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Engineering Hybrid Pseudomonads Capable of Utilizing a Wide Range of Aromatic Hydrocarbons and of Efficient Degradation of Trichloroethylene

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We constructed hybrid *Pseudomonas* strains in which the *bphA1* gene (coding for a large subunit of biphenyl dioxygenase) is replaced with the *todC1* gene (coding for a large subunit of toluene dioxygenase of *Pseudomonas putida* F1) within chromosomal biphenyl-catabolic *bph* gene clusters. Such hybrid strains gained the novel capability to grow on a wide range of aromatic hydrocarbons, and, more interestingly, they degraded chloroethenes such as trichloroethylene and *cis*-1,2-dichloroethylene very efficiently.

Biphenyl-utilizing bacteria, which include both gram-negative and gram-positive strains, have been isolated from various environmental samples (2–5, 7, 19, 30). The major catabolic pathway of biphenyl has been well documented in terms of degradation of polychlorinated biphenyls (Fig. 1) (8, 10, 13). Biphenyl dioxygenase in *Pseudomonas pseudoalcaligenes* KF707 is a multicomponent enzyme encoded by four genes, *bphA1A2A3A4*, where *bphA1* encodes a large subunit (BphA1) of terminal dioxygenase (an iron-sulfur protein), *bphA2* encodes a small subunit (BphA2) of terminal dioxygenase, *bphA3* encodes ferredoxin (BphA3), and *bphA4* encodes ferredoxin reductase (BphA4) (35). BphA1 and BphA2 are associated as a heterotetramer (referred as terminal dioxygenase) and catalyze direct introduction of two atoms of oxygen into the biphenyl ring. Ferredoxin and ferredoxin reductase act as an electron transfer system from NADH to reduce the terminal dioxygenase. Some *bph* genes isolated from gram-negative and gram-positive strains show significant similarities to those of KF707, but some are diversified and shuffled within the *bph* gene clusters (1, 6, 9, 18, 22, 24, 27, 35).

Toluene-utilizing bacteria have also been isolated widely from soil. Toluene can be metabolized by such bacteria via several routes. Among them, *Pseudomonas putida* F1 converts toluene via 2,3-dioxygenation (Fig. 1) (16, 17, 35, 44), in a fashion similar to that of biphenyl dioxygenase of *P. pseudoalcaligenes* KF707. The toluene catabolic operon (designated the *tod* operon) is similar to the KF707 *bph* gene cluster in terms of gene organization and the nucleotide sequences of the corresponding genes (Fig. 1) (35). The gene cluster *todC1C2BA* encodes a multicomponent toluene dioxygenase (44). As in the case of biphenyl dioxygenase, *todC1* encodes a large subunit (TodC1) of terminal dioxygenase (an iron-sulfur protein), *todC2* encodes a small subunit (TodC2) of terminal dioxygenase, *todB* encodes ferredoxin (TodB), and *todA* encodes ferredoxin reductase (TodA). The identities of amino acid sequences between corresponding subunits of biphenyl dioxygenase and toluene dioxygenase are as follows: BphA1 and TodC1, 65%; BphA2 and TodC2, 60%; BphA3 and TodB, 60%; BphA4 and TodA, 53%. Other corresponding Bph and Tod enzymes show high levels of similarity with each other (35, 41). Despite the similarities, biphenyl-utilizing KF707 does not grow on toluene, and toluene-utilizing F1 does not grow on biphenyl. Our previous studies revealed that the inability of KF707 to grow on toluene was due to a lack of initial oxidation activity of biphenyl dioxygenase toward toluene (12). In this context, the introduction of *todC1C2* or even *todC1* alone into KF707, in *trans*, permitted growth on toluene. On the other hand, the inability of F1 to grow on biphenyl was due to a lack of hydrolyzing activity of TodF (2-hydroxy-6-oxohepta-2,4-dienoic acid hydrolase, encoded by *todF*) toward the biphenyl *meta*-cleavage compound (2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid). The introduction of *bphD* (encoding the biphenyl *meta*-cleavage compound hydrolase) into F1, in *trans*, allowed it to grow on biphenyl (12). These results indicate that although *bph* and *tod* genes have a common ancestry, these genes have evolved adaptively over a long historical period and that the large subunit of terminal dioxygenase (encoded by *bphA1* and *todC1*) and the ring *meta*-cleavage compound hydrolase (encoded by *bphD* and *todF*) are critically involved in their discrete metabolic specificities.

Since it was found that biphenyl dioxygenase and toluene dioxygenase show a high degree of similarity (approximately 60%), we constructed a hybrid *bph-tod* gene cluster, by replacing the genes of large and/or small subunits of terminal dioxygenases (20). *Escherichia coli* cells expressing the hybrid dioxygenases composed of TodC1-BphA2A3A4, TodC1C2-BphA3A4, and BphA1-TodC2-BphA3A4 showed the capability of converting various aromatic hydrocarbons, including benzene, toluene, biphenyl, diphenylmethane, and naphthalene. These results indicate that the terminal dioxygenases TodC1-BphA2 and BphA1-TodC2 form functionally active dioxygenases associated with BphA3 (ferredoxin) and BphA4 (ferredoxin reductase). Interestingly, *E. coli* cells expressing the hybrid dioxygenase TodC1-BphA2A3A4 degraded trichloroethylene (TCE), which is among the most significant of environmental pollutants (11). The most promising approach for the polluted environment is bioremediation, which can be accomplished by exploring and engineering the catabolic capability of microorganisms. In this context, it is of great interest to construct hybrid *Pseudomonas* strains which have enhanced and expanded degradation capabilities for these environmental

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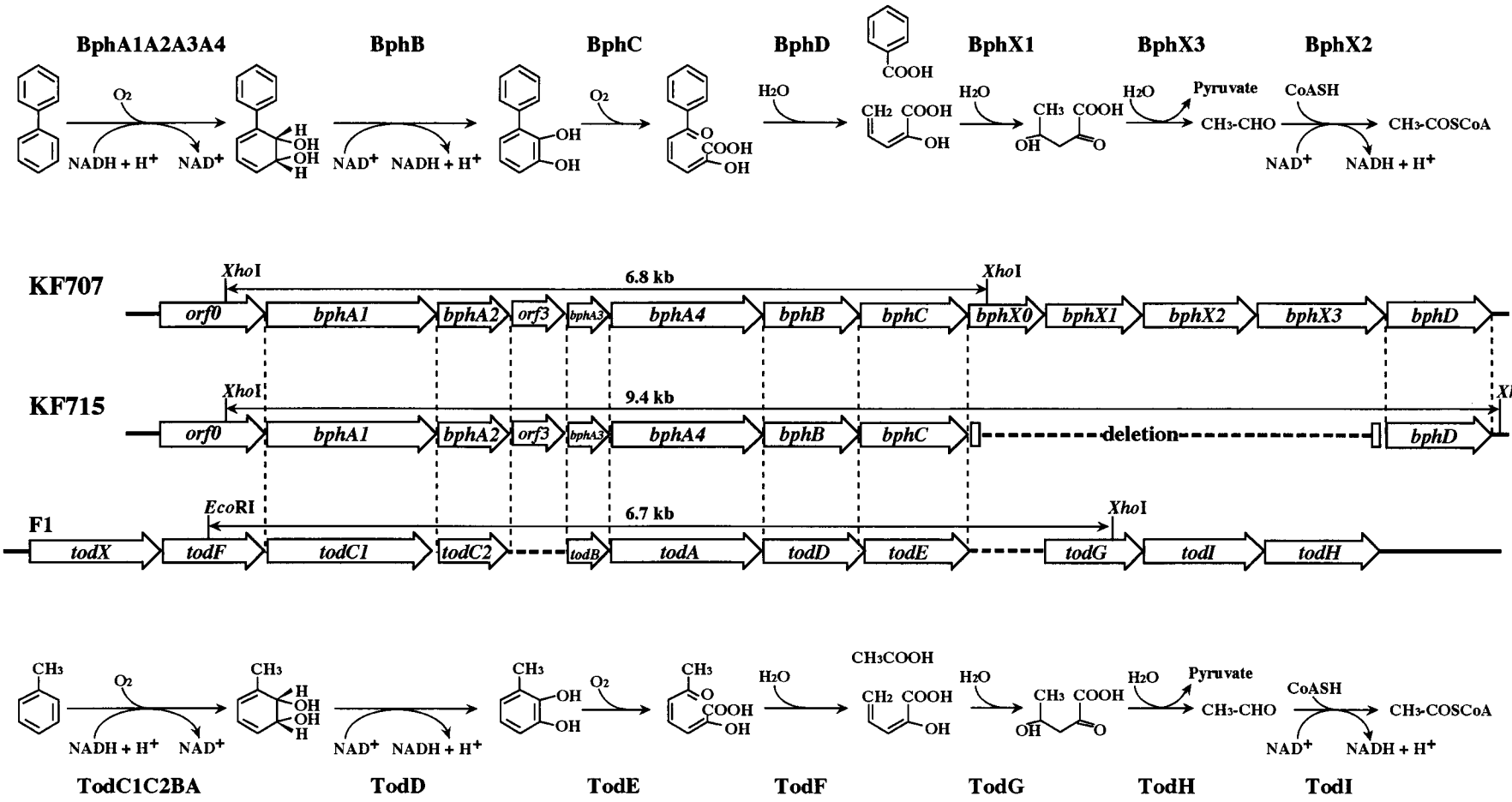

FIG. 1. Organization of *bph* genes in *P. pseudoalcaligenes* KF707 and *P. putida* KF715 and *tod* genes in *P. putida* F1. Genes and their products are as follows: *bphA1* and *todC1*, the large subunit of terminal dioxygenase; *bphA2* and *todC2*, the small subunit of terminal dioxygenase; *bphA3* and *todB*, ferredoxin; *bphA4* and *todA*, ferredoxin reductase; *bphB* and *todD*, dehydrogenase; *bphC* and *todE*, ring *meta*-cleavage dioxygenase; *bphX0*, glutathione *S*-transferase; *bphX1* and *todG*, 2-hydroxypenta-2,4-dienoate hydratase; *bphX2* and *todI*, acetaldehyde dehydrogenase; *bphX3* and *todH*, 4-hydroxy-2-oxovalerate aldolase; *bphD* and *todF*, ring *meta*-cleavage compound hydrolase; *todX*, membrane protein (18, 21, 25, 35, 41, 44). The functions of *orf0* and *orf3* are unknown. In *P. putida* KF715, parts of *bphX0* and *bphX3* remain, but the 3.3-kb DNA is deleted (21). Genes corresponding to *orf0* and *orf3* are absent in F1.

TABLE 1. Bacterial strains and plasmids used

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*	32
P. pseudoalcaligenes		
KF707	BP^+ Tol^-; wt	13, 34
KF707-S1	BP^+ Tol^+; single-crossover recombinant; Gm^r Cm^r	This study
KF707-D2	BP^+ Tol^+; double-crossover recombinant; Gm^s Cm^s	This study
P. putida		
KF715	BP^+ Tol^-; wt	18
KF715-S3	BP^+ Tol^+; single-crossover recombinant; Gm^r Cm^r	This study
KF715-D5	BP^+ Tol^+; double-crossover recombinant; Gm^s Cm^s	This study
F1	Tol^+ BP^-; wt	16, 17
Plasmids		
pSUPB30	Gm^r Cm^r; 14.0 kb	33
pJHF101	pUC118-*todC1*(F1)-*bphA2A3A4*(KF707); Ap^r; 8.6 kb	20
pASF101	pSUPB30-*todC1*(F1)-*bphA2A3A4*(KF707); Gm^r Cm^r; 15.3 kb	This study

[a] BP, biphenyl; Tol, toluene; wt, wild type; Gm, gentamicin; Cm, chloramphenicol; Ap, ampicillin; r, resistance; s, sensitive; +, growth; −, no growth.

pollutants. Here we report that hybrid *Pseudomonas* strains in which *bphA1* is replaced by *todC1* within the chromosomal *bph* operons have been successfully constructed and that the resultant strains show novel capabilities to grow on a wide range of aromatic hydrocarbons and degrade TCE and related compounds very efficiently.

MATERIALS AND METHODS

Strains and plasmids. The strains and plasmids used in this study are listed in Table 1. Biphenyl-utilizing *P. pseudoalcaligenes* KF707 (13, 35) and *P. putida* KF715 (18) were described previously (1, 2). Briefly, KF715 possesses a *bph* operon very similar to that of KF707, except that the *bphX* region, conferring the ability to convert 2-hydroxy-penta-2,4-dienoate to acetyl coenzyme A (21, 25), is deleted. The similarities of *bphC* and *bphD* between KF707 and KF715 were 92.4 and 96.0%, respectively (18). Although *bphA* regions of KF715 have not yet been completely sequenced, the nucleotide sequences of *orf0* and *bphA1A2 orf3A3A4* seem to be very similar, on the basis of restriction analyses (18). In KF707, *orf0* (738 bp) is located upstream of *bphA1*, and *orf3* (330 bp) is located between *bphA2* and *bphA3*, but their functions are unknown (35). Toluene-utilizing *P. putida* F1 was described previously (16, 17, 44) and provided by David T. Gibson (University of Iowa). All hybrid *Pseudomonas* strains listed in Table 1 were constructed by mating with *E. coli* S17-1 (chromosomally integrated RP4-2-Tc::Mu-Km::Tn*7*) (32) as the donor strain which carries pASF101 containing the hybrid *orf0-todC1-bphA2 orf3A3A4* gene cluster. pASF101 was constructed in this study as follows. pJHF101 contains the hybrid gene cluster *orf0-todC1-bphA2 orf3A3A4* in pUC118, as described previously (20). pJHF101 was cut with *Xho*I, and then the *Xho*I fragment containing *orf0-todC1-bphA2 orf3A3A4* was inserted into the *Xho*I-digested suicide vector, pSUPB30 (33), to get pASF101 (15.3 kb). Restriction enzymes *Xho*I and *Eco*RI and T4 DNA ligase were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan).

Media and growth conditions. Biphenyl-utilizing and toluene- and benzene-utilizing strains were grown at 30°C in a basal salts medium (14) supplemented with biphenyl, 4-methylbiphenyl, diphenylmethane, toluene, or benzene as the sole source of carbon and energy in the lid of an inverted petri dish. In the latter two cases, cotton soaked with toluene or benzene was placed into a small glass tube. Growth in liquid culture was carried out with the same medium containing 0.2% biphenyl, toluene as vapor, or 0.5% sodium succinate. An Erlenmeyer flask with a side arm was used for growth on toluene. Toluene-soaked cotton was placed into the side arm. *E. coli* strains were grown in Luria-Bertani (L) medium (31) or on agar medium (1.5% agar). Antibiotics were added when needed in order to select for the presence of plasmid and for the recombinant strains, as follows: gentamicin, 20 μg/ml for *E. coli* strains and 30 μg/ml for *Pseudomonas* strains, and ampicillin, 50 μg/ml for *E. coli* strains.

Mating and selection of recombinant strains. pASF101 containing *orf0-todC1-bphA2 orf3A3A4* was first introduced into *E. coli* S17-1 by transformation, and this strain was used as a donor strain. The S17-1 cells carrying pASF101 were filter mated overnight with biphenyl-utilizing *P. pseudoalcaligenes* KF707 and *P. putida* KF715. Since pASF101 cannot be replicated in *Pseudomonas* strains, single-crossover recombinants, in which *orf0* (upstream of the *todC1* insertion) or *bphA2 orf3A3A4* (downstream of the *todC1* insertion) was recombined, were first screened on basal salts agar medium supplemented with toluene as the sole carbon source and gentamicin. Such single-crossover recombinants were repeatedly subcultured in L broth to get double-crossover recombinants, in which a second internal recombination occurred between homologous *bph* regions on the chromosome, replacing *bphA1* with *todC1* within the *bph* gene cluster (see Fig. 2). The double-crossover recombinants were checked for growth on toluene and the loss of vector-borne gentamicin resistance.

Southern blot analyses. Digoxigenin-11-dUTP was used to label DNA by using the DIG DNA labeling and detection kit according to the instructions of the manufacturer (Boehringer Mannheim GmbH, Mannheim, Germany). Genomic DNAs of *Pseudomonas* strains listed in Table 1 were digested with *Xho*I or with *Xho*I and *Eco*RI and subjected to electrophoresis through 1.0% agarose gels. The digested DNA fragments were transferred onto nylon membranes (Zeta probe; Bio-Rad, Hercules, Calif.) as described elsewhere (34). Hybridization was performed with the digoxigenin-labeled *todC1* probe and the pSUPB30 vector probe.

Quantitative measurements of degradation of TCE and related compounds. Cells were grown in basal salts medium supplemented with 0.2% biphenyl, toluene as vapor, or 0.5% sodium succinate. When the optical density (OD) at 600 nm of the culture reached approximately 1.0 under these conditions, cells were harvested by centrifugation, washed once with fresh basal salts medium and resuspended to get an OD of 2.0 in the same medium supplemented with 20 mM glucose. Ten milliliters of the cell suspension was added to a glass vial (125 ml), which was sealed with a rubber septum and an aluminum crimp seal. TCE (Katayama Chemical Industries, Osaka, Japan) dissolved in *N,N′*-dimethylformamide was added to get 10 μg/ml (77 μM) by assuming that all of the TCE remains in the liquid phase. The vials were incubated at 30°C on a rotary shaker at 200 rpm. The degradation of TCE was monitored by measuring its concentration by the headspace method (11). Samples (100 μl) of the gas phase in each sealed vial were removed every hour and injected into a gas chromatograph (Shimadzu 9A) equipped with a flame ionization detector, fitted with a silicon DC-550 column (GL Sciences Inc., Kyoto, Japan), and operated at 100°C with a nitrogen gas flow of 30 ml/min.

RESULTS AND DISCUSSION

Construction of single- and double-crossover strains. Since we had previously constructed a hybrid gene cluster consisting of *todC1-bphA2 orf3A3A4* with pUC118 (designated pJHF101) (20), and since *E. coli* cells expressing the corresponding hybrid dioxygenase (TodC1-BphA2A3A4) showed the ability to degrade a wide range of aromatic hydrocarbons, we attempted to introduce *todC1* into two biphenyl-utilizing *Pseudomonas* strains. For this purpose a hybrid gene cluster consisted of *orf0-todC1-bphA2 orf3A3A4* was inserted into the suicide vector pSUPB30 as described in Materials and Methods, and the resultant plasmid, pASF101, was introduced into *E. coli* S17-1 (33). S17-1 carrying pASF101 and the parent biphenyl-utilizing strain, *P. pseudoalcaligenes* KF707, or another biphenyl-utiliz-

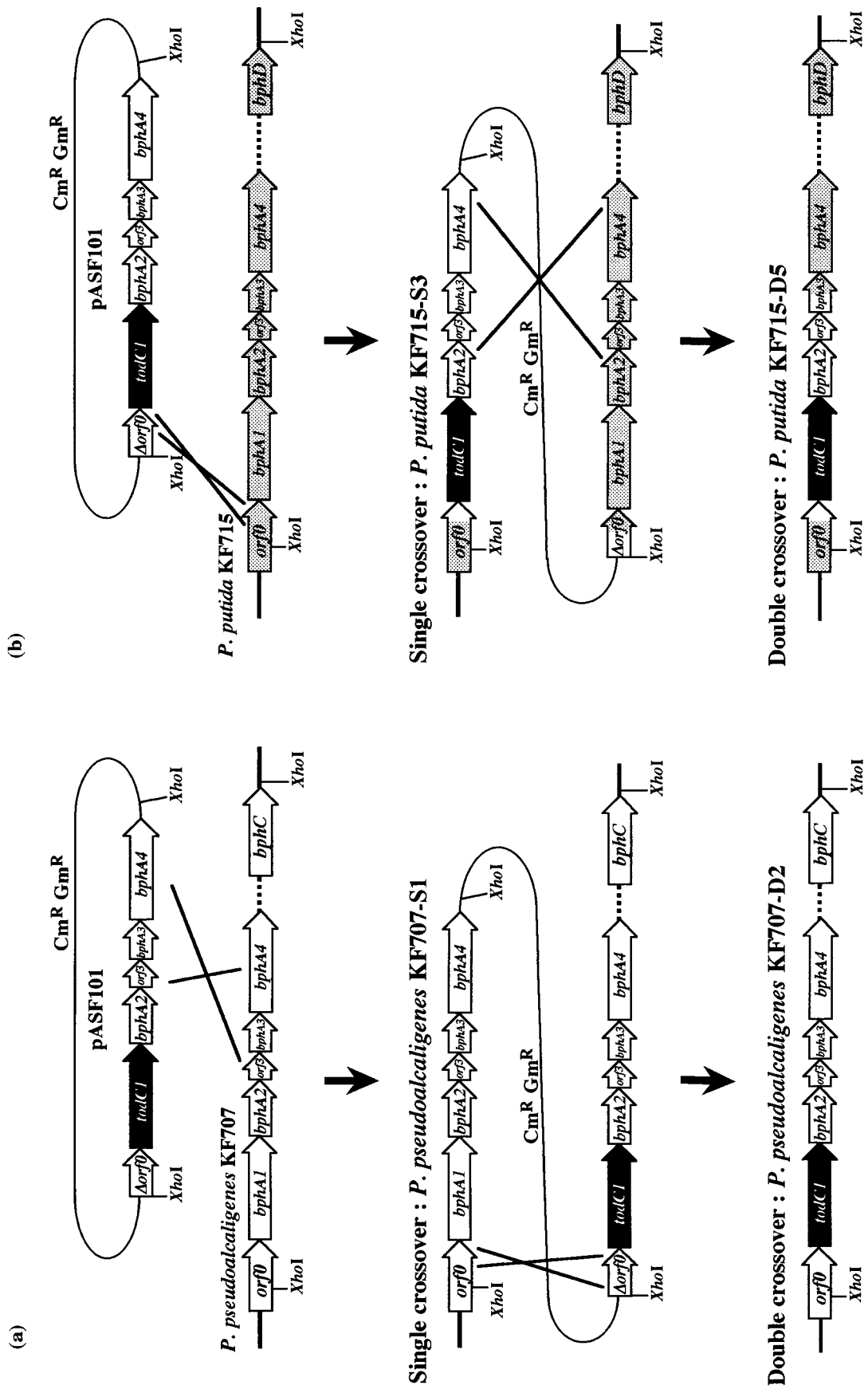

FIG. 2. Schematic representation of single- and double-crossover recombination between pASF101 and chromosomal *bph* gene clusters in *P. pseudoalcaligenes* KF707 (a) and *P. putida* KF715 (b).

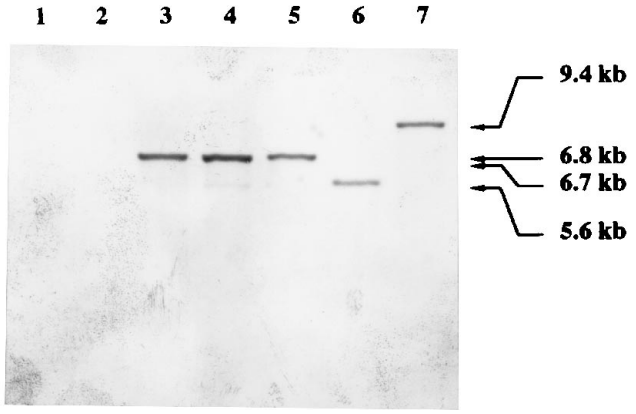

FIG. 3. Southern blot analyses of *todC1* in single- and double-crossover hybrid strains. Chromosomal DNA from each strain was digested by *Xho*I except that F1 chromosomal DNA was double digested by *Xho*I and *Eco*RI. The probe used digoxigenin-labeled *todC1*. Lanes: 1, KF707; 2, KF715; 3, F1; 4, KF707-S1; 5, KF707-D2; 6, KF715-S3; 7, KF715-D5. The arrows indicate the DNA fragments containing *todC1* (see Fig. 1 and 2).

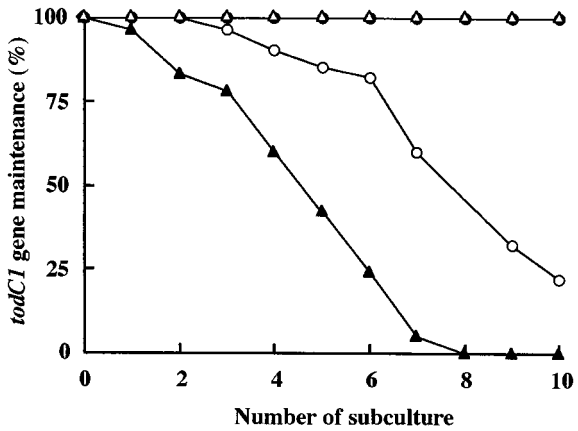

FIG. 4. Stability of chromosomal *bph-tod* hybrid genes in single- and double-crossover strains. ▲, KF707-S1; △, KF707-D2; ○, KF715-S3; ●, KF715-D5. The cells were successively subcultured every 24 h in L broth without any selective pressure. After every subculture, 100 colonies were examined for the presence of vector-borne gentamicin resistance and for growth on toluene.

ing strain, *P. putida* KF715, were filter mated overnight; colonies which could grow on toluene in the presence of gentamicin (20 μg/ml) were then selected. Several types of recombinants could be obtained when *orf0* (upstream of the *todC1* insertion) or *bphA2 orf3A3A4* (downstream of the *todC1* insertion) within pASF101 were recombined with the corresponding chromosomal *bph* regions in KF707 and KF715. Such upstream- or downstream-crossover strains could be distinguished by Southern blot analysis using *todC1* as the probe. In both strains, upstream crossover generated a 5.6-kb *Xho*I fragment containing *todC1*, since *Xho*I sites are present inside *orf0* and downstream of *bphA4* at the vector site of pASF101 (Fig. 2). While downstream-crossover strains generated a 6.8-kb *Xho*I fragment in KF707 (*Xho*I sites at *orf0* and downstream of *bphC*) and a 9.4-kb *Xho*I fragment in KF715 (*Xho*I sites in *orf0* and downstream of *bphD*) (Fig. 1 and 2). In double-crossover strains, a 6.8-kb *Xho*I fragment in KF707 and a 9.4-kb *Xho*I fragment in KF715 could be generated (Fig. 1 and 2). From KF707, downstream single-crossover strains were obtained (Fig. 2) at a frequency of 1.4 per 10^8 recipient cells. One such single-crossover recombinant (KF707-S1) was repeatedly subcultured in L broth to get a double-crossover strain by a second internal recombination between the homologous *bph* regions on the chromosome (Fig. 2a). Most of the second internal recombinations resulted in reversion to the original *bph* operon by losing *todC1*, because the chance of internal recombination between longer *bphA2 orf3A3A4* regions (2.8 kb) is much higher than that between shorter truncated *orf0* regions (0.5 kb). However, a few double-crossover strains which lost both vector-borne gentamicin and chloramphenicol resistances were obtained (Fig. 2a). One such strain, KF707-D2, could grow well on toluene. Southern blot analyses, using *todC1* as the probe, confirmed the presence of 6.8 kb of DNA (*Xho*I digest) for single-crossover recombinant KF707-S1 and 6.8 kb of DNA (*Xho*I digest) for double-crossover recombinant KF707-D2 (Fig. 3). pSUPB30 vector alone was also used as the probe and confirmed the presence of the rector in KF707-S1 and absence in KF707-D2 (data not shown). From *P. putida* KF715, we could get a single-crossover recombinant in which *orf0* on pASF101 was recombined with the corresponding region on the KF715 *bph* gene cluster (KF715-S3) (Fig. 3). Double-crossover strains were easily obtained from KF715-S3 by internal recombination between *bphA2 orf3A3A4* of KF707 and those of original KF715 within the chromosome; in these strains, *todC1* replaced *bphA1*, and certain parts of the KF707 *bphA2 orf3A3A4* region replaced the corresponding *bph* regions of KF715 (KF715-D5) (Fig. 2b). Southern blot analyses, using *todC1* as the probe, confirmed the presence of 5.6 kb of DNA (*Xho*I digest) for KF715-S3 and 9.4 kb of DNA (*Xho*I digest) for KF715-D5 (Fig. 3). Hence, it is worthwhile to note that double-crossover strain KF715-D5 possesses a hybrid gene cluster which consists of DNA segments from three different sources, namely, the *todC1* gene derived from toluene-utilizing *P. putida* F1, some of the *bphA2 orf3A3A4* region of *P. pseudoalcaligenes* KF707, and the remaining original KF715 *bph* genes (Fig. 2b).

Stability of *todC1* in single- and double-crossover strains. The stability of *todC1* within chromosomal *bph-tod* hybrid gene clusters was examined for single- and double-crossover strains (Fig. 4). As described previously, single-crossover strain KF707-S1, in which the *bphA2 orf3A3A4* region downstream from *todC1* was recombined, easily lost the *todC1* gene. On the other hand, single-crossover strain KF715-S3, in which the *orf0* region upstream from *todC1* was recombined, maintained *todC1* rather stably, compared with KF707-S1. Both double-crossover strains KF707-D2 and KF715-D5 maintained *todC1* stably for the repeated cultures in L broth without any selective pressure.

Growth characteristics of single- and double-crossover strains on various aromatic hydrocarbons. As shown in our previous work, the biphenyl-utilizing strains *P. pseudoalcaligenes* KF707 and *P. putida* KF715 could grow well on biphenyl, 4-methylbiphenyl, and diphenylmethane but not on single-ring hydrocarbons such as benzene and toluene (12). In contrast, *P. putida* F1 grew well on benzene and toluene but failed to grow on biphenyl and related compounds, forming yellow ring *meta*-cleavage compounds. On the other hand, both single- and double-crossover strains carrying *todC1* derived from both KF707 and KF715 grew well on benzene and toluene (Table 2). Single-crossover strains KF707-S1 and KF715-S3 grew on biphenyl as fast as the original KF707 and KF715. The double-crossover strains KF707-D2 and KF715-D5 still retained the ability to grow on biphenyl and other biphenyl-related compounds, but the growth was slower than that of the respective parent strains. This may reflect the lower-level activity of the

TABLE 2. Growth characteristics of parent strains and hybrid strains which possess the chromosomal *bph-tod* hybrid operon

Strain	Growth on carbon source[a]				
	BP	4-MeBP	DM	Tol	Ben
Wild type					
P. pseudoalcaligenes KF707	+++	++	+++	−	−
P. putida KF715	+++	++	+++	−	−
P. putida F1	−	−	−	+++	+++
Single crossover					
P. pseudoalcaligenes KF707-S1	+++	++	+++	++	++
P. putida KF715-S3	+++	++	+++	++	+
Double crossover					
P. pseudoalcaligenes KF707-D2	++	+	−	++	++
P. putida KF715-D5	++	+	+	+++	+++

[a] Growth was checked after 4 days of incubation at 30°C. Abbreviations: BP, biphenyl; 4-MeBP, 4-methylbiphenyl; DM, diphenylmethane; Tol, toluene; Ben, benzene. Symbols: +++, good growth; ++, moderate growth; +, poor growth; −, no growth or very poor growth.

TodC1-BphA2A3A4 hybrid dioxygenase toward biphenyl than that of the original biphenyl dioxygenase (20).

Biodegradation of TCE and related compounds by hybrid strains. We demonstrated previously that *E. coli* cells expressing hybrid dioxygenase composed of TodC1-BphA2A3A4 showed a remarkable ability to degrade TCE (11). The profiles of degradation of TCE by double-crossover strains derived from KF707 and KF715 are presented in Fig. 5. Both parent strains KF707 and KF715 grown on biphenyl failed to degrade TCE, and *P. putida* F1 grown on toluene degraded TCE at an initial degradation rate of 7.8 nmol/ml/h, which decreased over time (Fig. 5). Most interestingly, all single- and double-crossover strains carrying *todC1* on the chromosome displayed very efficient degradation of TCE and removed it almost completely. KF707-D2, for example, degraded 77 μM TCE (770 nmol/10 ml, 10 μg/ml) as fast as 49.2 nmol/ml/h, and completely degraded it in 4 h, when it grew on biphenyl. The cells grown on toluene and sodium succinate also showed degradation capabilities comparable to that of biphenyl-grown cells. Other single- and double-crossover strains also degraded TCE as efficiently as KF707-D2 when it grew on these three substrates. The expression of *bph* genes in KF707 and KF715 has not been elucidated yet, but we have evidence that both *bph* gene clusters are transcribed from at least two promoters upstream of *orf0* and that these transcriptions are constitutive rather than inducible (unpublished data).

Degradation of various chloroethenes by *P. putida* KF715-D5 was carried out in the same manner (Fig. 6). The degradation of *cis*-1,2-dichloroethylene was the fastest among chlorinated ethylenes tested. The initial rates of degradation of these compounds were as follows: *cis*-1,2-dichloroethylene, 98.9 nmol/ml/h; TCE, 65.8 nmol/ml/h; 1,1-dichloroethylene, 17.2 nmol/ml/h; *trans*-1,2-dichloroethylene, 10.2 nmol/ml/h; and tetrachloroethylene, 3.2 nmol/ml/h. The slight decrease of tetrachloroethylene in Fig. 6 is not likely due to the degradation, since the control experiment without the organism showed a similar decrease over time. Furthermore, the original strain, *P. putida* KF715, did not degrade any chloroethenes tested (data not shown). The substrate specificity of hybrid dioxygenase seems to be similar to that of toluene dioxygenase of *P. putida* F1 (39), although the rates of degradation of *cis*-1,2-dichloroethylene and TCE by hybrid *Pseudomonas* strains are much higher than those by *P. putida* F1, as described above.

It should be noted that recombinant *E. coli* cells have very high numbers of copies of pJHF101, which contains the hybrid *todC1-bphA2 orf3A3A4* gene cluster (11, 20), since the vehicle is high-copy-number plasmid pUC118 (43), whereas double-crossover hybrid strains of *Pseudomonas* have only one copy on the chromosome. Since the machineries of transcription and

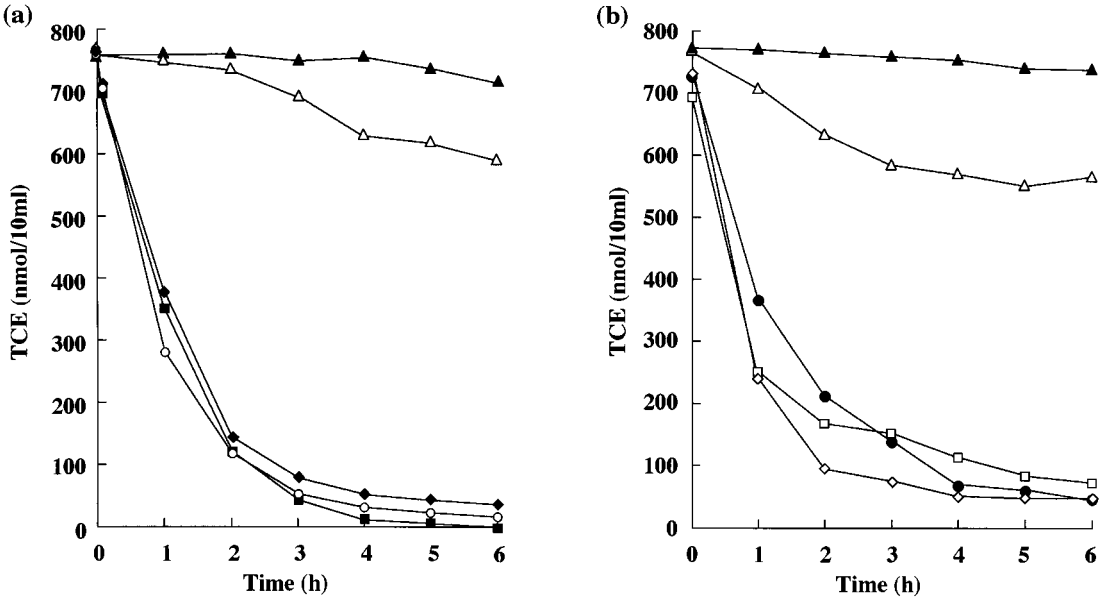

FIG. 5. Degradation of TCE by double-crossover strains *P. pseudoalcaligenes* KF707-D2 (a) and P. putida KF715-D5 (b). (a) ▲, *P. pseudoalcaligenes* KF707 grown with biphenyl; △, *P. putida* F1 grown with toluene; ■, KF707-D2 grown with biphenyl; ○, KF707-D2 grown with sodium succinate; ◆, KF707-D2 grown with toluene. (b) ▲, *P. putida* KF715 grown with biphenyl; △, *P. putida* F1 grown with toluene; □, KF715-D5 grown with biphenyl; ●, KF715-D2 grown with sodium succinate; ◇, KF715-D5 grown with toluene. Cells were suspended to get an OD of 2.0 at 600 nm in basal salts medium supplemented with 20 mM glucose.

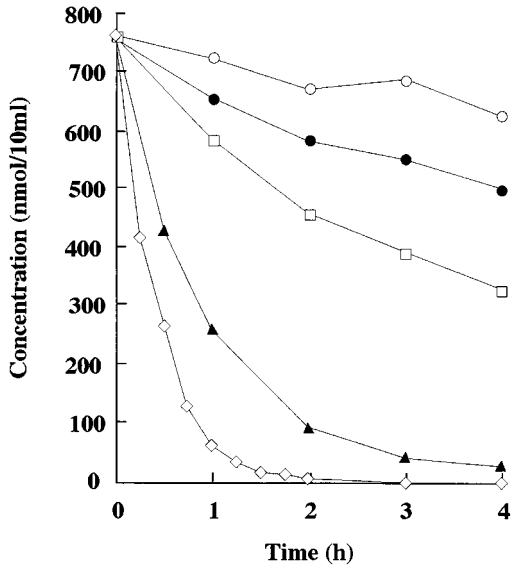

FIG. 6. Degradation of various chlorinated ethylenes by *P. putida* KF715-D5. Chlorinated ethylenes were used at 770 nmol/10 ml. Cell concentrations corresponded to an OD of 2.0 at 600 nm. ○, tetrachloroethylene; ●, *trans*-1,2-dichloroethylene; □, 1,1-dichloroethylene; ▲, TCE; ◇, *cis*-1,2-dichloroethylene.

translation are substantially the same in the original strain and the double-crossover strain, it is not likely that the increased expression of TodC1 is responsible for their increased activities in the degradation of TCE. In this sense, the TCE-degrading capability of hybrid *Pseudomonas* strains seems to be much greater than that of the recombinant *E. coli* strain. The explanation could be more efficient uptake and transport of TCE into the *Pseudomonas* cells than in the *E. coli* cells. Jahng and Wood (23) have cloned the soluble methane monooxygenase (sMMO) genes of *Methylosinus trichosporium* OB3b into *P. putida* F1. The maximum TCE degradation rate obtained with the recombinant strain was much lower than that of wild-type OB3b. It is postulated that the inconsistency in TCE degradation and lower maximum rate with *P. putida* F1 carrying sMMO genes may be due to inefficient ferrous ion addition to the sMMO apoenzyme. As in the case of hydroxylase of sMMO, TodC1 (an iron-sulfur protein) in the hybrid dioxygenase also contains FAD and a 2Fe-2S cluster (34, 42). *E. coli* may lack the cellular machinery responsible for transferring the necessary cofactor into TodC1, or such factors may be too short to cover overexpressed TodC1 in *E. coli*. If this is the case, some of the hybrid dioxygenase TodC1-BphA2A3A4 protein may not be functional in *E. coli* even if the recombinant *E. coli* cells overproduce hybrid dioxygenase. Another possibility is the formation of an inclusion body of foreign protein in *E. coli* cells, which may reduce the enzymatic activity.

In conclusion, we demonstrated in this study that replacement of the gene encoding the large subunit of biphenyl dioxygenase (*bphA1*) with that encoding the large subunit of toluene dioxygenase (*todC1*) within the chromosomal *bph* gene cluster could be successfully carried out by single- and double-crossover recombinations in the parent KF707 strain and another *Pseudomonas* strain which possesses a *bph* gene cluster similar to that of KF707; we have also shown that the resultant hybrid strains grew well on a wide range of aromatic hydrocarbons and, more interestingly, that they gained the novel capability to degrade xenobiotic compounds. The degradation of TCE and *cis*-1,2-dichloroethylene was very efficient at concentrations as high as 77 μM, which is comparable to that obtained with the efficient TCE degraders reported in the literature (23, 26, 28, 29, 37–40, 42, 45). The double-crossover strains maintained the foreign genes stably in the chromosome without any selective pressure. Furthermore, the hybrid dioxygenase could be expressed constitutively without induction. This kind of approach may provide an insight into evolution of catabolic genes and could be useful for breeding strains with enhanced capacities to degrade environmental pollutants (36).

REFERENCES

1. **Asturias, J. A., E. Díaz, and K. N. Timmis.** 1995. The evolutionary relationship of biphenyl dioxygenase from Gram-positive *Rhodococcus globerulus* P6 to multicomponent dioxygenases from Gram-negative bacteria. Gene **156:** 11–18.
2. **Barton, M. R., and R. L. Crawford.** 1988. Novel biotransformation of 4-chloro-biphenyl by a *Pseudomonas* sp. Appl. Environ. Microbiol. **54:**594–595.
3. **Bedard, D. L., R. Unterman, L. H. Bopp, M. J. Brennan, M. L. Haberl, and C. Johnson.** 1986. Rapid assay for screening and characterizing microorganisms for the ability to degrade polychlorinated biphenyls. Appl. Environ. Microbiol. **51:**761–768.
4. **Bedard, D. L., R. E. Wagner, M. J. Brennan, M. L. Haberl, and J. F. Brown, Jr.** 1987. Extensive degradation of Aroclors and environmentally transformed polychlorinated biphenyls by *Alcaligenes eutrophus* H850. Appl. Environ. Microbiol. **53:**1094–1102.
5. **Chung, S.-Y., M. Maeda, E. Song, K. Horikoshi, and T. Kudo.** 1994. A Gram-positive polychlorinated biphenyl-degrading bacterium, *Rhodococcus erythropolis* strain TA421, isolated from a termite ecosystem. Biosci. Biotechnol. Biochem. **58:**2111–2113.
6. **Erickson, B. D., and F. J. Mondello.** 1992. Nucleotide sequencing and transcriptional mapping of the genes encoding biphenyl dioxygenase, a multicomponent polychlorinated biphenyl-degrading enzyme in *Pseudomonas* strain LB400. J. Bactriol. **174:**2903–2912.
7. **Furukawa, K.** 1982. Microbial degradation of polychlorinated biphenyls, p. 33–57. *In* A. M. Chakrabarty (ed.), Biodegradation and detoxification of environmental pollutants. CRC Press, Inc., Boca Raton, Fla.
8. **Furukawa, K.** 1994. Molecular genetics and evolutionary relationship of PCB-degrading bacteria. Biodegradation **5:**289–300.
9. **Furukawa, K., N. Hayase, K. Taira, and N. Tomizuka.** 1989. Molecular relationship of chromosomal genes encoding biphenyl/polychlorinated biphenyl catabolism: some soil bacteria possess a highly conserved *bph* operon. J. Bacteriol. **171:**5467–5472.
10. **Furukawa, K., S. Hayashida, and K. Taira.** 1991. Gene-specific transposon mutagenesis of the biphenyl/polychlorinated biphenyl-degradation-controlling *bph* operon in soil bacteria. Gene **98:**21–28.
11. **Furukawa, K., J. Hirose, S. Hayashida, and K. Nakamura.** 1994. Efficient degradation of trichloroethylene by a hybrid aromatic ring dioxygenase. J. Bacteriol. **176:**2121–2123.
12. **Furukawa, K., J. Hirose, A. Suyama, T. Zaiki, and S. Hayashida.** 1993. Gene components responsible for discrete substrate specificity in the metabolism of biphenyl (*bph* operon) and toluene (*tod* operon). J. Bacteriol. **175:**5224–5232.
13. **Furukawa, K., and T. Miyazaki.** 1986. Cloning of gene cluster encoding biphenyl and chlorobiphenyl degradation in *Pseudomonas pseudoalcaligenes*. J. Bacteriol. **166:**392–398.
14. **Furukawa, K., J. R. Simon, and A. M. Chakrabarty.** 1983. Common induction and regulation of biphenyl, xylene/toluene, and salicylate catabolism in *Pseudomonas paucimobilis*. J. Bacteriol. **154:**1356–1362.
15. **Galli, E., P. Barbieri, and G. Bestetti.** 1992. Potential of pseudomonads in the degradation of methylbenzene, p. 268–276. *In* E. Galli, S. Silver, and B. Witholt (ed.), *Pseudomonas*: molecular biology and biotechnology. American Society for Microbiology, Washington, D.C.
16. **Gibson, D. T., M. Hensley, H. Yoshioka, and T. J. Mabry.** 1970. Formation of (+)-cis-2,3-dihydroxy-1-methylcyclohexa-4,6-diene from toluene by *Pseudomonas putida*. Biochemistry **9:**1626–1630.
17. **Gibson, D. T., G. J. Zylstra, and S. Chauhan.** 1990. Biotransformations catalyzed by toluene dioxygenase from *Pseudomonas putida* F1, p. 121–132. In S. Silver, A. M. Chakrabarty, B. Iglewski, and S. Kaplan (ed.), *Pseudomonas*: biotransformations, pathogenesis, and evolving biotechnology. American Society for Microbiology, Washington, D.C.
18. **Hayase, N., K. Taira, and K. Furukawa.** 1990. *Pseudomonas putida* KF715 *bphABCD* operon encoding biphenyl and polychlorinated biphenyl degradation: cloning, analysis, and expression in soil bacteria. J. Bacteriol. **172:**1160–1164.
19. **Hickey, W. J., V. Brenner, and D. D. Focht.** 1992. Mineralization of 2-chloro- and 2,5-dichlorobiphenyl by *Pseudomonas* sp. strain UCR2. FEMS Microbiol. Lett. **98:**175–180.
20. **Hirose, J., A. Suyama, S. Hayashida, and K. Furukawa.** 1994. Construction

of hybrid biphenyl (*bph*) and toluene (*tod*) genes for functional analysis of aromatic ring dioxygenase. Gene **138:**27–33.
21. **Hofer, B., S. Backhaus, and K. N. Timmis.** 1994. The biphenyl/polychlorinated biphenyl-degradation locus (*bph*) of *Pseudomonas* sp. LB400 encodes four additional metabolic enzymes. Gene **144:**9–16.
22. **Hofer, B., L. D. Eltis, D. N. Dowling, and K. N. Timmis.** 1993. Genetic analysis of a *Pseudomonas* locus encoding a pathway for biphenyl/polychlorinated biphenyl degradation. Gene **130:**47–55.
23. **Jahng, D., and T. K. Wood.** 1994. Trichloroethylene and chloroform degradation by a recombinant pseudomonad expressing soluble methane monooxygenase from *Methylosinus trichosporium* OB3b. Appl. Environ. Microbiol. **60:**2473–2482.
24. **Kikuchi, Y., Y. Yasukochi, Y. Nagata, M. Fukuda, and M. Takagi.** 1994. Nucleotide sequence and functional analysis of the *meta*-cleavage pathway involved in biphenyl and polychlorinated biphenyl degradation in *Pseudomonas* sp. strain KKS102. J. Bacteriol. **176:**4269–4276.
25. **Kimura, N., H. Kato, A. Nishi, and K. Furukawa.** 1996. Analysis of substrate range of biphenyl-catabolic enzymes. Biosci. Biotechnol. Biochem. **60:**220–223.
26. **Little, C. D., A. V. Palumbo, S. E. Herbes, M. E. Lidstrom, R. L. Tyndall, and P. J. Gilmer.** 1988. Trichloroethylene biodegradation by a methane-oxidizing bacterium. Appl. Environ. Microbiol. **54:**951–956.
27. **Masai, E., A. Yamada, J. M. Healy, T. Hatta, K. Kimbara, M. Fukuda, and K. Yano.** 1995. Characterization of biphenyl catabolic genes of gram-positive polychlorinated biphenyl degrader *Rhodococcus* sp. strain RHA1. Appl. Environ. Microbiol. **61:**2079–2085.
28. **Oldenhuis, R., J. Y. Oedzes, J. J. van der Waarde, and D. B. Janssen.** 1991. Kinetics of chlorinated hydrocarbon degradation by *Methylosinus trichosporium* OB3b and toxicity of trichloroethylene. Appl. Environ. Microbiol. **57:**7–14.
29. **Oldenhuis, R., R. L. J. M. Vink, D. B. Janssen, and B. Witholt.** 1989. Degradation of chlorinated aliphatic hydrocarbons by *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase. Appl. Environ. Microbiol. **55:**2819–2826.
30. **Pettigrew, C. A., A. Breen, C. Corcoran, and G. S. Sayler.** 1990. Chlorinated biphenyl mineralization by individual populations and consortia of freshwater bacteria. Appl. Environ. Microbiol. **56:**2036–2045.
31. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
32. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**784–791.
33. **Simon, R., J. Quandt, and W. Klipp.** 1989. New derivatives of transposon Tn*5* suitable for mobilization of replicons, generation of operon fusions and induction of genes in Gram-negative bacteria. Gene **80:**161–169.
34. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
35. **Taira, K., J. Hirose, S. Hayashida, and K. Furukawa.** 1992. Analysis of *bph* operon from the polychlorinated biphenyl-degrading strain of *Pseudomonas pseudoalcaligenes* KF707. J. Biol. Chem. **267:**4844–4853.
36. **Timmis, K. N., R. J. Steffan, and R. Unterman.** 1994. Designing microorganisms for the treatment of toxic compounds. Annu. Rev. Microbiol. **48:**525–557.
37. **Tsien, H.-C., G. A. Brusseau, R. S. Hanson, and L. P. Wacket.** 1989. Biodegradation of trichloroethylene by *Methylosinus trichosporium* OB3b. Appl. Environ. Microbiol. **55:**3155–3161.
38. **Uchiyama, H., T. Nakajima, O. Yagi, and T. Nakahara.** 1992. Role of heterotrophic bacteria in complete mineralization of trichloroethylene by *Methylocystis* sp. strain M. Appl. Environ. Microbiol. **58:**3067–3071.
39. **Wackett, L. P., and D. T. Gibson.** 1988. Degradation of trichloroethylene by toluene dioxygenase in whole-cell studies with *Pseudomonas putida* F1. Appl. Environ. Microbiol. **54:**1703–1708.
40. **Wackett, L. P., M. J. Sadowsky, L. M. Newman, H.-G. Hur, and S. Li.** 1994. Metabolism of polyhalogenated compounds by a genetically engineered bacterium. Nature (London) **368:**627–629.
41. **Wang, Y., M. Rawlings, D. T. Gibson, D. Labbe, H. Bergeron, R. Brousseau, and P. C. K. Lau.** 1995. Identification of a membrane protein and truncated LysR-type regulator associated with the toluene degradation pathway in *Pseudomonas putida* F1. Mol. Gen. Genet. **246:**570–579.
42. **Winter, R. B., K.-M. Yen, and B. D. Ensley.** 1989. Efficient degradation of trichloroethylene by a recombinant *Escherichia coli*. Bio/Technology **7:**282–285.
43. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
44. **Zylstra, G. J., and D. T. Gibson.** 1989. Toluene degradation by *Pseudomonas putida* F1: nucleotide sequence of the *todC1C2BADE* genes and their expression in *Escherichia coli*. J. Biol. Chem. **264:**14940–14946.
45. **Zylstra, G. J., L. P. Wackett, and D. T. Gibson.** 1989. Trichloroethylene degradation by *Escherichia coli* containing the cloned *Pseudomonas putida* F1 toluene dioxygenase genes. Appl. Environ. Microbiol. **55:**3162–3166.