***Pseudomonas putida* CE2010 can degrade biphenyl by a mosaic pathway encoded by the *tod* operon and *cmtE*, which are identical to those of *P. putida* F1 except for a single base difference in the operator–promoter region of the *cmt* operon**

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***Pseudomonas putida* CE2010 can assimilate biphenyl despite its high similarity to *P. putida* F1. Biphenyl degradation in strain CE2010 was achieved using a mosaic of pathways consisting of the *cmt* and *tod* operons. CmtE hydrolysed 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid, the *meta*-cleavage product of 2,3-dihydroxybiphenyl. This enzyme was expressed differently in strains CE2010 and F1. A *cmtE* disruption mutant, a *tod* operon disruption mutant and a *cmt* operon disruption mutant were unable to utilize biphenyl. The introduction of the *cmtE* gene enabled the *cmt* operon disruption mutant to grow on biphenyl. A single base difference was found in the *cmt* promoter–operator region in strain CE2010, compared with that of strain F1. CymR protein was purified from *Escherichia coli* and binding assays were performed, the results of which suggested that the protein bound less strongly to the CE2010 operator sequence than to the F1 operator sequence. Exchanging the F1 promoter–operator fragment into strain CE2010 resulted in a loss of biphenyl degradation capacity. These results indicate that *cmtE* is not effectively repressed by CymR in strain CE2010, leading to low constitutive expression and, therefore, low growth on biphenyl.**

Keywords: biphenyl, mosaic pathway, biodegradation, *Pseudomonas putida*

INTRODUCTION

Aromatic hydrocarbon-degrading bacteria are widely distributed in nature and have acquired various kinds of metabolic pathways through the course of evolution. Biphenyl-degrading bacteria have been isolated from the environment. The major catabolic pathway is the most common pathway for biphenyl degradation (Catelani *et al.*, 1973; Hayase *et al.*, 1990; Kimbara *et al.*, 1989; see Fig. 2).

Abbreviations: 2,3-DHBP, 2,3-dihydroxybiphenyl; 2,3-DHBD, 2,3-dihydroxybiphenyl dioxygenase; HPDA, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid; HOHD, 2-hydroxy-6-oxo-hepta-2,4-dienoic acid; Ap, ampicillin; Cb, carbenicillin; Km, kanamycin; Tc, tetracycline.

The DDBJ accession numbers for the sequences reported in this paper are AB042508 and AB042509.

Similar reactions are found in different catabolic pathways. The benzene/toluene degrader *Pseudomonas putida* BE81 has *bnz* genes (Irie *et al.*, 1987). *Pseudomonas* sp. mt-2 has a *xyl* operon encoding the catabolic enzymes required for growth on xylene and toluene (Harayama & Rekik, 1990). It is postulated that a number of catabolic genes involved in the degradation of aromatic compounds share a common ancestry and form gene superfamilies (Meer *et al.*, 1992).

Catabolic genes in bacteria are often clustered in operons and the comparison of gene organization of catabolic operons among various bacteria has demonstrated that functional clusters of genes often move as units from one genetic element to another (Meer *et al.*, 1992). The order of the genes responsible for the oxidation of benzoate to catechol on the chromosome of *Acinetobacter calcoaceticus* (*benABCD*) is the same as that on the TOL

plasmid of *P. putida* (xylXYZL) (Harayama *et al.*, 1991). The genetic organization of the *meta* operons of the TOL and NAH plasmids are very similar, although regulator XylS belongs to the AraC family and NahR belongs to the LysR family (Lorenzo *et al.*, 1996). Gene movements or pathway shuffling are ways for bacteria to expand their substrate specificity and to adapt to the environment.

From the viewpoint of the expansion of substrate specificity as a functional unit, the co-evolution of several enzymes is often necessary because the catabolic pathways consist of multiple enzymic steps. If the range of compounds which might be degraded by the particular functional unit is limited by a single enzyme with narrow substrate specificity, the recruitment of another enzyme is sufficient to expand the substrate range of the pathway. *P. putida* F1 grows well on toluene/benzene but not on biphenyl (Gibson *et al.*, 1968). Despite the discrete substrate specificity, the *bph* and *tod* operons are very similar, not only in terms of gene organization and pathway, but also in terms of the sizes and the deduced amino acid sequences of the gene products (Zylstra *et al.*, 1988; Lau *et al.*, 1994). The inability of *P. putida* F1 to grow on biphenyl is due to the lack of TodF activity for biphenyl *meta*-cleavage of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (HPDA) (Furukawa *et al.*, 1993). The introduction of the *bphD* gene derived from *Pseudomonas pseudoalcaligenes* KF707 into *P. putida* F1 permits its growth on biphenyl. It might be one of the strategies of natural evolution that the expansion of substrate specificity and the adaptation to a new substrate are achieved by enzyme recruitment.

Recently, *P. putida* CE2010 was isolated from refinery wastewater and was found to be capable of degrading PCB/biphenyl in the presence of an organic solvent (Ohta *et al.*, 1996). *P. putida* CE2010 is very similar to *P. putida* F1, except for its ability to degrade biphenyl. In this study we analysed the mechanisms involved in the biphenyl degradation pathway in strain CE2010. *P. putida* CE2010 degrades biphenyl using a mosaic of pathways consisting of *tod* and *cmt* operons. Biphenyl degradation is achieved by the recruitment of CmtE together with the *tod* pathway in *P. putida* CE2010. We found that a single base difference in the operator–promoter region of the *cmt* operon, CmtE and the *tod* operon are all essential for biphenyl degradation in this strain.

METHODS

Bacterial strains, plasmids and culture conditions. Biphenyl-utilizing *P. putida* CE2010 has been described previously (Ohta *et al.*, 1996), as has toluene-utilizing *P. putida* F1 (Gibson *et al.*, 1968) and this strain was obtained from ATCC (Manassas, VA, USA). *P. putida* CE2010 was grown on C medium, which contained (l⁻¹): 5 g (NH₄)₂SO₄, 5.87 g KH₂PO₄, 0.3 g MgSO₄·7H₂O, 2 g NaCl, 0.03 g CaCl₂, 0.01 g FeSO₄·7H₂O, 0.6 mg NiSO₄·7H₂O, 0.1 g yeast extract and 2 ml trace elements solution. The trace elements solution contained (l⁻¹): 4 mg MoO₃, 28 mg ZnSO₄·5H₂O, 2 mg CuSO₄·5H₂O, 4 mg H₃BO₃, 4 mg MnSO₄·5H₂O and 4 mg

CoCl₂·6H₂O. Biphenyl was added to C medium at a final concentration of 0.5% as sole carbon source and cultures were incubated on a reciprocal shaker at 120 r.p.m. at 30 °C. *Escherichia coli* JM109 {Δ(lac–proAB) endA1 gyrA96 hsdR17 λ⁻ relA1 supE44 thi [F' lacI^qZΔM15 proAB traD36]} and MV1184 {ara⁻, Δ(lac–proAB) Δ(stl–racA)306::Tn10 φ80dlacZΔM15 rpsL⁻ thi⁻ [F' lacI^qZΔM15 proAB traD36]} were used as recipient strains for transformation experiments. *E. coli* JM109 and MV1184 were routinely grown at 37 °C in Luria–Bertani (LB) broth containing ampicillin (Ap; 50 µg ml⁻¹) or IPTG (500 µM) when necessary. pUC118 and pUC119 vectors were used to clone the *bphC* gene. All recombinant *Pseudomonas* strains were constructed by tri-parental mating with *E. coli* JM109 (as the donor strain which carried one of the recombinant plasmids constructed containing the *cmt*_{CE2010} or *cym*_{CE2010} genes) and *E. coli* HB101A(pRK2013) as a helper strain. The transconjugants were obtained by spreading the mixture on *Pseudomonas* medium (Difco) with kanamycin (Km; 25 µg ml⁻¹) or tetracycline (Tc; 25 µg ml⁻¹).

Biphenyl- and toluene-utilizing strains were grown at 30 °C in C medium containing biphenyl or toluene as sole carbon source. Antibiotics were added at the following concentrations to select for the presence of the plasmid and the knock-out strains: Ap at 50 µg ml⁻¹ for *E. coli* strains and at 300 µg ml⁻¹ for *Pseudomonas* strains; Tc at 12.5 µg ml⁻¹ for *E. coli* strains and at 25 µg ml⁻¹ for *Pseudomonas* strains; Km at 25 µg ml⁻¹ for *E. coli* and *Pseudomonas* strains.

Enzyme assays. 2,3-Dihydroxybiphenyl dioxygenase (2,3-DHBD) activity was determined by measuring the formation of *meta*-cleavage reaction products at 434 nm using a Hitachi model 330 spectrophotometer equipped with a water-cooled cell holder and a Haake circulating water bath. Activity assays were performed at 30 °C using reaction mixtures containing 200 µM 2,3-dihydroxybiphenyl (2,3-DHBP) as the substrate in 50 mM Tris/HCl (pH 7.5). One unit of enzyme activity was defined as the amount of enzyme that catalysed the formation of 1 µmol product min⁻¹ at 30 °C. The molecular extinction coefficient of the product under these assay conditions was taken to be 13 200 cm⁻¹ M⁻¹ (Maeda *et al.*, 1995). The relative ring cleavage activities were determined from the extinction coefficient of the ring fission products formed from the following substrates: catechol (λ_{max} 375 nm; ε 36 000 cm⁻¹ M⁻¹).

HPDA hydrolysis activity (BphD-like activity) was assayed spectrophotometrically by monitoring the decrease in A₄₃₄. Specific activity was defined as the number of enzyme units (mg protein)⁻¹. HPDA was prepared by incubation of recombinant *E. coli* (carrying the *bphC* gene) with 2,3-DHBP immediately before assaying hydrolase activity. Following the incubation period, the cells were removed by centrifugation and the supernatant was filtered using a 40 µm pore-size filter (Amicon). The protein concentration was assayed using a protein assay kit (Bio-Rad), according to the method of Bradford (1976).

Purification of an enzyme hydrolysing HPDA. Chromatography was performed on an FPLC system (Amersham Pharmacia Biotech). Wet *P. putida* CE2010 cells (6.2 g) were suspended in 20 ml 30 mM Tris/HCl buffer, pH 7.5 (Buffer A), and disrupted by sonication (Branson TW3). The supernatant fluid was prepared by centrifugation at 28 000 g for 30 min as a crude extract. To the mixture, 3.5 g (NH₄)₂SO₄ was added, the resulting precipitate was removed by centrifugation at 28 000 g for 20 min and the supernatant fraction was resuspended in 40 ml Tris/HCl buffer, pH 7.5,

Fig. 1. (a) Cloning and localization of *P. putida* CE2010 gene 1 and gene 2 encoding the enzyme that catalyses ring meta-cleavage of 2,3-DHBP. The restriction map of the *tod* operon is from Zylstra & Gibson (1989) and those of the *cmt* operons are from Eaton (1996, 1997). The location of the genes encoding *cmt* or *tod* pathway enzymes is indicated at the top. Below the map the DNA fragments cloned and the subclones obtained are shown.

containing 1 M $(\text{NH}_4)_2\text{SO}_4$. The $(\text{NH}_4)_2\text{SO}_4$ fraction was applied to a Phenyl Sepharose column (1.6×15 cm; Amersham Pharmacia Biotech) previously equilibrated with Buffer A containing 1 M $(\text{NH}_4)_2\text{SO}_4$. The enzyme was eluted with a linear gradient mixture of 200 ml Buffer A containing 1 M $(\text{NH}_4)_2\text{SO}_4$ and Buffer A at a flow rate of 1 ml min^{-1} . The active fractions were collected, dialysed against Buffer A and applied to a MonoQ column (0.5×10 cm; Amersham Pharmacia Biotech) equilibrated with Buffer A. The enzyme was eluted with a linear gradient mixture of 20 ml Buffer A and 20 ml Buffer A containing 0.5 M NaCl at a flow rate of 0.5 ml min^{-1} . Eluted fractions were subjected to a 12.5% SDS-PAGE analysis. Molecular mass standard proteins used were bovine serum albumin (M_r 66000), ovalbumin (46000), carbonic anhydrase (30000), trypsin inhibitor (21500) and lysozyme (14300).

Gel filtration methods. The native mass of the protein which hydrolyses HPDA was estimated by gel filtration through a Sephacryl S200HR column (1.6×100 cm; Amersham Pharmacia Biotech; flow rate 15 ml h^{-1}) in Buffer A containing 150 mM NaCl. The calibration proteins were albumin (M_r 67000), ovalbumin (43000), chymotrypsinogen A (25000) and ribonuclease A (13700).

DNA manipulations. Restriction analysis and recombinant DNA techniques were performed by standard methods (Sambrook *et al.*, 1989).

Cloning of the gene that encoding 2,3-DHBD activity. Genomic DNA from *P. putida* CE2010 and *P. putida* F1 was prepared by a procedure based on the method described by Wilson (1990). *P. putida* CE2010 chromosomal DNA was digested partially with *Sau3AI* or *HindIII* and DNA fragments

were fractionated by agarose gel electrophoresis. The DNA fragments were ligated to pUC118, which had been digested with *Bam*HI or *Hind*III, or pKS13 which had been digested with *Bam*HI, and treated with alkaline phosphatase. The resulting plasmids were introduced into *E. coli* JM109 by transformation and the cells were plated on LB agar medium containing Ap and IPTG. The resulting cosmid library was introduced into *E. coli* VCS257 using the Gigapack II Packaging Extracts Kit (Stratagene) and the cells were plated on LB agar medium containing Tc. Clones that expressed meta-cleavage dioxygenase activity were identified by spraying colonies with a solution containing 20 mM 2,3-DHBP in acetone. Positive clones turned yellow. A 9.5 kb *Sau3AI* fragment (pYO-C5) and 7.4 kb *Hind*III fragment (pYO1009) were obtained from the plasmid in the positive clones.

Construction of a *cmtE*_{CE2010}-deficient mutant of strain CE2010. The *cmtE*_{CE2010} gene of pYO250 was disrupted by insertion of a blunt-ended 1.4 kb *Eco*RI-*Sty*I fragment of pBR322, which carries the Tc resistance gene, into the *Kpn*I site (pCM250) (Fig. 1). The inserted fragment of pCM250 was moved to pKTYMCS (Table 1) and introduced into strain CE2010 by tri-parental mating with pRK2013 as a helper plasmid. All recombinant *Pseudomonas* strains were constructed by tri-parental mating with *E. coli* JM109 (as the donor strain carrying one of the recombinant plasmids constructed containing the *cmtE*_{CE2010} or *cymE*_{CE2010} genes) and *E. coli* HB101(pRK2013) as a helper strain. The transconjugants were obtained by spreading the mixture on *Pseudomonas* medium (Difco) with Tc ($25 \mu\text{g ml}^{-1}$). A Tc^r transconjugant was selected from among the transformed cells. Insertion of the 1.4 kb fragment into the *cmtE*_{CE2010} gene on the chromosome was confirmed by Southern blot analysis

Table 1. Bacterial strains, plasmids and oligonucleotides used in this study

Strain/plasmid	Relevant characteristics	Source/reference
Strain		
<i>P. putida</i> CE2010	Biphenyl/PCB-degrading bacterium	Ohta <i>et al.</i> (1996)
<i>P. putida</i> F1	Toluene-degrading bacterium	Gibson <i>et al.</i> (1968)
<i>E. coli</i> JM109	Host strain for DNA manipulation	Toyobo
Plasmids		
pUC118	Cloning vector; Ap ^r	Toyobo
KS + Bluescript	Cloning vector; Ap ^r	Toyobo
pBR322	Cloning vector; Ap ^r , Tc ^r	Toyobo
pMW218	Cloning vector; Km ^r	Nippon Gene
pKK233-3	Expression vector; Ap ^r	Pharmacia-Amersham
pGEX-4T-1	GST gene fusion vector	Pharmacia-Amersham
pMMB67EH	IncQ, <i>tac</i> promoter; Ap ^r , Cb ^r	Fürste <i>et al.</i> (1986)
pRK2013	Helper plasmid	Fürste <i>et al.</i> (1986)
pKS13	Broad-host-range cosmid vector	Kimbara <i>et al.</i> (1989)
pTER3400	1.2 kb Km ^r gene from pMW218 and 0.9 kb <i>rrnB</i> terminator gene from pKK233-3 inserted into <i>HincII</i> -digested pUC118	This study
pKTY320::Tn5	Suicide vector	Nagata <i>et al.</i> (1993)
pKTYΔEH	pKTY320::Tn5 digested with <i>EcoRI</i> and <i>HindIII</i> , blunt-ended and re-ligated	This study
pKTYMCS	0.2 kb <i>PvuII</i> fragment (containing multi-cloning sites) from Bluescript SK + inserted into <i>PvuII</i> -digested pKTYΔEH	This study
pMTC67EH	1.4 kb fragment from pBR322 (Tc ^r) inserted into <i>HindIII</i> site of pMMB67EH; Ap ^r , Tc ^r	This study
pYO-C5	9.5 kb <i>Sau3AI</i> fragment from <i>P. putida</i> CE2010 inserted into pUC118	This study
pYO1009	7.4 kb <i>HindIII</i> fragment from <i>P. putida</i> CE2010 inserted into pUC118	This study
pYOS13	27 kb <i>Sau3AI</i> fragment from <i>P. putida</i> CE2010 inserted into pKS13	This study
pYO-B1	2.0 kb <i>SphI</i> fragment from pYO-C5 inserted into <i>SphI</i> -digested pUC118	This study
pYO-C10	1.6 kb <i>XhoI</i> and <i>SphI</i> fragment from pYO-C5 inserted into <i>HincII</i> -digested pUC118	This study
pYO320	3.0 kb <i>XhoI</i> – <i>HindIII</i> fragment from pYO-C5 inserted into <i>XhoI</i> / <i>HindIII</i> -digested Bluescript KS +	This study
pKT101	2.1 kb fragment containing a Km ^r gene and a <i>rrnB</i> terminator gene from pTER3400 inserted into <i>SphI</i> -digested and blunt-ended pYO320	This study
pYO-B2	3.4 kb <i>Sall</i> fragment from pYO1009 inserted into <i>Sall</i> -digested pUC118	This study
pYO250	2.0 kb <i>XhoI</i> fragment from pYOS13 inserted into <i>HincII</i> -digested pUC118	This study
pCM250	1.4 kb fragment from pBR322 (Tc ^r) inserted into <i>KpnI</i> site of pYO250, <i>cmtE</i> ::Tc ^r ; Ap ^r , Tc ^r	This study
pYO400	pYO1009 digested with <i>Sall</i> and re-ligated (3.3 kb); Ap ^r	This study
pKT301	2.1 kb fragment containing a Km ^r gene and a <i>rrnB</i> terminator gene from pTER3400 inserted into <i>EcoRV</i> -digested pYO400	This study
pMTC101	0.9 kb PCR fragment carrying <i>cmtE</i> in pMTC67EH cloning vector; Ap ^r , Tc ^r	This study
pCE1352	1.5 kb PCR fragment carrying a part of <i>cymE</i> , <i>cmt</i> promoter region and a part of <i>cmtA1</i> from <i>P. putida</i> CE2010 in Bluescript KS +	This study
pF1352	1.5 kb PCR fragment carrying a part of <i>cymE</i> , <i>cmt</i> promoter region and a part of <i>cmtA1</i> from <i>P. putida</i> F1 in Bluescript KS +	This study
pCP1352	1.4 kb fragment from pBR322 (Tc ^r) inserted into <i>MluI</i> site of pCE1352	This study
pFP1352	1.4 kb fragment from pBR322 (Tc ^r) inserted into <i>MluI</i> site of pF1352	This study
pGSTcymR	0.6 kb PCR fragment carrying <i>cymR</i> in pGEX-4T-1; Ap ^r	This study
Oligonucleotide		
CEPro	AGGCCCTAGCAACTTGACAGGTGAATTCGAGGCGGATGA TTTTTTTTTGA AAAACAAACACACAATCTGGTCAGTTTGTGTA TTATAAGTAAAAACACCCACGT	
F1Pro	AGGCCCTAGCAACTTGACAGGTGAATTCGAGGCGGATGA TTTTTTTTTGA AAAACAAACACACAATCTGGTCTGTTTGTGTA TTATAAGTAAAAACACCCACGT	
PCR primer		
CYM10589	CTTCGTTGTTGTATGAGGGCTTGC	
CYM10391	ACCTGCTTTACACATCGGGCAC	
CMT11921	ATACGGCAAGTGGCTCTCCTCT	
CUT11941	TTGGATAGCTGCGGCCTTTCAT	
CMT18558	GAATTCTATAGAGAGGTATTTATG	
CMT19470	GAATTCACAGGTTTCGCAATCACGTT	
CYMRN	GAATTCATGAGTCCAAAGAGAAGAACACAGGCAGA	
CYMRC	GAATTCCTAGCGCTTGAATTTTCGCGTACCGCTCT	

(Southern, 1975; Anderson & Young, 1985) using a 2.0 kb *Xho*I fragment from pYO250 as probe. To confirm double crossover mutants, the insertion mutants were streaked on LB plates containing Ap (300 µg ml⁻¹) (Wang *et al.*, 1995). Wild-type *P. putida* CE2010 was tolerant to Ap (100 µg ml⁻¹). The single crossover mutants grew well on the plates, since they were derived from the integration of the whole plasmid (containing the Ap resistance gene from pKTYMCS) into the *P. putida* CE2010 chromosome. But the double crossover mutants did not grow or grew slowly on the plates. These phenotypes showed the correlation with their crossover type through the insertion mutants.

Construction of *tod*_{CE2010}-operon-deficient and *cmt*_{CE2010}-operon-deficient mutants of strain CE2010. pYO320 carried partial *todX*_{CE2010}, *todF*_{CE2010} and *todC1*_{CE2010} genes and pYO400 carried *cmtAa*_{CE2010} and *cmtAb*_{CE2010} (Fig. 1). A blunt-ended 2.1 kb *Eco*RI and *Pvu*I fragment of pTER3400, which carried a Km^r gene and an *rrnB* terminator gene, was inserted into the *Sph*I site of pYO320 (pKT101), or into the *Eco*RV site of pYO400 (pKT301). The inserted fragment was moved to pKTYMCS. The inserted fragments were introduced into strain CE2010 as described above. A Km^r strain was selected from among the transformed cells. Insertion of the Km^r gene and the *rrnB* terminator gene into the *todC1*_{CE2010} gene or the *cmtAa*_{CE2010} gene on the chromosome was confirmed by Southern blot analysis using a 2.0 kb *Eco*RV fragment from pYO320 or a 2.0 kb *Eco*RI fragment from pYO400, respectively, as a probe. Double crossover mutation was confirmed as described above.

Cloning of the *cmtE*_{CE2010} gene into a broad-host-range vector. A 0.9 kb fragment containing the *cmtE*_{CE2010} gene was amplified from strain CE2010 DNA by PCR using CMT18558 and CMT19470 as primers. pMTC101 was constructed by insertion of the amplified fragment into the *Eco*RI site of the cloning vector pMTC67EH (Fig. 1). Strains CE2010 and F1 were transformed with pMTC67EH and pMTC101 by tri-parental mating with pRK2013 as a helper plasmid.

Replacement of the *cmt* promoter region. A 1.5 kb fragment containing a part of *cymE*, the *cmt* promoter region and a part of *cmtA1* was amplified from the DNA of strain CE2010 or strain F1 by PCR with CYM10589 and CMT11921 as primers (Table 1 and Fig. 1). The PCR fragment was inserted into the *Sma*I site of Bluescript KS+ after being blunt-ended with Klenow fragment (pCE1352 and pF1352). A blunt-ended 1.4 kb fragment which carried the Tc resistance gene was inserted into the *Mlu*I site of pCE1352 and pF1352 in the direction opposite to that of the *cmt* promoter (pCP1352, pFP1352; Fig. 1). The inserted fragment was moved to pKTYMCS and introduced into strain CE2010 and strain F1 as described above. Tc^r strains were selected from among the transformed cells. Insertion of a 2.9 kb fragment into the *cmt* promoter region on the chromosome of each strain was confirmed by Southern blot analysis using a 1.5 kb fragment from pCE1352 as a probe. Double crossover mutation was confirmed as described above. The replaced *cmt* promoter region was amplified from recombinant strain CE2010 and strain F1 by PCR with CYM10391 and CMT11941 as primers. The PCR fragment was subcloned into the *Sma*I site of Bluescript KS+ after being blunt-ended and the nucleotide sequence was confirmed by the dideoxy sequencing method.

Overexpression of CymR and DNA binding. CymR was overproduced as a fusion protein using a commercial glutathione *S*-transferase (GST) system. A 620 bp fragment containing *cymR* was amplified from strain CE2010 by PCR with CYMRN and CYMRC as the primers and inserted into

pGEX-4T-1 (Pharmacia Amersham) at the *Eco*RI site. The resulting plasmid, encoding the CymR fusion to GST, was designated pGSTcymR. A 50 kDa fusion protein was produced in *E. coli* JM109 cells induced with 1 mM IPTG for 4 h. The fusion protein was purified according to the manufacturer's instructions.

The DNA binding assay was carried out as follows. Two 100 bp fragments of double-stranded DNA, one containing the *cmt* promoter region of *P. putida* CE2010 (CEPro; Table 1) and the other containing that of *P. putida* F1 (F1Pro), were synthesized as probes and labelled at one end with digoxigenin (Boehringer Mannheim). Assay mixtures containing 1 µg CymR and varying amounts of labelled DNA (40 fmol, 200 fmol) in 20 µl binding buffer (20 mM HEPES, pH 7.6, 1 mM EDTA, 10 mM (NH₄)₂SO₄, 1 mM DTT, 0.2%, w/v, Tween 20, 30 mM KCl, 1 µg Poly[d(I-C)], 1 ng poly-L-lysine) were incubated on ice for 20 min. The samples were separated by electrophoresis in a 6% polyacrylamide gel containing 0.2 × TBE (89 mM Tris/HCl, pH 8.0, 89 mM boric acid, 2 mM EDTA). The probe DNA was transferred to a Hybond-N membrane (Amersham). Detection of the digoxigenin-labelled probe was carried out by means of the chemiluminescence detection system using the Boehringer Mannheim kit according to the instruction manual. Imaging of the chemiluminescence was performed using Fuji RX film by exposure for 1 h.

RESULTS

Screening of the library for *meta*-cleavage dioxygenase expression and identification of the clones

A gene (gene 1) encoding an enzyme that catalyses ring *meta*-cleavage of 2,3-DHBP was subcloned from pYO-C5 as a 2.0 kb *Sph*I fragment into pUC118 (pYO-B1) (Fig. 1). A second gene (gene 2) encoding an enzyme that catalyses ring *meta*-cleavage of 2,3-DHBP was subcloned from pYO1009 as a 3.4 kb *Sal*I fragment into pUC118 (pYO-B2). Sequence analysis of two clones revealed that gene 1 was 100% identical to *todE*_{F1}, encoding the toluene dihydroxy dioxygenase of *P. putida* F1, and gene 2 was 100% identical to *cmtC*_{F1}, encoding the cumate dioxygenase of *P. putida* F1. A cosmid library containing approximately 5000 clones was tested for *meta*-cleavage dioxygenase activity as described above. Twenty-one colonies were positive. Cosmid DNA was prepared from each of these clones and genomic DNA was extracted from *P. putida* CE2010 and *P. putida* F1. Southern hybridization analysis was carried out using *todE*_{CE2010} (encoded by gene 1) and *cmtC*_{CE2010} (encoded by gene 2) as probes. pYOS13 contained a 27 kb fragment and hybridized with both *todE*_{CE2010} and *cmtC*_{CE2010}. Under stringent conditions, its hybridization pattern with the two probes showed no notable difference compared to that in the case of *P. putida* F1 (Fig. 1). It was confirmed that *P. putida* CE2010 could utilize toluene, cumate or cymene as sole carbon source. These results indicate that *P. putida* CE2010 possesses the *tod*_{CE2010} operon, the *cmt*_{CE2010} operon and the *cym*_{CE2010} operon and that the location of the *cmt*_{CE2010} and *tod*_{CE2010} operons in *P. putida* CE2010 is the same as that in *P. putida* F1.

Fig. 2. Metabolic pathways encoded by the *tod* and *bph* operons. TodC1C2BA, components of toluene dioxygenase; TodD, *cis*-toluene dihydrodiol dehydrogenase; TodE, 3-methylcatechol 2,3-dioxygenase; TodF, 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase; BphA1A2A3A4, components of biphenyl dioxygenase; BphB, 2,3-dihydroxy-4-phenylhexa-4,6-diene dehydrogenase; BphC, 2,3-DHBD; BphD, HPDA hydrolase.

Metabolic pathway encoded by the *tod* and *bph* operons

Toluene-utilizing *P. putida* F1 could not grow on biphenyl, but toluene-grown *P. putida* F1 cells did convert biphenyl into the *meta*-cleavage product HPDA (Furukawa *et al.*, 1993). These observations suggested that toluene dioxygenase (TodC1C2BA), toluene dihydrodiol dehydrogenase (TodD) and 3-methylcatechol dioxygenase (TodE) are all active in the conversion of biphenyl to HPDA. However, 2-hydroxy-6-oxo-hepta-2,4-dienoic acid (HOHD) hydrolase (TodF) did not act on HPDA (Fig. 2).

The *todF*_{CE2010} gene of strain CE2010 was subcloned from pYO-C5 as a 1.6 kb *Xho*I-*Sph*I fragment into pUC118 (pYO-C10) and sequence analysis showed that the DNA sequence of the *todF*_{CE2010} gene in *P. putida* CE2010 was 100% identical to that of *todF*_{F1} in *P. putida* F1. *E. coli* carrying pYO-C10 expressed no detectable HPDA hydrolysis activity. Thus, it seems that *P. putida* CE2010 possesses an enzyme that hydrolyses HPDA, which is different from TodF_{CE2010}, and that this enzyme enables *P. putida* CE2010 to assimilate HPDA. Cell lysates prepared from *P. putida* CE2010 grown on biphenyl showed HPDA hydrolysis activity [0.019 units mg⁻¹ (1 unit = 1 µmol HPDA hydrolysed min⁻¹) and also weak HPDA hydrolysis activity (0.002 units mg⁻¹) when grown on glucose. HPDA hydrolysis activity increased 10-fold when *P. putida* CE2010 was grown with biphenyl as sole carbon source. However, cell

lysates prepared from *P. putida* F1 grown on glucose did not show HPDA hydrolysis activity.

Purification of an enzyme hydrolysing HPDA from *P. putida* CE2010

The enzyme which hydrolyses HPDA was purified as described in Methods. The results are summarized in Table 2. The purity of the enzyme obtained was estimated to be > 95% by SDS-PAGE (not shown). The apparent molecular mass of the purified enzyme, as determined by SDS-PAGE, was 30 kDa. Gel-permeation chromatography under non-denaturing conditions on Superose S200HR, calibrated with globular proteins, indicated an apparent molecular mass of 60 kDa. This implies a dimeric structure of the enzyme in its native form. The N-terminal amino acid sequence of the purified enzyme was determined to be NH₂-NLSLDLLGATIRFVDTKAFGTIRIA. This sequence information was used to identify the corresponding gene. The sequence identified was identical to that of CmtE_{F1}, 2-hydroxy-6-oxo-7-methylcat-2,4-dienoate hydrolase, of *P. putida* F1 (Eaton, 1996).

The *cmtE*_{CE2010} gene is required for growth of strain CE2010 on biphenyl

To confirm whether CmtE_{CE2010} hydrolyses HPDA, the *cmtE*_{CE2010} gene was subcloned from pYOS13 as a 2.0 kb *Xho*I fragment into pUC118, yielding plasmid

Table 2. Purification of an enzyme which hydrolyses HPDA from *P. putida* CE2010

Step	Volume (ml)	Total protein (mg)	Total activity (units)*	Recovery (%)	Specific activity (units mg ⁻¹)	Purification (-fold)
Cell extract	22	860	16.3	100	0.019	—
(NH ₄) ₂ SO ₄	40	320	10.3	64.4	0.033	1.7
Phenyl Sepharose	36	25.3	9.7	59.5	0.441	23.3
MonoQ	4	0.5	0.3	1.8	0.593	31.3

* One unit = 1 µmol HPDA hydrolysed min⁻¹.

pYO250. Subclone pYO250 expressed detectable HPDA hydrolysis activity (0.13 units mg⁻¹). This result indicates that the CmtE_{CE2010} hydrolyses HPDA.

To confirm the involvement of CmtE_{CE2010} in biphenyl utilization in *P. putida* CE2010, the *cmtE*_{CE2010} gene in the chromosome was selectively interrupted by insertion of a Tc^r gene from pBR322 into a unique *Kpn*I site, thus interrupting only CmtE_{CE2010}. Chromosomal integration was confirmed for one of the insertional mutants by Southern blot analysis of the *cmtE*_{CE2010} region using a 2.0 kb *Xho*I fragment from pYO250 as a probe. *P. putida* CE2010(*cmtE*_{CE2010}::Tc^r) did not grow on biphenyl; there were no colonies visible even after 7 d incubation, whereas colonies of *P. putida* CE2010 were visible within 2 d. Also, this mutant could not utilize cumate as sole carbon source. These results indicate that CmtE_{CE2010} enables *P. putida* CE2010 to utilize biphenyl as a carbon source.

Both *cmtE*_{CE2010} and the *tod*_{CE2010} operon are required for growth with biphenyl

It was shown that CmtE_{CE2010} hydrolyses HPDA and that it contributes to biphenyl utilization in *P. putida* CE2010. It was expected that the *cmt*_{CE2010} operon was involved in biphenyl utilization in this strain. The *cmt*_{F1} operon encodes the enzymes required for *p*-cumate metabolism in *P. putida* F1 (Eaton, 1996). *CmtAaAbAcAd*_{F1} encode the components of the *p*-cumate 2,3-dioxygenase (ferredoxin reductase, the large subunit of the terminal dioxygenase, the small subunit of the terminal dioxygenase and ferredoxin, respectively), *cmtC*_{F1} encodes 2,3-dihydroxy-*p*-cumate 3,4-dioxygenase, *cmtB*_{F1} encodes 2,3-dihydroxy-2,3-dihydro-*p*-cumate dehydrogenase and *cmtE*_{F1} encodes 2-hydroxy-6-oxo-7-methylocta-2,4-dienoate hydrolase. These genes show similarity in terms of gene organization as well as size and homology to the corresponding genes for *bph* metabolism enzymes. The *cmt*_{F1} operon also encodes *cmtFHG*_{F1} whose products convert isobutyrate to tricarboxylic acid cycle intermediates. The *cmtFHG*_{F1} genes show homology to *bphEGF*, respectively, which encode enzymes involved in the downstream portion of the biphenyl metabolic pathway. Thus, it might be possible that the *cmt*_{CE2010} operon, not the *tod*_{CE2010} operon, is entirely responsible

for the ability of *P. putida* CE2010 to utilize biphenyl. To test whether the *tod*_{CE2010} operon in *P. putida* CE2010 is required for biphenyl degradation, the *tod*_{CE2010} operon was disrupted. When a Km^r gene and an *rrnB* terminator gene from *E. coli* were inserted into the *Sph*I site of the *todC1*_{CE2010} gene in *P. putida* CE2010 as described in Methods, the insertion mutant could not grow on biphenyl or toluene as sole carbon source. Chromosomal integration was confirmed by Southern blot analysis of the *todC1*_{CE2010} region using a 2.0 kb fragment (containing the *todC1*_{CE2010} gene) from pYO-C5 as probe (not shown). It seems likely that all or part of both the *tod*_{CE2010} operon and the *cmt*_{CE2010} operon are required for degradation of biphenyl by *P. putida* CE2010. It has been reported that the failure of *P. putida* F1 to grow on biphenyl is simply due to lack of HPDA hydrolase activity in *P. putida* F1 grown on toluene (Furukawa *et al.*, 1993). Introduction of *bphD* (the KF707 HPDA hydrolase gene) into *P. putida* F1 permitted growth on biphenyl. To confirm whether only the *cmtE*_{CE2010} gene in addition to the *tod*_{CE2010} operon is required for biphenyl degradation in *P. putida* CE2010, the *cmt*_{CE2010} operon in *P. putida* CE2010 was disrupted and then an intact *cmtE*_{CE2010} gene was introduced. When a Km^r gene and an *rrnB* terminator gene from *E. coli* were inserted into the *Eco*RV site of the *cmtAa*_{CE2010} gene in *P. putida* CE2010, the loss of biphenyl utility was observed. However, when pMTC101, *cmtE*_{CE2010} in pMTC67EH, was introduced into this insertion mutant, the ability to utilize biphenyl as sole carbon source for growth was restored with IPTG as the inducer. When *P. putida* F1 was transformed with pMTC101, it could utilize biphenyl as sole carbon source with IPTG as the inducer (data not shown). These results indicate that the *cmtE*_{CE2010} gene and the *tod*_{CE2010} operon are required for biphenyl utilization in the case of *P. putida* CE2010.

Sequence analysis of the *cmt*_{CE2010} operon in *P. putida* CE2010

The *cmtE*_{CE2010} gene in *P. putida* CE2010 is expressed in the presence of biphenyl, whereas that in *P. putida* F1 is not. Chromosomal DNA was isolated from *P. putida* CE2010 and from *P. putida* F1, then digested with various kinds of restriction enzymes and subjected to Southern hybridization with the *cmtE*_{CE2010} gene as a

Fig. 3. Sequence of the *cmt* promoter–operator region and its potential hairpin structure. The arrow indicates the position of the single base difference between the sequences of *P. putida* CE2010 and *P. putida* F1. The hairpin is between -30 and -10 in *P. putida* F1 and has a free energy value of -13.1 kcal. The change to T at -7 (indicated by the arrow) in *P. putida* CE2010 would reduce this to -8.8 kcal. The sequence is numbered according to Eaton (1997).

probe. The hybridization patterns showed no apparent difference between *P. putida* CE2010 and *P. putida* F1. Using pYOS13, we analysed the entire sequence of the *cmt*_{CE2010} operon from 11231 to 24937 (numbering according to Eaton, 1997) and a part of the *tod*_{CE2010} operon from 1 to 3545 (numbering according to Wang *et al.*, 1995) in *P. putida* CE2010. A single base difference was found upstream of the *cmt*_{CE2010} operon (Fig. 3). In *P. putida* F1, this region is the putative operator–promoter region of the *cmt*_{F1} operon and is located between the putative -35 , -10 (σ^{70}) promoter (Harley & Reynolds, 1987) and the beginning of *cmtAa*_{F1} (Eaton, 1997). It was proposed that this is a recognition site (operator) for CymR, the putative repressor protein regulating expression of the *cmt*_{F1} and *cym*_{F1} operons, and forms a hairpin structure (Eaton, 1997). In the case of *P. putida* F1, the hairpin is between -87 and -62 , where -1 is the base preceding the A of the ATG initiation codon of *cmtAa*_{F1}, and it has a free energy value of -13.1 kcal ($1 \text{ cal} = 4.184 \text{ J}$). In *P. putida* CE2010 the single base difference was located at the base of a putative hairpin structure (Fig. 3). In the case of *P. putida* CE2010 the value would be reduced to -8.8 kcal. This single base difference seems to generate instability in the hairpin structure and this is likely to affect the interaction with CymR_{CE2010}. We also analysed the sequence of *cymR*_{CE2010} in *P. putida* CE2010 and there was no difference compared to *cymR*_{F1} in *P. putida* F1.

Assay of the binding of CymR to the *cmt* promoter region

To compare the binding of CymR to the promoter region of the *cmt* operon of *P. putida* CE2010 and that of *P. putida* F1, a gel shift assay was performed using purified CymR and two synthetic DNA fragments. CymR was prepared as described in Methods. dsDNA fragments (100 bp) corresponding to the *cmt* promoter region of *P. putida* CE2010 (CEPro) and that of *P. putida*

Fig. 4. Comparison of the binding of CymR to the *cmt* promoter region of *P. putida* F1 (F1Pro) and *P. putida* CE2010 (CEPro). A gel shift assay was performed as described in Methods. Purified CymR was incubated with CEPro (a) or F1Pro (b). Lanes: 1, digoxigenin-labelled CEPro (a) or F1Pro (b) (40 fmol) alone; 2, 3, purified CymR (1 μ g) incubated with 40 (lane 2) or 200 fmol (lane 3) digoxigenin-labelled CEPro (a) or F1Pro (b); 4, purified CymR (1 μ g) was incubated with 200 fmol digoxigenin-labelled CEPro (a) or F1Pro (b) and 2 pmol unlabelled CEPro (a) or F1Pro (b).

F1 (F1Pro) were synthesized and labelled with digoxigenin at the 3' terminus. A mixture of these DNA fragments was used in the binding studies, incubated with the purified CymR.

A specific DNA–CymR complex was observed when labelled F1Pro was incubated with CymR (Fig. 4b). The retarded band disappeared upon addition of a 10-fold molar excess of unlabelled F1Pro. These results indicate that CymR binds to this region specifically. In contrast, a weak DNA–CymR complex band was observed when labelled CEPro was incubated with CymR (Fig. 4a). The single base difference in the *cmt*_{CE2010} promoter region of *P. putida* CE2010, compared to that of *P. putida* F1, resulted in a decreased amount of retarded DNA band. These results indicate that CymR binds less strongly to the *cmt*_{CE2010} promoter region of *P. putida* CE2010 than to the *cmt*_{F1} promoter region of *P. putida* F1. Thus, the observed single base difference affects the binding of the CymR regulator to this region. It is expected that the regulation of the *cmt*_{CE2010} operon in *P. putida* CE2010 is different from that in *P. putida* F1.

Exchanging the *cmt* promoter region between *P. putida* CE2010 and *P. putida* F1

To further elucidate the influence of the *cmt* promoter–operator region on biphenyl utilization *in vivo*, the *P. putida* CE2010 *cmt*_{CE2010} promoter region was replaced with the *P. putida* F1-type *cmt*_{F1} promoter region and vice versa. The recombinant *P. putida* CE2010 could not

Table 3. Exchange of *cmt* promoter–operator region between *P. putida* CE2010 and *P. putida* F1

The mutants were streaked on C medium plates containing Tc with the carbon sources indicated. The plates were incubated for 7 d at 30 °C.

C source	<i>P. putida</i> CE2010		<i>P. putida</i> F1	
	CE pro	F1 pro	CE pro	F1 pro
Toluene	+	+	+	+
Biphenyl	+	–	–	–
Cumate	+	+	+	+
Cymene	+	+	+	+

utilize biphenyl as sole carbon source (Table 3). This result supports the view that the *cmt*_{CE2010} promoter region in *P. putida* CE2010 is essential for its ability to utilize biphenyl. On the other hand, when the *P. putida* CE2010-type *cmt*_{CE2010} promoter region was introduced into *P. putida* F1, the recombinant *P. putida* F1 could not grow on biphenyl as sole carbon source as determined after 10 d incubation.

DISCUSSION

Biphenyl degrader *P. putida* CE2010 is an organic-solvent-tolerant bacterium capable of growth on toluene in LB medium and capable of degrading biphenyl in a two-phase system. *P. putida* CE2010 was found to have both the *tod*_{CE2010} operon and the *cmt*_{CE2010} operon and to utilize toluene, cumate or cymene as sole carbon source. The location of the *cmt*_{CE2010} and the *tod*_{CE2010} operons is the same as that in *P. putida* F1. *P. putida* CE2010 is very similar to *P. putida* F1 in this aspect.

Results obtained through purification of the HPDA-hydrolysing protein of *P. putida* CE2010 and determination of its N-terminal sequence indicate that CmtE_{CE2010} is involved in biphenyl utilization in *P. putida* CE2010. CmtE_{F1} encodes 2-hydroxy-6-oxo-7-methylocta-2,4-dienoate hydrolase (Eaton, 1996). The degree of amino acid sequence homology between CmtE_{CE2010} and other BphD-like hydrolases is relatively low. CmtE showed 27–33 % identity with BphD_{KKS102} (Kimbara *et al.*, 1989), BphD_{LB400} (Hofer *et al.*, 1993), BphD_{KF715} (Hayase *et al.*, 1990), BphD_{B356} (Ahmad *et al.*, 1995), BphD_{RHA1} (Masai *et al.*, 1997), BphD_{M5} (Lau *et al.*, 1996), TodF_{F1} (Menn *et al.*, 1991) and XylF_{mt2} (Horn *et al.*, 1991). The amino acid sequence identities between KKS102, LB400, KF715 and B-356 BphDs and those between XylF and TodF of *Pseudomonas* strains are much higher (69 and 53 %, respectively). The sequences of BphD_{LB400} and BphD_{M5} are only 42 % identical and that of BphD_{RHA1} is only 30 % identical to both of these. From the viewpoint of substrate specificity, some BphD proteins hydrolyse both HPDA and HOHD and show relatively broad substrate specificities. BphD_{LB400} hydrolyses relatively well

analogues of HPDA that contain at least a two-carbon group at C-6 (Seah *et al.*, 1998). The specificities of the enzyme for HPDA and 2-hydroxy-6-oxo-7-methylocta-2,4-dienoate differ only by a factor of five. So it may not be surprising that CmtE_{CE2010} can hydrolyse HPDA despite having relatively low homology with BphD.

The phenotype of the CmtE_{CE2010} disruption mutant and that of the *tod*_{CE2010} operon disruption mutant indicate that *P. putida* CE2010 degrades biphenyl by means of a mosaic of pathways consisting of enzymes encoded by the *tod*_{CE2010} and *cmt*_{CE2010} operons. The *cmt*_{CE2010}-operon-disrupted mutant could not utilize biphenyl, but when *cmt*_{CE2010} was introduced into the mutant, it was able to grow on biphenyl. These results indicate that *cmt*_{CE2010} and the *tod*_{CE2010} operon are responsible for degradation of biphenyl in the case of *P. putida* CE2010. In the case of *P. putida* F1, the range of compounds that can be degraded by the *tod*_{F1} pathway is limited by TodF_{F1}, which shows narrow substrate specificity. In *P. putida* CE2010, the recruitment of only one enzyme, CmtE_{CE2010}, which *P. putida* CE2010 has, is sufficient to expand the substrate specificity of the *tod*_{CE2010} pathway to include biphenyl.

Sequence analysis of the *cmt*_{CE2010} operon in *P. putida* CE2010 showed that there was a single base difference in the region upstream of the *cmt*_{CE2010} operon as compared to that in *P. putida* F1. In *P. putida* F1, this region was expected to be the CymR recognition site (Eaton, 1997). Eaton demonstrated that CymR is a repressor protein regulating expression of the *cym*_{F1} and *cmt*_{F1} operons. In *P. putida* CE2010, the single base difference was located within a predicted hairpin structure in the promoter region and was expected to destabilize the structure.

Results of the gel shift assay indicate that CymR binds less strongly to the *cmt*_{CE2010} promoter region derived from *P. putida* CE2010 than to the corresponding region from *P. putida* F1. These results indicate that the single base difference in the *cmt*_{CE2010} promoter region affects the binding of CymR to this region. It seems likely that, in *P. putida* CE2010, due to the single base difference in the promoter region, CymR does not efficiently repress the *cmt*_{CE2010} promoter, resulting in expression of the *cmt*_{CE2010} operon. These results explain the constitutive expression of CmtE_{CE2010} at a comparatively low level in *P. putida* CE2010 cultured with glucose as carbon source.

When the *cmt*_{CE2010} promoter in *P. putida* CE2010 was replaced with the *P. putida* F1-type *cmt*_{F1} promoter, the recombinant *P. putida* CE2010 could not utilize biphenyl as sole carbon source. This result supports the view that the single base difference in the *cmt*_{CE2010} promoter region of *P. putida* CE2010 is essential for its ability to degrade biphenyl. On the other hand, when the *cmt*_{CE2010} promoter from *P. putida* CE2010 was introduced into *P. putida* F1, the recombinant *P. putida* F1 could not grow on biphenyl as sole carbon source as determined after 7 d. However, after 2 weeks, several colonies appeared, whereas the wild-type *P. putida* F1

could not grow on biphenyl even when incubated for more than 2 weeks (data not shown). These findings suggest that the single base difference is necessary, but not sufficient for the expression of the *cmt* operon in *P. putida* F1 with biphenyl. It is suspected that the introduction of the *cmt*_{CE2010} promoter in *P. putida* F1 cannot cause the expression of CmtE in the modified F1 strain to the same level as in strain CE2010. Other factors in addition to the single base difference in the *cmt*_{CE2010} promoter region might be required for *P. putida* F1 to utilize biphenyl. This may explain why the wild-type *P. putida* F1 rarely turns into a biphenyl utilizer spontaneously. *P. putida* CE2010 is very similar to *P. putida* F1, but not identical. It is also suspected that the regulation of *cmt* operon expression in *P. putida* F1 is not always identical to that in *P. putida* CE2010.

Expression of the *tod* operon is regulated by the TodST system, a two-component system (Lau *et al.*, 1997). The *todS* gene encodes a sensory hybrid kinase and the *todT* gene product is an activator of *tod* structural genes. The toluene utilizer *P. putida* F1 converted biphenyl into the yellow *meta*-cleavage product HPDA through the action of the components of the *tod* pathway (Furukawa *et al.*, 1993). Although these authors did not mention the regulation of the expression of the *tod* operon in the presence of biphenyl, this indicates that in *P. putida* F1 biphenyl was transported into the cell and the Tod enzymes were expressed in the presence of biphenyl. It is possible that production of the *meta*-cleavage product is due to low constitutive expression of Tod enzymes, or the induced expression of Tod enzymes by biphenyl or an intermediate of the pathway in *P. putida* CE2010 as well as *P. putida* F1.

P. putida CE2010 showed weak HPDA hydrolysis activity (0.002 units mg⁻¹) when grown on glucose. It is expected that in *P. putida* CE2010, due to the single base difference in the promoter region, the expression of *cmt* is caused by ineffective repression by CymR, leading to low, constitutive expression.

When *P. putida* CE2010 was grown with biphenyl as sole carbon source, HPDA hydrolysis activity increased 10-fold. This indicates that in *P. putida* CE2010 the expression of the *cmt* operon is induced in the presence of biphenyl. In *P. putida* F1, CymR is a repressor protein regulating expression of the *cym* and *cmt* operons. CymR is 100% identical in CE2010 and F1. In *P. putida* F1, *p*-cumate acts as an effector of both the *cym* and *cmt* operons, whereas *p*-cymene is not an effector. Since the structures of *p*-cumate and biphenyl are quite different, biphenyl is not expected to be an effector of the *cmt* operon in *P. putida* CE2010. An intermediate of the pathway consisting of *cmt* and the *tod* operon might be an effector in *P. putida* CE2010. There is a possibility that in *P. putida* CE2010 regulatory proteins other than CymR are also involved in regulating expression of the *cmt*_{CE2010} operon in the presence of biphenyl. In *P. putida* F1, such mechanisms might not exist or may not be modulated by biphenyl or the intermediates or the products.

Our study serves to demonstrate how, with a slight change in a regulatory region, two distinct degradation pathways with distinct substrate spectra can expand substrate specificity via a mosaic of the pathways. It is also expected that not only the hydrolysis step, but also an earlier step, the *meta*-cleavage or later steps in the degradation pathway may expand substrate specificity in *P. putida* CE2010 as a result of the mosaic of pathways. Our results suggest that catabolic enzymes and catabolic gene regulators exhibit considerable evolutionary potential. Bacteria could acquire broad substrate specificities with a hybrid pathway consisting of the products of two distinct operons.

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