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Gene Components Responsible for Discrete Substrate Specificity in the Metabolism of Biphenyl (*bph* Operon) and Toluene (*tod* Operon)

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***bph* operons coding for biphenyl-polychlorinated biphenyl degradation in *Pseudomonas pseudoalcaligenes* KF707 and *Pseudomonas putida* KF715 and *tod* operons coding for toluene-benzene metabolism in *P. putida* F1 are very similar in gene organization as well as size and homology of the corresponding enzymes (G. J. Zylstra and D. T. Gibson, J. Biol. Chem. 264:14940–14946, 1989; K. Taira, J. Hirose, S. Hayashida, and K. Furukawa, J. Biol. Chem. 267:4844–4853, 1992), despite their discrete substrate ranges for metabolism. The gene components responsible for substrate specificity between the *bph* and *tod* operons were investigated. The large subunit of the terminal dioxygenase (encoded by *bphA1* and *todC1*) and the ring *meta*-cleavage compound hydrolase (*bphD* and *todF*) were critical for their discrete metabolic specificities, as shown by the following results. (i) Introduction of *todC1C2* (coding for the large and small subunits of the terminal dioxygenase in toluene metabolism) or even only *todC1* into biphenyl-utilizing *P. pseudoalcaligenes* KF707 and *P. putida* KF715 allowed them to grow on toluene-benzene by coupling with the lower benzoate *meta*-cleavage pathway. Introduction of the *bphD* gene (coding for 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase) into toluene-utilizing *P. putida* F1 permitted growth on biphenyl. (ii) With various *bph* and *tod* mutant strains, it was shown that enzyme components of ferredoxin (encoded by *bphA3* and *todB*), ferredoxin reductase (*bphA4* and *todA*), and dihydrodiol dehydrogenase (*bphB* and *todD*) were complementary with one another. (iii) *Escherichia coli* cells carrying a hybrid gene cluster of *todC1bphA2A3A4BC* (constructed by replacing *bphA1* with *todC1*) converted toluene to a ring *meta*-cleavage 2-hydroxy-6-oxo-hepta-2,4-dienoic acid, indicating that TodC1 formed a functional multicomponent dioxygenase associated with BphA2 (a small subunit of the terminal dioxygenase in biphenyl metabolism), BphA3, and BphA4.**

The relationships among the different aromatic pathways and gene clusters often reveal that evolutionary changes were involved in the development of metabolic routes (23–25, 28, 30). Such evolution could be directed from various genetic events, such as gene transfer, mutation, deletion, duplication, and recombination. Biphenyl-utilizing bacteria are widely distributed in the natural environment (5, 9, 10). They are mostly aerobic, gram-negative soil bacteria. They cometabolize polychlorinated biphenyls (PCBs) to chlorobenzoic acids (1, 4, 6, 10, 14, 19). We have previously cloned the genes coding for the conversion of biphenyl to benzoic acid from two *Pseudomonas* strains: *bphABCXD* genes from *Pseudomonas pseudoalcaligenes* KF707 (15) and *bphABCD* genes from *Pseudomonas putida* KF715 (26). The principal metabolic route of biphenyl-PCB by bacteria is presented in Fig. 1 (15, 33). In the first metabolic step, molecular oxygen is introduced at the 2,3 position to produce a dihydrodiol (compound II in Fig. 1) by the action of a multicomponent enzyme, biphenyl dioxygenase (the product of a gene cluster in the *bphA* region, BphA). The dihydrodiol is then dehydrogenated to 2,3-dihydroxybiphenyl (23OHBP) (Wako Pure Chemical, Tokyo, Japan) (compound III) by a dihydrodiol dehydrogenase (the product of *bphB*, BphB). 23OHBP is cleaved at the 1,2 position by the 23OHBP dioxygenase (the product of *bphC*, BphC) to yield 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HPDA) (compound IV), which is eventually hydrolized to benzoic acid (compound V) and 2-hydroxy-pent-2,4-dienoic acid (compound X) by HPDA hydrolase (the product of *bphD*, BphD). The *bphX* region, which exists in *P. pseudoalcaligenes* KF707 but not in *P. putida* KF715, has been sequenced, and three open reading frames were found which could be involved in further metabolism of 2-hydroxy-pent-2,4-dienoic acid (compound X) to acetyl coenzyme A (Fig. 1) (18). The overall homology of the *bphC* genes in *P. pseudoalcaligenes* KF707 and *P. putida* KF715 at the DNA level was as high as 92.4%, and the corresponding amino acid homology was 91.4% (26).

P. putida F1 grows well on toluene-benzene but not on biphenyl (20, 21). The initial oxidation of toluene is carried out by a multicomponent enzyme system (35, 36). Nucleotide sequence determination of the 6.8-kb fragment which includes *bphABC* revealed that the gene organization as well as the size and homology of the corresponding enzymes between the biphenyl-PCB degrader *P. pseudoalcaligenes* KF707 and the toluene-benzene degrader *P. putida* F1 was highly conserved despite the discrete substrate specificities of the strains (33, 35). The *bphA* region coding for a multicomponent enzyme, biphenyl dioxygenase, consisted of five open reading frames, of which four were similar to *todC1C2BA* genes coding for the corresponding enzymes catalyzing the initial toluene dioxygenation (Fig. 2). The products of *bphA1*, *bphA2*, *bphA3*, and *bphA4* corresponded with the products of *todC1* (coding for a large subunit of terminal dioxygenase), *todC2* (a small subunit of terminal dioxygenase), *todB* (ferredoxin), and *todA* (ferredoxin reductase), respectively (33, 35). The nucleotide sequences of

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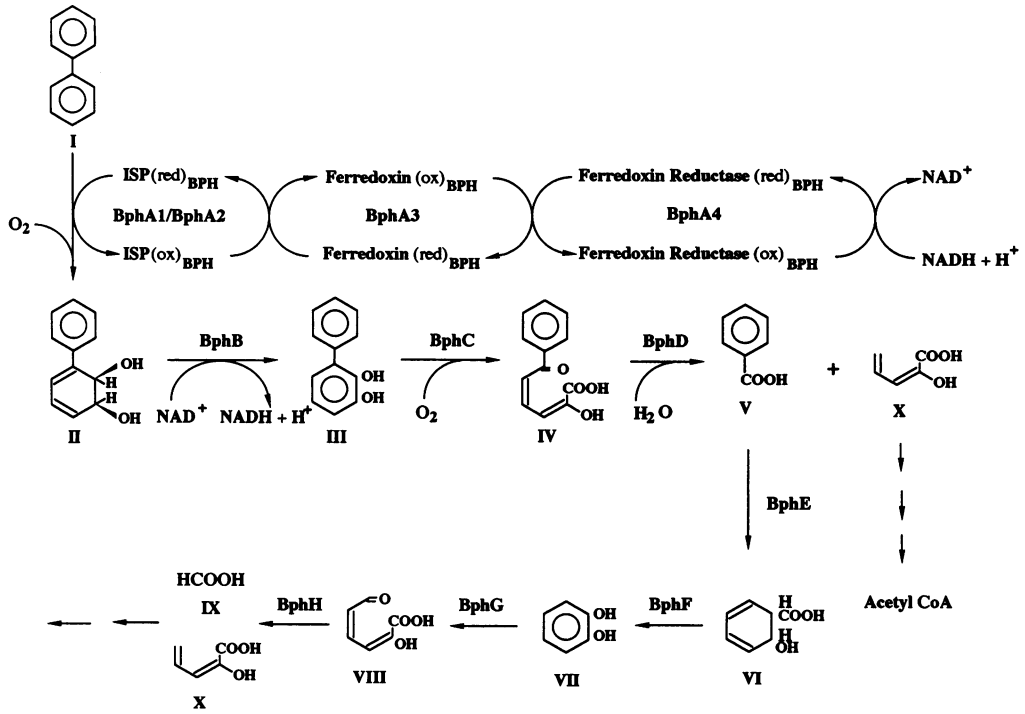

FIG. 1. Catabolic pathway for degradation of biphenyl in *P. pseudoalcaligenes* KF707. BphB, 2,3-dihydroxy-4-phenylhexa-4,6-diene dehydrogenase; BphE, benzoate oxidase; BphF, 2-hydroxy-3-carboxyhexa-4,6-diene hydrolase; BphG, catechol-2,3-dioxygenase.

bphAEFG genes coding for a multicomponent biphenyl dioxygenase from an American isolate of *Pseudomonas* sp. strain LB400 (7) were almost identical to those of *bphA1A2A3A4* of *P. pseudoalcaligenes* KF707 (97.4% overall homology). The identities of amino acid sequences of the corresponding pairs BphA1 and TodC1, BphA2 and TodC2, BphA3 and TodB, BphA4 and TodA, BphB and TodD, and BphC and TodE were between 53 and 65% (34). On the other hand, the level of similarity between BphD (*P. putida* KF715) and TodF (29) was relatively low (35.1%). Furthermore, *bphD* is located downstream of *bphX*, but *todF* is located just upstream of *todC1* (Fig. 2). On the basis of these findings, we were interested in asking what gene components in the *bph* and *tod* operons are responsible for the substrate specificity or interchangeable in the metabolism of biphenyl-PCB and toluene-benzene.

MATERIALS AND METHODS

Strains and plasmids. The strains and plasmids used in this study are listed in Table 1. Biphenyl-utilizing *P. pseudoalcaligenes* KF707 and *P. putida* KF715 were described previously (15, 26). Strains KF733, KF748, and KF744 are mutants of KF707 in which transposon Tn*5*-B21 is inserted

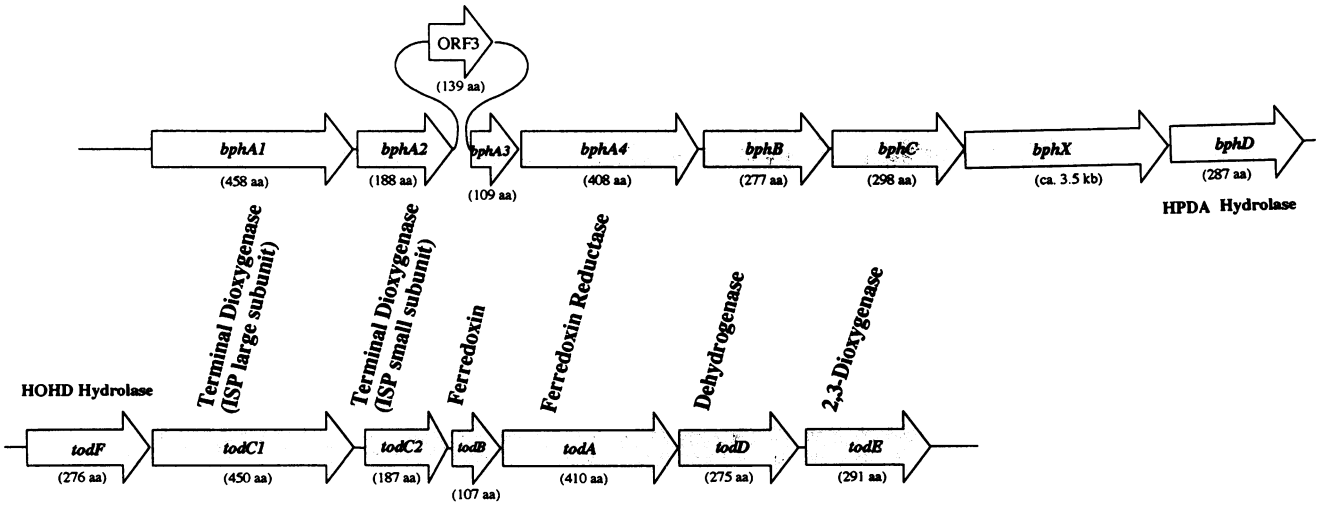

FIG. 2. Organization of *bph* operon in *P. pseudoalcaligenes* KF707 (33) and comparison with *tod* operon in *P. putida* F1 (35).

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
E. coli		
S17-1	*pro thi recA hsdR*, chromosomally integrated RP4-2-Tc::Mu-Km::Tn*7*	31
JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* Δ(*lac proAB*) [F'*proAB lacI*^{q} DM15 *traD36*]	34
P. pseudoalcaligenes		
KF707	BP$^+$ Tol$^-$, wt	15
KF707(pDTG351)	BP$^+$ Tol$^+$, pDTG351, Sm^r	This study
KF707(pMLC1C2)	BP$^+$ Tol$^+$, pMLC1C2, Gm^r	This study
KF707(pMLC1)	BP$^+$ Tol$^+$, pMLC1, Gm^r	This study
KF733	BP$^-$ Tol$^-$, *bphA*::Tn*5*-B21, Tc^r	13
KF733(pDTG351)	BP$^+$ Tol$^+$, *bphA*::Tn*5*-B21, pDTG351, Tc^r Sm^r	This study
KF733(pMLC1C2)	BP$^-$ Tol$^-$, *bphA*::Tn*5*-B21, pMLC1C2, Tc^r Gm^r	This study
KF733(pMLC1)	BP$^-$ Tol$^-$, *bphA*::Tn*5*-B21, pMLC1, Tc^r Gm^r	This study
KF744	BP$^-$ Tol$^-$, *bphC*::Tn*5*-B21, Tc^r	13
KF744(pDTG351)	BP$^+$ Tol$^+$, *bphC*::Tn*5*-B21, pDTG351, Tc^r Sm^r	This study
KF744(pMLC1C2)	BP$^-$ Tol$^+$, *bphC*::Tn*5*-B21, pMLC1C2, Tc^r Gm^r	This study
KF744(pMLC1)	BP$^-$ Tol$^+$, *bphC*::Tn*5*-B21, pMLC1, Tc^r Gm^r	This study
KF748	BP$^-$ Tol$^-$, *bphB*::Tn*5*-B21, Tc^r	13
KF748(pDTG351)	BP$^+$ Tol$^+$, *bphB*::Tn*5*-B21, pDTG351, Tc^r Sm^r	This study
KF748(pMLC1C2)	BP$^-$ Tol$^-$, *bphB*::Tn*5*-B21, pMLC1C2, Tc^r Gm^r	This study
KF748(pMLC1)	BP$^-$ Tol$^-$, *bphB*::Tn*5*-B21, pMLC1, Tc^r Gm^r	This study
P. putida		
KF715	BP$^+$ Tol$^-$, wt	26
KF715(pDTG351)	BP$^+$ Tol$^+$, pDTG351, Sm^r	This study
KF715(pMLC1C2)	BP$^+$ Tol$^+$, pMLC1C2, Sm^r	This study
KF715(pMLC1)	BP$^+$ Tol$^+$, pMLC1, Sm^r	This study
KF791	BP$^-$ Tol$^-$, Δ(*bphABCD bphEFGH*)	This study
KF791(pDTG351)	BP$^-$ Tol$^-$, pDTG351, Sm^r	This study
KF791(pMLC1C2)	BP$^-$ Tol$^-$, pMLC1C2, Sm^r	This study
KF791(pMLC1)	BP$^-$ Tol$^-$, pMLC1, Sm^r	This study
KF796	BP$^-$ Tol$^-$, Δ(*bphABCD*)	This study
KF796(pDTG351)	BP$^-$ Tol$^+$, pDTG351, Sm^r	This study
KF796(pMLC1C2)	BP$^-$ Tol$^-$, pNLC1C2, Sm^r	This study
KF796(pMLC1)	BP$^-$ Tol$^-$, pMLC1, Sm^r	This study
P. putida		
F1	Tol$^+$ BP$^-$, wt	20
F1(pMFB2)	Tol$^+$ BP$^-$, pMFB6, Sm^r	This study
F1(pMFB4)	Tol$^+$ BP$^-$, pMFB4, Sm^r	This study
F1(pMFB6)	Tol$^+$ BP$^-$, pMFB6, Sm^r	This study
F1(pMFB8)	Tol$^+$ BP$^+$, pMFB8, Sm^r	This study
F1(pNHF715)	Tol$^+$ BP$^+$, pNHF715, Sm^r	This study
F39/D	Tol$^-$ BP$^-$ *todD*	22
F39/D(pMFB2)	Tol$^+$ BP$^-$, pMFB2, Sm^r	This study
F39/D(pMFB4)	Tol$^+$ BP$^-$, pMFB4, Sm^r	This study
F39/D(pMFB6)	Tol$^+$ BP$^-$, pMFB6, Sm^r	This study
F39/D(pMFB8)	Tol$^-$ BP$^-$, pMFB8, Sm^r	This study
F39/D(pNHF715)	Tol$^+$ BP$^+$, pNHF715, Sm^r	This study
Plasmids		
pBluescript II KS+	*lacZ* Ap^r, 3.0 kb	Stratagene
pHSG396	*lacZ* Cm^r, 2.2 kb	Takara Shuzo
pKTF18	pUC118-*bphA1A2A3A4BC* (KF707)	33
pYF680	pHSG396-*bphD* (KF707)	26
pJHF10	pUC118-*todC1* (F1) *bphA2A3A4BC* (KF707)	This study
pML122	Nm^r Gm^r, 11.4 kb	27
pBSC1C2	pBluescript KS+-*todC1C2*	This study
pHSGC1C2	pHSG396-*todC1C2*	This study
pUC351	pUC118-*todC1C2BADE*	This study
pDTG351	pKT230-*todC1C2BADE*	36
pMLC1C2	pML122-*todC1C2*	This study
pMLC1	pML122-*todC1*	This study
pMFB2	pKT230-*bphABC* (KF707)	15
pMFB4	pKT230-*bphA* (KF707)	15
pMFB5	pKT230-*bphC* (KF707)	15
pMFB6	pKT230-*bphAB* (KF707)	16
pMFB8	pKT230-*bphD* (KF707)	26
pNHF715	pKT230-*bphABCD* (KF715)	26

[a] BP, biphenyl; Tol, toluene; wt, wild type; Sm^r, streptomycin resistance; Gm^r, gentamicin resistance; Tc^r, tetracycline resistance; Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance.

into *bphA1*, *bphB*, and *bphC*, respectively, as described previously (13). Strain KF796 is a KF715 mutant in which the upper *bphABCD* operon was deleted spontaneously (18). This strain had lost the ability to grow on biphenyl but grew on benzoate by using the *meta*-cleavage pathway. Strain KF791 is another KF715 mutant in which both upper *bphABCD* and lower benzoate *meta*-pathway genes (putative *bphEFGH*) were deleted, but it grew on benzoate by the *ortho*-cleavage pathway (18). Toluene-utilizing *P. putida* F1 and the *todD* (coding for toluene dihydrodiol dehydrogenase) mutant F39/D were described previously (20–22) and were provided by David T. Gibson, Department of Microbiology, University of Iowa, Iowa City. All recombinant *Pseudomonas* strains listed in Table 1 were constructed by mating with *Escherichia coli* S17-1 (chromosomally integrated RP4-2-Tc::Mu-Km::Tn*7*) (31) as the donor strain which carries respective recombinant plasmids containing a variety of *bph* genes or *tod* genes.

pMFB2 containing *bphABC* (KF707), pMFB4 containing *bphA* (KF707), pMFB6 containing *bphAB* (KF707), pMFB8 containing *bphD* (KF707), and pNHF715 containing *bphABCD* (KF715) were constructed with a broad-host-range plasmid vector, pKT230 (3), as described previously (15, 26). pKTF18 was constructed by introducing *bphABC* (KF707) into pUC118 (33). pDTG351 is a recombinant plasmid in which *todC1C2BADE* is inserted into pKT230 (35, 36). pMLC1C2, pMLC1, and pJHF10 were constructed in this study as described below.

Media and growth conditions. Biphenyl- and toluene-benzene-utilizing strains were grown at 30°C in a basal salts agar medium (15) supplemented with biphenyl, toluene, or benzene as a sole source of carbon and energy in the lid of an inverted petri dish. Cotton was soaked with toluene or benzene and placed into a small glass tube that was sealed with vinyl tape. Growth in liquid culture was carried out with the same medium, with 0.1% biphenyl added directly to the medium. An Erlenmeyer flask with a side arm was used for the growth on toluene. Toluene-soaked cotton was placed into the side arm. Cell growth was monitored by measuring turbidity at 660 nm. *E. coli* strains were grown in L broth (10 g of Bacto Tryptone, 5 g of yeast extract, 5 g of NaCl in 1 liter of distilled water) or on an L-agar plate (1.5% agar). Antibiotics were added at the following concentrations when needed in order to select for the presence of plasmids: streptomycin, 100 μg/ml for *E. coli* strains and 300 μg/ml for *Pseudomonas* strains; ampicillin, 30 μg/ml, or chloramphenicol, 20 μg/ml, for *E. coli* strains; and gentamicin, 10 μg/ml for *E. coli* strains and 20 μg/ml for *Pseudomonas* strains.

DNA amplification and construction of pMLC1C2, pMLC1, and pJHF10. The *todC1C2* genes were amplified by the polymerase chain reaction (Takara Shuzo, Kyoto, Japan). The primer of 5'TCTCTCGAGCTCGAAAAGTG-AGAAG ACAATGA3' including the upstream sequence of the *todC1* gene in which the *Sac*I site (underlined) and start codon of *todC1* (boldface letters) were introduced was synthesized by a model 392 Applied Biosystems, Inc., synthesizer. The reverse primer for the 3' end of the *todC2* gene was synthesized to be 3'TCAAAGAAGAAGATCCACAAATTTCT-C GTG5', in which a *Dra*I site (underlined) and a stop codon (boldface letters) were included. For the amplification of *todC1*, the same primer for the *todC1* upstream sequence was used and the reverse primer for the 3' end of *todC1* was synthesized as 3'-CTTCCGCTGTGCGACTTAGTCTAGAA CGAA-5', in which the *Bgl*II site (underlined) and a stop codon (boldface letters) were included. The reaction was performed with a total volume of 50 μl which contained polymerase chain reaction buffer (Takara Shuzo), 50 ng of plasmid pDTG351 as template DNA, 100 μM (each) deoxynucleoside triphosphate, 1 μM (each) oligoprimer, and 0.5 U of *Taq* DNA polymerase. Amplification of DNA was carried out for 20 cycles under the following conditions: denaturation, 95°C for 30 s; primer annealing, 55°C for 30 s; and primer extension, 72°C for 1 min. The amplified DNA (ca. 2 kb) including *todC1C2* was purified by SUPREC-02 (Takara Shuzo). The purified DNA was double digested with *Sac*I and *Dra*I and ligated into a plasmid vector, pBluescript II KS+ (Stratagene, La Jolla, Calif.), at the *Sac*I and *Sma*I sites (pBSC1C2). pBSC1C2 was then cut with *Sac*I and *Kpn*I. The *Sac*I-*Kpn*I fragment, including *todC1C2*, was inserted into pHSG396 (Takara Shuzo) at the same restriction sites to get pHSGC1C2 (4.2 kb), from which the *Sac*I-*Xho*I fragment (*todC1C2*) was cut out and ligated into a broad-host-range plasmid vector, pML122 (27), at the same restriction site to get pMLC1C2. pMLC1 was constructed from pMLC1C2 by removing the 0.5-kb *Hin*dIII fragment which includes the *todC2* gene.

pJHF10 containing the hybrid *todC1bphA2A3A4BC* gene cluster was constructed as follows. pKTF18 is a recombinant plasmid in which the *bphA1A2A3A4BC* (KF707) gene cluster is inserted into pUC118 (33). Since the unique *Bgl*II site is present in the flanking region between *bphA1* and *bphA2*, *bphA1* was removed by being cut out with *Sac*I, the unique *Sac*I site right after the ATG codon of *bphA1*, and *Bgl*II. The amplified DNA (1.4-kb *todC1* DNA) was double digested with *Sac*I and *Bgl*II. The *Sac*I-*Bgl*II fragment containing *todC1* was then ligated with *Sac*I–*Bgl*II-digested pKTF18 to get pJHF10, in which *bphA1* is replaced with *todC1*, forming a hybrid gene cluster of *todC1bphA2A 3A4BC*.

Enzyme assay. Cells of *P. pseudoalcaligenes* KF707, *P. putida* KF715, *P. putida* F1, and their mutant strains were grown on biphenyl, succinate, or benzoate as the sole carbon source in basal salts agar medium. The cells were scraped off the agar with 50 mM phosphate buffer (pH 7.5) and washed once. The washed cells were suspended in a small amount of the same buffer containing 10% ethanol and were disrupted by sonication (Tomy UD-201). The supernatant, after being centrifuged at 88,000 × *g*, was used as the crude extract. HPDA was prepared from 2,3OHBP by the resting cells of *Pseudomonas aeruginosa* PAO1161 carrying pMFB5, which contains *bphC* (KF707) (15). After complete conversion of 2,3OHBP to HPDA, the reaction mixture was centrifuged to remove the cells and the yellow supernatant was used as the substrate for the assay of HPDA hydrolase. The molar extinction coefficient of 22,000 (at 434 nm) of HPDA was employed (11). The activity of HPDA hydrolase was assayed by measuring the decrease in A_{434}. 2-Hydroxy-6-oxo-hepta-2,4-dienoic acid (HOHD) was prepared from 3-methylcatechol (Aldrich Chemical Company, Inc., Milwaukee, Wis.) by using *E. coli* JM109 cells carrying pUC351, which contains *todC1C2BADE* (36). The molar extinction coefficient of 13,800 (at 388 nm) of HOHD was employed (8). The activity of HOHD hydrolase was assayed by measuring the decrease in A_{388}. One unit of enzyme activity was defined as the amount that catalyzed 1 μmol of the product per min at 30°C.

Conjugal transfer of recombinant plasmids. The recombinant plasmids (Table 1) were first introduced into *E. coli* S17-1 by transformation (31). The S17-1 cells carrying various *bph* genes or *tod* genes were then filter mated with *P. pseudoalcaligenes* KF707 and the transposon mutants, with *P. putida* KF715 and the *bph* deletion mutants, and with *P. putida* F1 and the *todD* mutant listed in Table 1. The

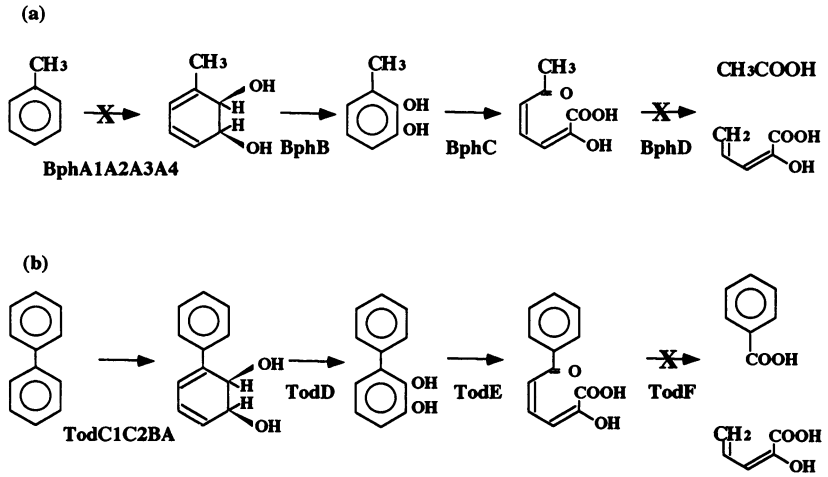

FIG. 3. Metabolism of toluene by biphenyl degrader *P. pseudoalcaligenes* KF707 (a) and that of biphenyl by toluene degrader *P. putida* F1 (b). X, inability of the enzyme to carry out conversion.

Pseudomonas transconjugants were screened on basal salts agar medium supplemented with succinate as a sole carbon source and the appropriate antibiotic: streptomycin at a concentration of 300 μg/ml for the transconjugants carrying the pMFB series plasmids, pNHF715, and pDTG351 or gentamicin at 20 μg/ml for the transconjugants carrying pMLC1C2 and pMLC1.

RESULTS

Metabolism of toluene by biphenyl degrader *P. pseudoalcaligenes* KF707 and that of biphenyl by toluene degrader *P. putida* F1. The biphenyl-utilizing strains KF707 and KF715 did not grow at the expense of toluene as a sole source of carbon and energy. In order to investigate the inability of KF707 to utilize toluene, *E. coli* JM109 cells carrying pKTF18 containing *bphA1A2A3A4BC* (KF707) were incubated with toluene, resulting in no conversion of toluene (Fig. 3). The same cells, however, converted toluene-*cis*-dihydrodiol into the ring *meta*-cleavage HOHD. These results indicate that BphA (biphenyl dioxygenase) does not convert toluene, but BphB (biphenyl dihydrodiol dehydrogenase) converts toluene-*cis*-dihydrodiol to 3-methylcatechol and BphC (23OHBP dioxygenase) cleaves 3-methylcatechol to HOHD. HPDA hydrolase produced from *E. coli* JM109 cells carrying pYF860 containing *bphD* (KF707) failed to hydrolyze HOHD (Fig. 3).

On the other hand, toluene-utilizing *P. putida* F1 did not grow on biphenyl, but toluene-grown F1 cells did convert biphenyl into the *meta*-cleavage HPDA. It was also observed that *E. coli* JM109 cells carrying pUC351 containing *todC1C2BADE* converted biphenyl to HPDA. These results indicate that toluene dioxygenase (TodC1C2BA), toluene dihydrodiol dehydrogenase (TodD), and 3-methylcatechol dioxygenase (TodE) are all active for the conversion of biphenyl to HPDA. However, HOHD hydrolase (TodF) did not act on HPDA (Fig. 3).

Growth characteristics of biphenyl utilizer *P. pseudoalcaligenes* KF707 and the *bph* transposon mutants, which carry recombinant plasmids containing various *tod* genes. In order to elucidate the critical components in the *bph*-encoded enzymes for toluene metabolism, a variety of *tod* genes were introduced into *P. pseudoalcaligenes* KF707 (wild type) and the transposon mutants KF733 (*bphA*::Tn*5*-B21), KF748 (*bphB*::Tn*5*-B21), and KF744 (*bphC*::Tn*5*-B21). When pDTG351 (*todC1C2BADE* coding for the conversion of toluene into the *meta*-cleavage HOHD) was introduced, all transconjugants gained a novel capability of growth on toluene (Table 2 and Fig. 4). Although BphD hydrolase did not act on HOHD, BphH hydrolase encoded by the putative *bphH* gene in the benzoate *meta*-cleavage pathway operon (Fig. 1) hydrolyzed HOHD, resulting in the growth of these transconjugants on toluene. More importantly, the introduction of pMLC1C2 containing *todC1C2* into KF707 (wild type) and KF744 (*bphC*::Tn*5*-B21) allowed the recombinant strains to grow on toluene (Table 2). These results indicate that TodC1C2 (the large and small subunits of toluene terminal dioxygenase) forms a multicomponent dioxygenase associated with two other components of ferredoxin (BphA3) and ferredoxin reductase (BphA4) and that this hybrid enzyme composed of TodC1C2BphA3A4 is functional for initial dioxygenation of toluene. Furthermore, the introduction of pMLC1 containing only *todC1* into KF707 (wild type) and KF744 resulted in the weak growth on toluene. However, the introduction of pMLC1C2 (*todC1C2*) or pMLC1 (*todC1*) into KF733 (*bphA1*::Tn*5*-B21) or KF748 (*bphB*::Tn*5*-B21) did not support the growth of these recombinant strains on toluene. This inability to grow could be due to the lack of more than one essential enzyme component for the initial toluene metabolism, since KF733 failed to produce BphA1, BphA2, BphA3, BphA4, BphB, and BphC and KF748 failed to produce BphB and BphC because of the polar effect of transposon insertion. All of the toluene-growing cells carrying the *tod* genes grew on benzene as well.

The recombinant strains KF733, KF748, and KF744 carrying pDTG351 grew not only on toluene-benzene, but on biphenyl also (Table 2), since pDTG351 containing *todC1C2BADE* confers the ability to convert biphenyl into the *meta*-cleavage HPDA. The activities of BphD (HPDA hydrolase) and BphH (HOHD hydrolase) in the parent KF707 and its transposon mutants are presented in Table 3. The HPDA hydrolase seems to be expressed constitutively even in the transposon mutants. An unknown promoter may be located somewhere upstream of the *bphD* gene, possibly in the *bphX* region. Constitutive expression of *bphD* allows

TABLE 2. Growth characteristics of recombinant strains carrying *bph* and *tod* genes

Plasmid	Growth[a]																	
	P. pseudoalcaligenes								*P. putida*									
	KF707		KF733		KF748		KF744		KF715		KF796		KF791		F1		F39/D	
	BP	Tol	BP	Tol	BP	Tol	BP	Tol	BP	Tol	BP	Tol	BP	Tol	BP	Tol	BP	Tol
None	+++	−	−	−	−	−	−	−	+++	−	−	−	−	−	−	+++	−	−
pDTG351 (*todC1C2BADE*)	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	−	++	−	−				
pMLC1C2 (*todC1C2*)	+++	++	−	−	−	−	−	+	+++	+	−	−	−	−				
pMLC1 (*todC1*)	+++	+	−	−	−	−	−	+	+++	+	−	−	−	−				
pMFB2 (*bphABC*)															−	+++	−	+++
pMFB6 (*bphAB*)															−	+++	−	+++
pMFB4 (*bphA*)															−	+++	−	−
pMFB8 (*bphD*)															+++	+++	−	−
pNHF715 (*bphABCD*)															+++	+++	+++	+++

[a] Growth was checked after 4 days of incubation at 30°C. Symbols: +++, good growth; ++, moderate growth; +, poor growth; −, no growth. All Tol$^+$ strains grew on benzene as well. Boldface items indicate the novel acquisition of growth capability by the strain by the introduction of hybrid plasmids. BP, biphenyl; Tol, toluene.

these transposon mutants carrying pDTG351 to grow on biphenyl. Although KF744 (*bphC*::Tn*5*-B21) carrying *todC1C2* grew on toluene-benzene, the same recombinant strain failed to grow on biphenyl but accumulated 23OHBP, indicating that catechol dioxygenase (putative BphG) in the benzoate *meta*-cleavage pathway is not able to act on 23OHBP.

Growth characteristics of biphenyl utilizer *P. putida* KF715 and the *bph* deletion mutants which carry the recombinant plasmids containing various *tod* genes. Another biphenyl utilizer, *P. putida* KF715, carries the *bphABCD* operon, which is similar to the *bphABCXD* operon of *P. pseudoalcaligenes* KF707 except that the *bphX* region (ca. 3.3 kb) is missing between *bphC* and *bphD*. The parent strain, KF715, also gained the capability to grow on toluene, as with KF707 when pDTG351 (*todC1C2BADE*) or pMLC1C2 (*todC1C2*) was introduced. KF715 carrying pMLC1 (*todC1*) also showed weak growth on toluene. The mutant strain, KF796, in which the upper *bphABCD* operon is spontaneously deleted from the genome gained the ability to grow on toluene but not on biphenyl when pDTG351 was introduced (Table 2 and Fig. 4). Since KF796 still retains the benzoate *meta*-cleavage pathway, toluene *meta*-cleavage HOHD can be metabolized further, but BphH cannot convert biphenyl *meta*-cleavage HPDA. This might be the reason why KF796(pDTG351) grew on toluene but failed to grow on biphenyl. On the other hand, the mutant KF791, in which both upper *bphABCD* and lower benzoate *meta*-cleavage pathway genes (putative *bphEFGH* [Fig. 1]) are spontaneously deleted, failed to grow on either biphenyl or toluene (Table 2 and Fig. 4). Because KF791(pDT351) lacks both HPDA hydrolase and HOHD hydrolase, the yellow *meta*-cleavage HPDA or HOHD accumulated from biphenyl or toluene, respectively.

Growth characteristics of toluene utilizer *P. putida* F1 and the *todD* mutant, which carry recombinant plasmids containing various *bph* gene components. The toluene utilizer *P. putida* F1 converted biphenyl into the yellow *meta*-cleavage HPDA, so that the introduction of pMFB8 containing the *bphD* (KF707 HPDA hydrolase gene) permitted growth on biphenyl (Fig. 4). The *todD* mutant F39/D gained the ability to grow on toluene but not on biphenyl when pMFB6 containing the *bphAB* gene cluster was introduced (Table 2). These results indicate that *bphB* (biphenyl dihydrodiol de-

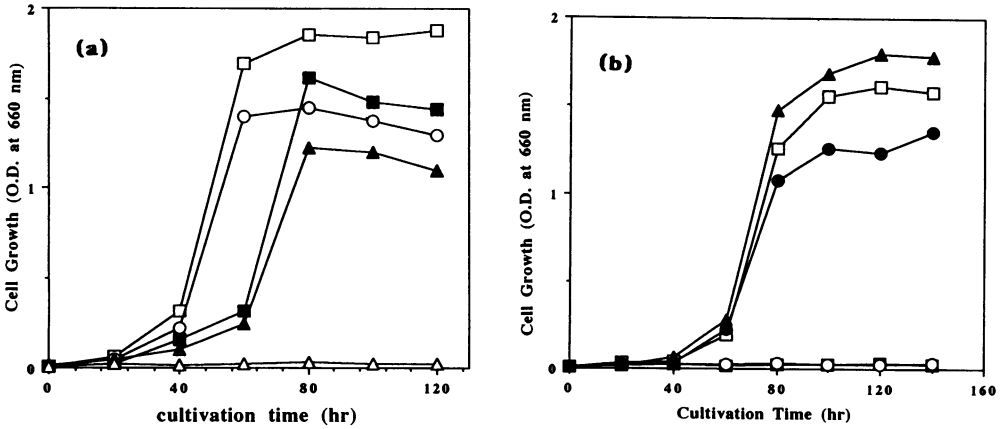

FIG. 4. (a) Growth on toluene of *P. pseudoalcaligenes* KF707 (□), its transposon mutants KF744 (■) and *P. putida* KF715 (○), and its *bph* deletion mutants KF796 (▲) and KF791 (△), which all carry pDTG351 (*todC1C2BADE*). (b) Growth on biphenyl of *P. putida* F1 and its *todD* mutant F39/D, which carry pMFB2 (*bphABC*), pMFB8 (*bphD*), or pNHF715 (*bphABCD*). ■, F1(pMFB2); ▲, F1(pMFB8); □, F1(pNHF715); ○, F39/D(pMFB2); ●, F39/D(pNHF715).

TABLE 3. HPDA and HOHD hydrolase activities in *P. pseudoalcaligenes* KF707, *P. putida* KF715, *P. putida* F1, and their mutant and recombinant strains[a]

Strain	Activity on the following substrate:							
	BP		Suc		BA		Tol	
	HPDAH	HOHDH	HPDAH	HOHDH	HPDAH	HOHDH	HPDAH	HOHDH
KF707	803	529	549	116	489	386		
KF733			165	135	294	420		
KF748			244	140	259	217		
KF744			302	188	356	324		
KF715	327	532	92	389	83	297		
KF796			<10	181	<10	208		
KF791			<10	<10	<10	<10		
F1			<10	177			<10	1,490
F1(pMFB8)			550	130			387	867

[a] Enzyme activities were measured as microunits per microgram of protein. HPDAH, HPDA hydrolase; HOHDH, HOHD hydrolase; BP, biphenyl; Suc, succinate; BA, benzoic acid; Tol, toluene. KF733, KF748, and KF744 are the transposon mutants of KF707. KF796 and KF791 are *bph* deletion mutants (Table 1).

hydrogenase gene) is complementary with *todD* (toluene dihydrodiol dehydrogenase gene). The failure to grow on biphenyl is simply due to the lack of HPDA hydrolase in this recombinant strain. In this context, the introduction of pNHF715 containing the *bphABCD* (KF715) gene cluster supported the growth of F39/D on biphenyl (Fig. 4).

Enzyme system encoded by a hybrid gene cluster of *todC1bphA2A3A4BC*. *E. coli* JM109 cells carrying pKTF18 (33) containing the *bphA1A2A3A4BC* gene cluster (KF707) converted biphenyl quickly to the *meta*-cleavage HPDA, but the same cells did not convert toluene to HOHD (Table 4). On the other hand, it was found that *E. coli* JM109 carrying pJHF10 containing the hybrid gene cluster of *todC1 bphA2A3A4BC* (constructed by replacing *bphA1* with *todC1*) gained the novel capability to convert toluene to the *meta*-cleavage HOHD. The conversion rate of toluene to HOHD by JM109(pJHF10) was ca. 40% of that by JM109(pUC351) (with pUC351 containing *todC1C2BADE*) (Table 4). JM109 (pJHF10) still retained the ability to convert biphenyl to the *meta*-cleavage HPDA, but it did so more slowly than JM109(pKTF18) (with pKTF18 containing *bphA1A2A3 A4BC*) did (Table 4). The hybrid multicomponent dioxygenase composed of TodC1BphA2A3A4 thus became active for toluene but lost some activity for biphenyl compared with the original biphenyl dioxygenase.

DISCUSSION

Despite the discrete substrate specificities of the biphenyl-PCB degrader *P. pseudoalcaligenes* KF707 and the toluene-benzene degrader *P. putida* F1, the *bph* and the *tod* operons are very similar not only in gene organization but also in size and sequence of the deduced amino acids, particularly in the regions coding for the initial oxidation steps (Fig. 2). Identities in the amino acid sequences are as follows: large subunit of the terminal dioxygenases (BphA1 and TodC1), 65%; small subunit of the terminal dioxygenases (BphA2 and TodC2), 60%; ferredoxins (BphA3 and TodB), 60%; ferredoxin reductases (BphA4 and TodA), 53%; dihydrodiol dehydrogenases (BphB and TodD), 60%; and ring *meta*-cleavage dioxygenases (BphC and TodE), 55% (33). However, some significant discrepancies are also noticeable (Fig. 2). Open reading frame 3 in the *bph* operon (KF707) is missing in the counterpart of the *tod* operon. The function of open reading frame 3 has not been elucidated yet, but site-specific deletion of open reading frame 3 from the *bphA* region allowed the region to still retain the ability of biphenyl oxidation in *E. coli* (33). Further discrepancies can be seen with the hydrolases. *bphD* is located downstream of *bphX* (26), but *todF* is located just upstream of *todC1* (29). It is thus of significant value to know which components are critical in the metabolism of biphenyl and toluene and which are interchangeable. In the present study, we have identified the components responsible for the substrate specificity of biphenyl and toluene metabolism. TodC1 was critical for the initial oxidation of toluene. The introduction of *todC1C2* into the biphenyl-PCB degraders *P. pseudoalcaligenes* KF707 and *P. putida* KF715 resulted in the growth of these recombinant strains on toluene-benzene. However, the introduction of only *todC1* led these biphenyl degraders to poor growth on the same substrates. Such a difference in growth between a *todC1C2* carrier and a *todC1* carrier might be due to the fact that the affinity between BphA1 and BphA2 is stronger than that between TodC1 and BphA2. However, the enzyme system encoded by a hybrid gene cluster of *todC1bphA2A3A4BC* in *E. coli* clearly demonstrated that TodC1BphA2A3A4 formed a functionally active hybrid dioxygenase in the initial oxidation of toluene to dihydrodiol. Toluene dihydrodiol could then be converted to 3-methylcatechol by BphB (biphenyl dihydrodiol dehydrogenase). Further conversion of 3-methylcatechol to the *meta*-cleavage HOHD could be conducted by BphG (catechol 2,3-dioxygenase in benzoate *meta*-cleavage pathway [Fig. 1]). BphD hydrolases from KF707 and KF715 did not hydrolyze HOHD, but BphH (hydrolase encoded by a putative *bphH* in Fig. 1) hydrolyzed HOHD. Thus, the introduction of

TABLE 4. Conversion of biphenyl and toluene by *E. coli* JM109 cells carrying pJHF10 compared with that by JM109 carrying pKTF18 or pUC351

Plasmid carried by *E. coli* JM109[a]	Formation[b] of:	
	HPDA from 0.5 mM biphenyl	HOHD from 0.5 mM toluene
pKTF18 (*bphA1A2A3A4BC*)	1.52	<0.01
pUC351 (*todC1C2BADE*)	0.41	1.72
pJHF10 (*todC1bphA2A3A4BC*)	0.52	0.70

[a] The resting cells of *E. coli* JM109 carrying their respective recombinant plasmids were adjusted to an optical density of 0.5 at 660 nm in 50 mM phosphate buffer (pH 7.5). Parentheses enclose hybrid gene clusters in plasmids.

[b] After the cells were removed, the formation of HPDA or HOHD was determined as A_{434} per hour or A_{388} per hour, respectively.

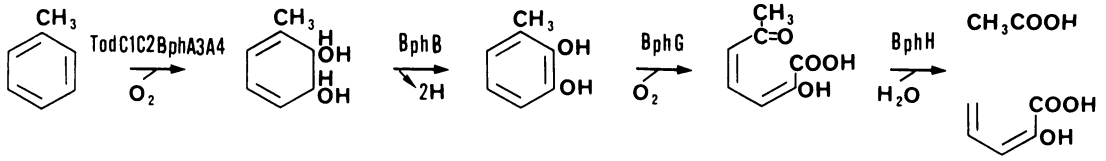

FIG. 5. Hybrid pathway for toluene metabolism in *P. pseudoalcaligenes* KF707, which carries pMLC1C2 (*todC1C2*).

todC1C2 or even only *todC1* into the biphenyl-PCB degraders KF707 and KF715 resulted in the growth of these recombinant strains on toluene-benzene by the combined catabolic pathways encoded by the upper *bph* genes and the lower benzoate *meta*-cleavage pathway genes (Fig. 5). It was previously shown that toluene dioxygenase (TodC1C2BA) possesses very relaxed substrate specificity to oxidize a variety of aromatic compounds which include biphenyls (22). The dihydrodiol dehydrogenases of TodD and BphB were exchangeable with each other (Table 2). The inability of *P. putida* F1 to grow on biphenyl is due to the lack of TodF (HOHD hydrolase) activity for biphenyl *meta*-cleavage HPDA (Table 3). The amino acid sequence homology between TodF and BphD (HPDA hydrolase of *P. putida* KF715) was 35.1% (29). This value is considerably lower than that of 53 to 65% between the other corresponding Bph and Tod components. Moreover, BphD is a tetramer composed of an identical subunit, but TodF is a homodimer (29). These differences may reflect the discrete substrate specificities of the two hydrolases.

The chromosomal *bph* genes in various natural isolates show a variety of genetic diversities (12). Some biphenyl strains possess a *bphABCXD* operon almost identical to that of *P. pseudoalcaligenes* KF707, and some strains possess *bph* genes with different degrees of homology. Notwithstanding the apparent enzymatic similarities of 2,3-dihydroxybiphenyl dioxygenase (the product of *bphC*) of *P. pseudoalcaligenes* KF707 and *P. paucimobilis* Q1 (11, 32), the homology between BphC (KF707) and BphC (Q1) is much lower (38%) than the corresponding value of 55% between BphC (KF707) and TodE (33, 35). BphC (KF707) possesses only weak activity for catechol, but BphC (Q1) shows significant activity for the same compound (32). It is postulated that many degraders of aromatics could be involved in the final degradation of plant lignin, which is massively distributed in the environment and which consists of many polymerized aromatic moieties (17). This idea coincides with the fact that a number of catabolic genes involved in the degradation of aromatic compounds share a common ancestry and form gene superfamilies (2, 23, 28). The genetic diversity or shuffling of catabolic operons among soil bacteria is of particular interest from the viewpoint of how microorganisms gain the novel catabolic activities for xenobiotics, which include many chemicals of man-made origin.

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