each other during the course of evolution.

ACKNOWLEDGMENTS

We thank K. Furukawa for providing us with his unpublished data and for his help and advice during the course of this research. Helpful discussions with K. Tanemura, N. Nishikawa, and the other members of the Engineering Research Institute of the Tokyo Electric Power Co. are gratefully acknowledged.

This work was partly supported by a fund from the Tokyo Electric Power Co.

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