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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

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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) *Pst*I fragment of a 29-kb cosmid DNA insert was subcloned into pUC18 at the *Pst*I 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 *Pst*I 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 Tn*5* 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 2,3-dihydroxybiphenyl 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 cloning of an operon, *bphABC*, from *P. pseudoalcaligenes*, but *bphD* was not contained in this operon.

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 KH_2PO_4 (1.7 g/liter), Na_2HPO_4 (9.8 g/liter), $(NH_4)_2SO_4$ (1.0 g/liter), $MgSO_4 \cdot 7H_2O$ (0.1 g/liter), $FeSO_4 \cdot 7H_2O$ (0.95 mg/liter), MgO (10.75 mg/liter), $CaCO_3$ (2.0 mg/liter), $ZnSO_4 \cdot 7H_2O$ (1.44 mg/liter), $CuSO_4 \cdot 5H_2O$ (0.25 mg/liter), $CoSO_4 \cdot 7H_2O$

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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	*met nal*	12
E. coli		
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[a])	9
MV1184	*ara* Δ*(lac-pro) str thi* φ80 *lacIZ*Δ*M15* Δ*(srl-recA)*:: Tn*10* (F′ *traD proAB lacI*^{q}*Z*Δ*M15)*	32
Plasmids		
pRK2013	Km^r Tra$^+$ ColE1 replicon	28
pUC18	Ap^r *lacZ′* pMB9 replicon	32
pMFY40	Ap^r Tc^r Mob$^+$ RSF1010 replicon	12
pKTY320[b]	Ap^r Cm^r Mob$^+$ p15A replicon	This laboratory
pKS13	Tc^r *cos* Mob$^+$ RK2 replicon	This study
pKH1	*bphCD* in pKS13	This study
pKH10	11-kb *Bam*HI 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::Tn*5*) phage has the following genotype: λ *b221 rex*::Tn*5* *c*I857 *O*am29 *P*am80.

[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.

(0.28 mg/liter), H_3BO_3 (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 ^{32}P-labeled, nick-translated DNA was performed as described by Southern (31).

Tn*5* mutagenesis was performed as described by de Bruijn and Lupski (9). The 3.2-kb subcloned fragment was inserted into pKTY320, and the λ::Tn*5* 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 Tn*5* 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-dihydroxybiphenyl 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

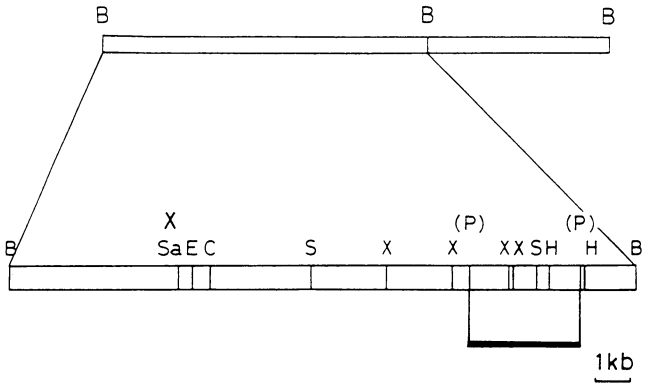

FIG. 1. Cloning of a 29-kb fragment from the genome of *Pseudomonas* strain KKS102 by using a cosmid vector (pKS13) and the restriction map of the 18-kb *Bam*HI fragment in it. The fragment consisted of the two *Bam*HI fragments (11 and 18 kb). The heavy line between the two Ps represents a 3.2-kb *Pst*I 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, *Hin*dIII; 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 *Bam*HI (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 *Bam*HI fragments conferred 23DBDO activity on *E. coli*. However, 23DBDO activity was observed when pKH101, constructed by inserting a 3.2-kb *Pst*I fragment into a vector (pUC18) at the *Pst*I 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 *Pst*I 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

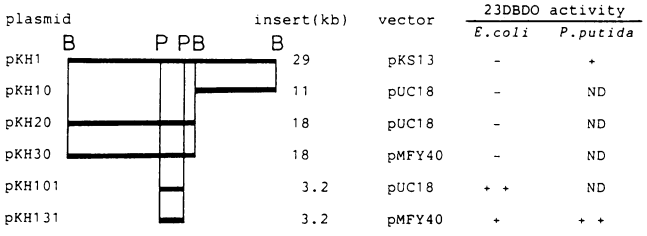

FIG. 2. Subcloning of a gene for 23DBDO. A variety of plasmids were constructed by using the restriction fragments obtained from the original 29-kb insert in pKH1. The names of the constructed plasmids and the structures and the sizes of the inserted DNA fragments are shown at the left. The 23DBDO activity 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, *Bam*HI; P, *Pst*I.

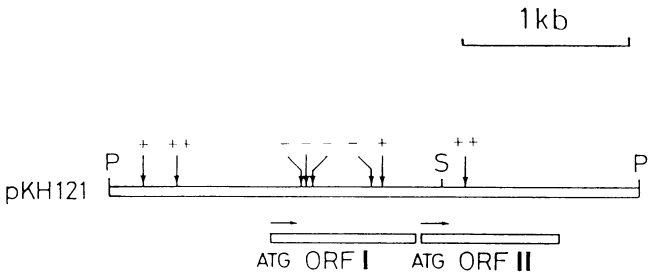

FIG. 3. Positions of Tn*5* insertion in the subcloned 3.2-kb fragment and the effect of insertion on 23DBDO activity. Vertical arrows indicate the Tn*5* insertion sites, and + and − represent the presence and the absence, respectively, of 23DBDO activity in each strain carrying a plasmid with Tn*5* insertion 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, *Pst*I; S, *Sma*I.

nick-translated ^{32}P-labeled 3.2-kb *Pst*I 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).

Tn*5* mutagenesis. Tn*5* 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 *Pst*I 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 Tn*5* 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 Tn*5* 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

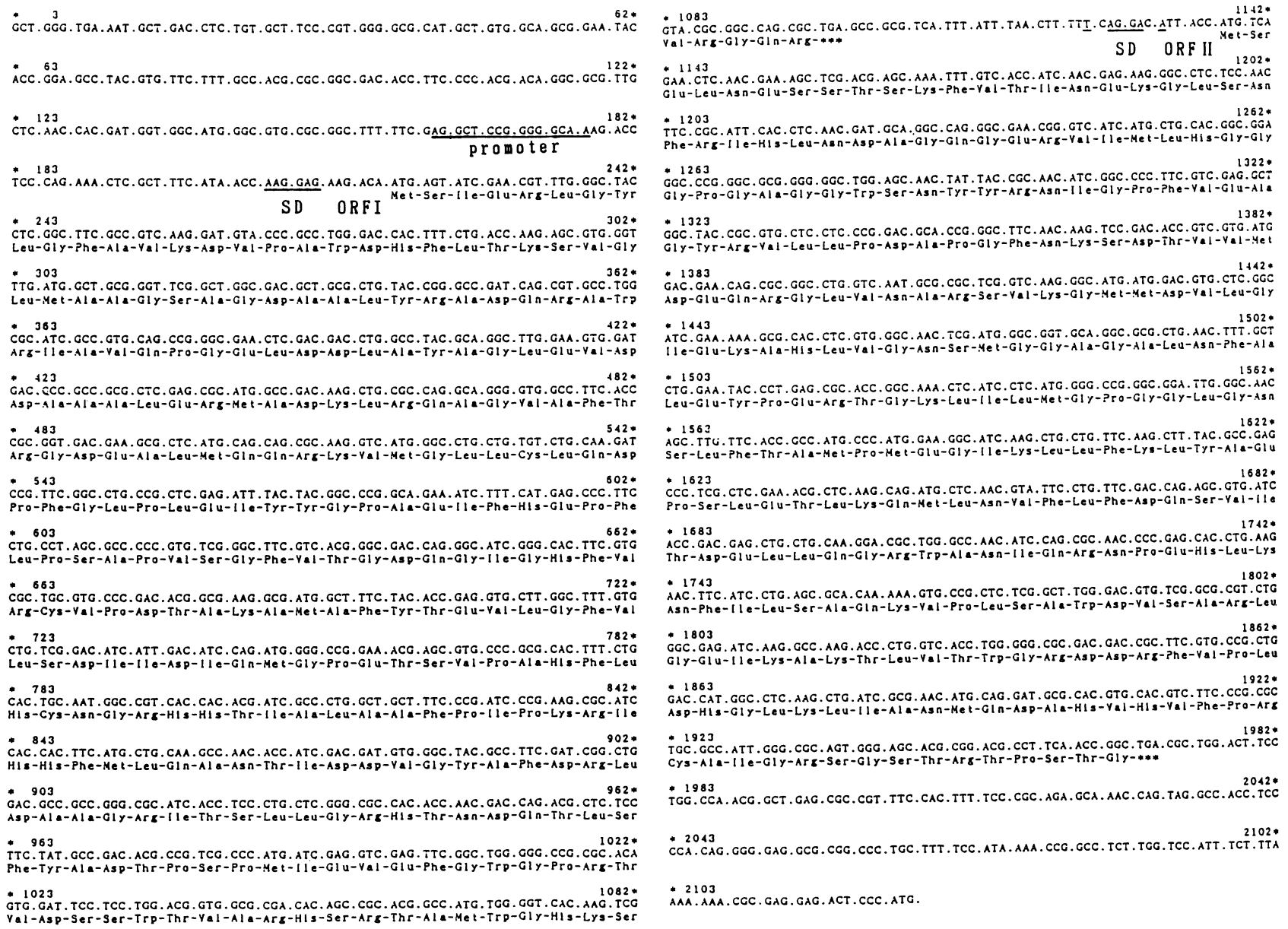

FIG. 4. Nucleotide sequence of the middle part of the 3.2-kb fragment. The deduced amino acid sequences of ORFI and ORFII are shown. Possible Shine-Dalgarno (SD) sequences and a promoterlike sequence are underlined.

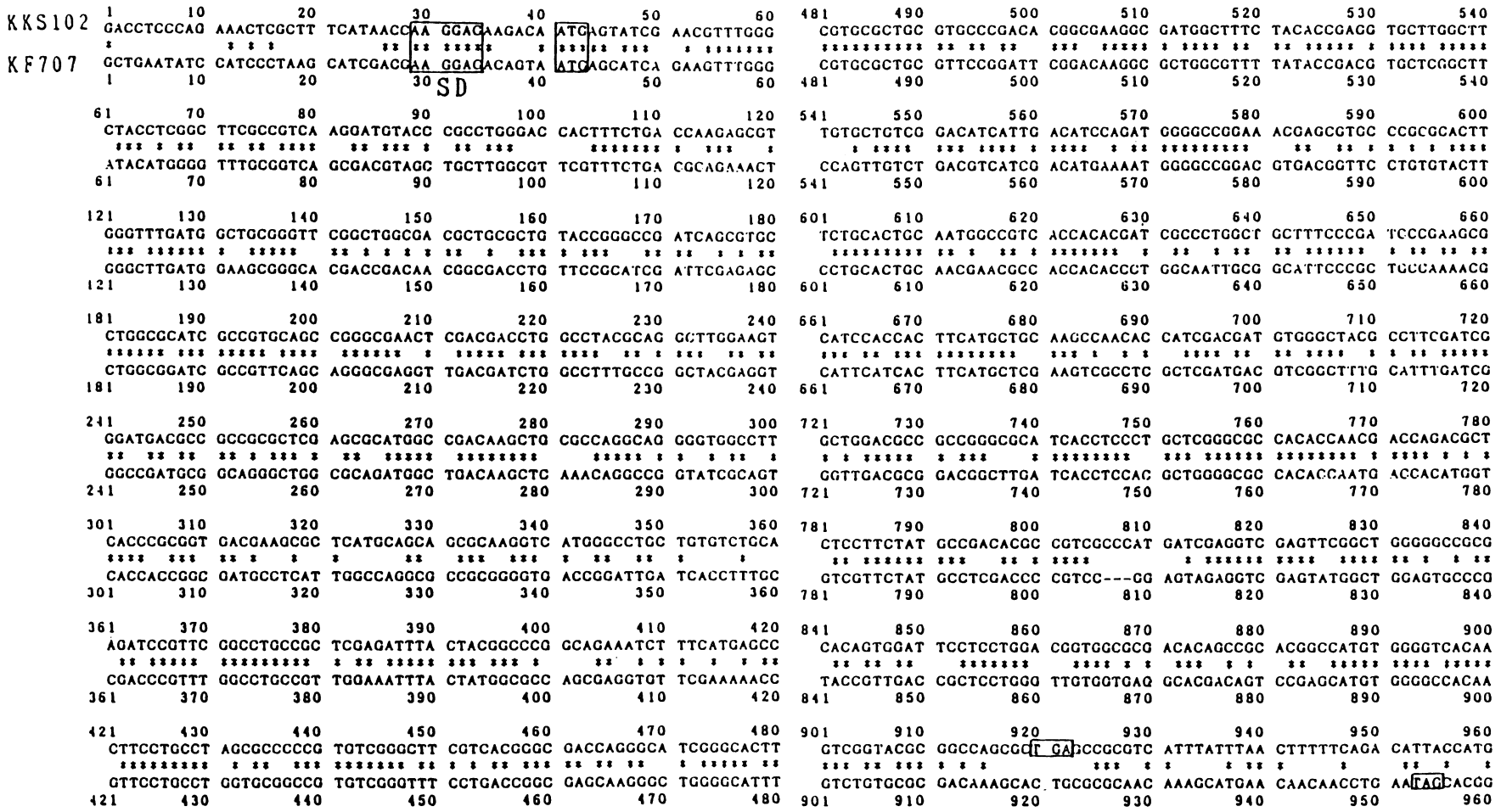

FIG. 5. Comparison of the nucleotide sequence of ORFI with that of the *bphC* gene of *P. pseudoalcaligenes* KF707. The nucleotide sequence of ORFI is shown from the downstream region of the putative promoter sequence indicated in Fig. 4 together with that of *bphC*. A possible Shine-Dalgarno (SD) sequence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

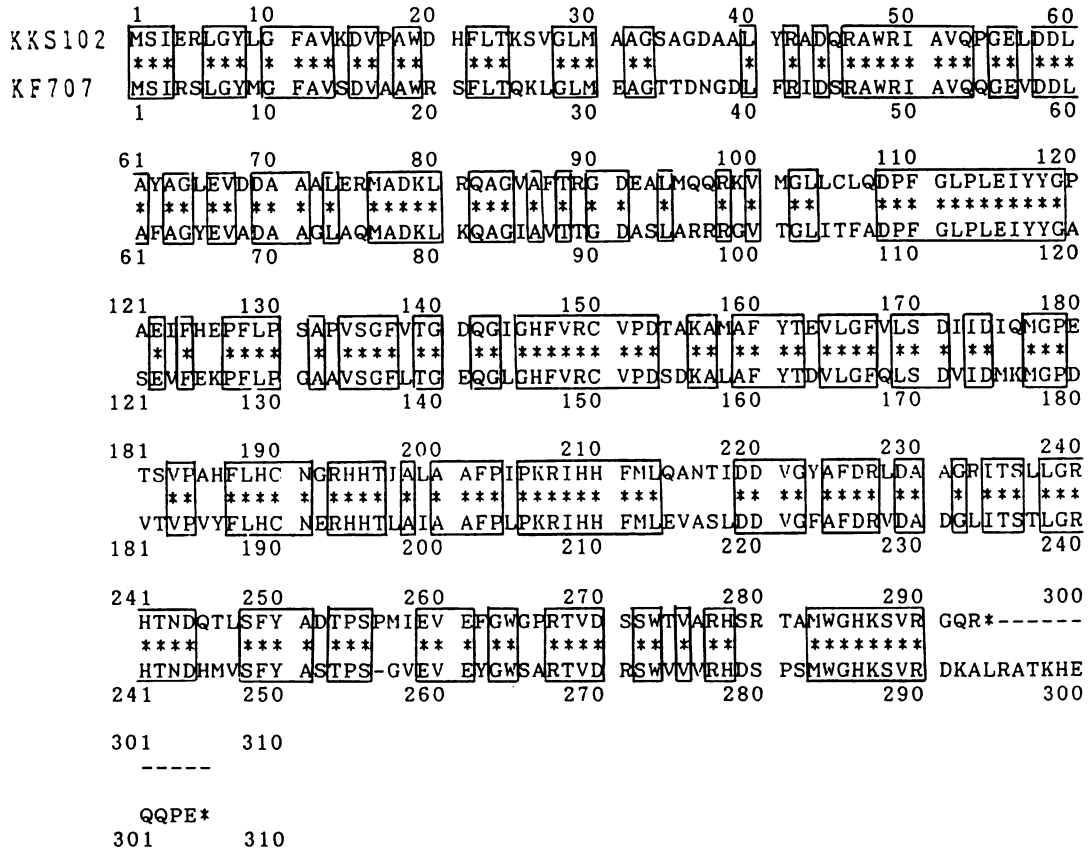

FIG. 6. Comparison of the deduced amino acid sequence coding for 23DBDO of *Pseudomonas* strain KKS102 with that of *P. pseudoalcaligenes* KF707 (the gene product of *bphC*). Homologous amino acids are boxed.

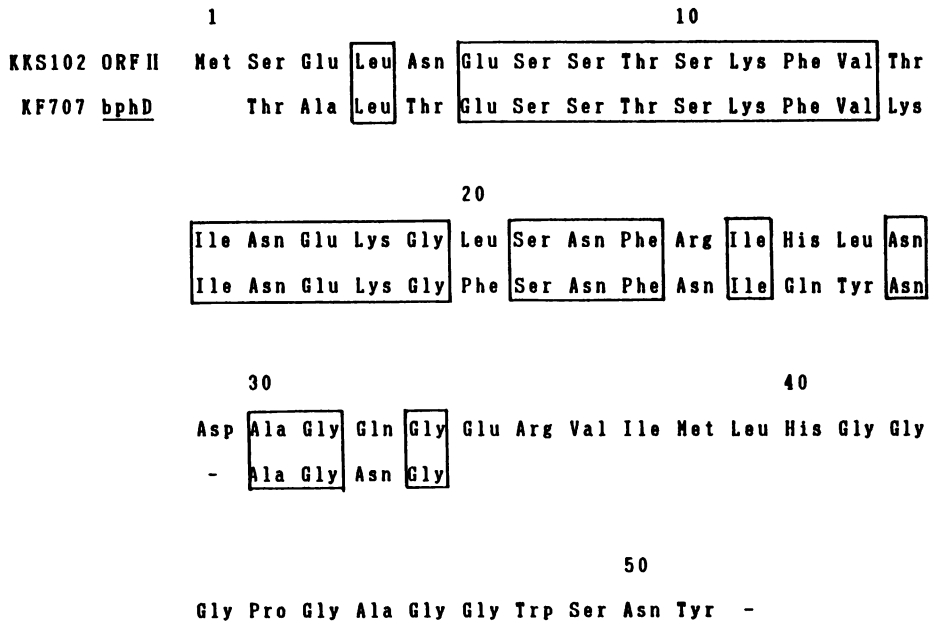

FIG. 7. Comparison of the N-terminal amino acid sequence of ORFII of *Pseudomonas* strain KKS102 (deduced from the nucleotide sequence) with the N-terminal amino acid sequence of *meta* cleavage compound hydrolase (a gene product of *bphD*) of *P. pseudoalcaligenes* KF707. Homologous amino acids are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

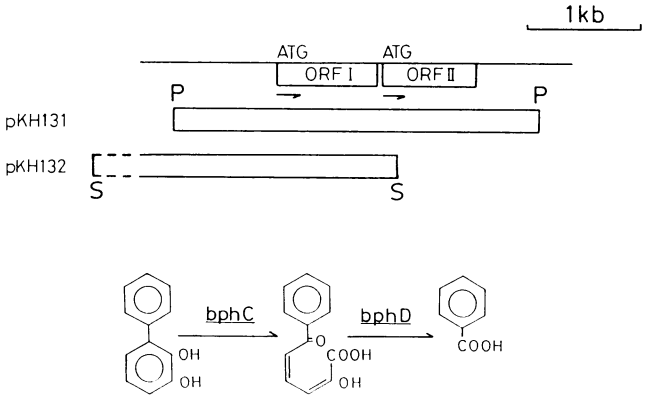

FIG. 8. Structures of DNA fragments in pKH131 and pKH132. pKH131 has the complete ORFI and ORFII in a broad-host-range plasmid. pKH132 has the complete ORFI and a part of ORFII in the same plasmid. The positions of ORFI and ORFII are indicated, with the initiation codon ATG in front of each ORF and the 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 *Sma*I fragment derived from the middle of the 18-kb *Bam*HI 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 Tn*5* insertion mutation analysis shown in Fig. 3 indicated that an insertion of Tn*5* 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.

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