

Cloning and Expression in *E. coli* of the Genes Responsible for Degradation of 4-Chlorobenzoate and 4-Chlorocatechol from *Pseudomonas* sp. Strain S-47

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Pseudomonas sp. strain S-47 can grow on 4-chlorobenzoate (4CBA) and transform 4CBA to 4-chlorocatechol (4CC) under aerobic conditions, which is subsequently degraded to produce 2-hydroxypent-2,4-dienoate (2H-2,4DA). The upper steps for conversion of 4CBA to 4CC are recognized to be conducted by the benzoate-1,2-dioxygenase (B12O) system encoded by *benABC* and *benD*. The ensuing *meta*-cleavage reaction of 4CC is catalyzed by catechol 2,3-dioxygenase (C23O) encoded by the *xylE* gene. The *benABCD* and the *xylE* genes were cloned from the chromosome of *Pseudomonas* sp. S-47 into pCS1 (48.7 kb), pCS101 (24.4 kb), pCS201 (17.7 kb), and pCS202 (6.7 kb) recombinant plasmids, and were well expressed in *E. coli* XL1-Blue host cells. The *Pst*I-insert (4.0 kb) of pCS202 was found to contain the *benABCD* and *xylE* genes and to have 2 *Eco*RV, 1 *Sph*I, and 3 *Sac*II restriction sites.

Key words: *benABCD*, *xylE* 4-chlorobenzoate, 4-Chlorocatechol, dioxygenase, *Pseudomonas* sp. S-47

Aromatic hydrocarbons are biodegraded by oxidation reactions under aerobic conditions to produce catechol or catecholic compounds as common intermediates (3, 11). They are ultimately utilized for carbon and energy sources via the tricarboxylic acid cycle (3, 11, 21). Benzoate is also converted by aerobic bacteria to the corresponding *cis*-diol 2-hydro-1,2-dihydroxybenzoate (2H-1,2DHBA), and then to catechol, which is further degraded by one of two pathways (12). Catechol can be cleaved between the two hydroxyl groups via the *ortho* pathway (4). On the other hand, catechol cleavage via the *meta* pathway occurs at an extradiol position with respect to the hydroxyl groups, and subsequent reactions lead to the formation of 2-hydroxymuconic semialdehyde (or 5-chloro-2-hydroxymuconic semialdehyde from 4-chlorocatechol) as shown in Fig. 1, which is then degraded to pyruvate and acetaldehyde (8, 9).

The genes responsible for the conversion of benzoate and chlorobenzoate to the corresponding 2H-1,2DHBA have been isolated from aerobic bacteria and named *benABC* in *Acinetobacter calcoaceticus* (12) and *xylXYZ* in *Pseudomonas putida* mt2 (7).

The *benD* gene responsible for the conversion of 2H-1,2DHBA to catechol was also reported from *Acinetobacter calcoaceticus* (12). The *benABC* genes are generally called the benzoate-1,2-dioxygenase (B12O) system (12, 13). The *xylE* gene coding for catechol 2,3-dioxygenase (C23O) has been studied in various aerobic bacteria which are capable of degrading xylene (7), biphenyl (19), naphthalene (5), and phenol (18). Neidle *et al.* (12, 13) and other investigators (2, 4, 21) reported that the metabolite, 1, 2-dihydrodiol-4-chlorobenzoate (1,2DH-4CBA), produced by the benzoate dioxygenase system (*benABC*), could be transformed to catechol by a dehydrogenase encoded by the *benD* gene. Therefore, *benABC* (or *xylE*), *benD* (or *xylL*), and *xylE* are known to function consecutively in degradation of benzoate and related aromatic compounds. There have been several different nomenclatures (*ben*, *xyl*, *bph*, *nah*, *dmp*, *tod*, and *bnz*) for the isofunctional genes specifying the catabolic pathway of benzoate and related aromatic compounds as shown in Table 1.

Aromatic *meta*-ring dioxygenases for benzoate and related aromatics are known to be complexes formed by three- or four-protein subunits (12). The dioxygenases encoded by *benABC* (12), *xylXYZ* (7), *bphXYZ* (19), *nahAcAdAbAa* (5), and *todC1C2AB* (21)

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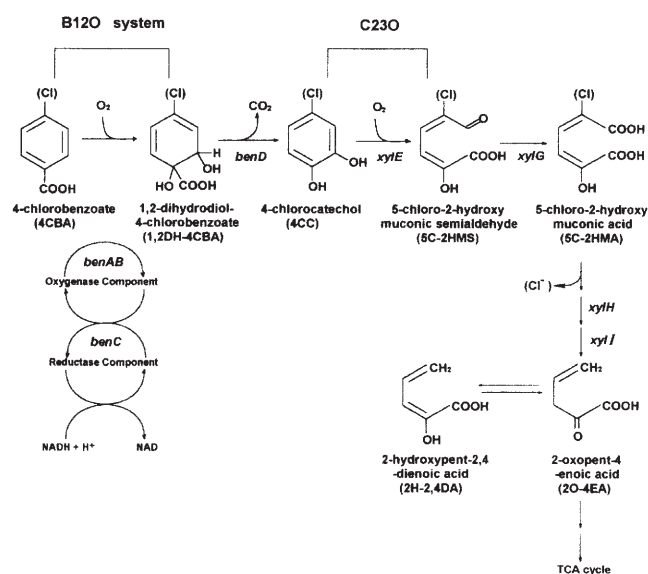


Fig. 1. Pathway for biodegradation of 4-chlorobenzoate by *Pseudomonas* sp. S-47. The genes coding for the enzymes responsible for each step are indicated as follows: *benABC*, benzoate 1,2-dioxygenase (B12O); *benD*, benzoate *cis*-diol dehydrogenase; *xylE*, catechol 2,3-dioxygenase (C23O); *xylG*, 2-hydroxymuconic semialdehyde dehydrogenase; *xylH*, 4-oxalocrotonate isomerase; *xylI*, 4-oxalocrotonate decarboxylase.

are multicomponent enzymes consisting of a short electron transport system with two or three different subunits as shown in Fig. 1.

The *meta*-cleavage C23O enzyme is recognized to be encoded by *xylE* (3), *nahH* (5), *todE* (21), *bphE* (19), and *dmpB* (18) in a similar way. The C23O isolated from *Pseudomonas putida* mt2 was reported to consist of a homo-subunit that contains ferrous ion as a functional cofactor (1). Such characteristics of the C23O were similar to those of dioxygenases including NahH, TodE, and BphE produced from different strains (3, 5).

Pseudomonas sp. S-47 is a bacterial strain that was isolated from contaminated wastes by Seo *et al.* (17). The strain is capable of transforming 4-chlorobenzoate (4CBA) to 4-chlorocatechol (4CC) under aerobic conditions, which is subsequently degraded by *meta*-ring cleavage to produce 5-chloro-2-hydroxymuconic semialdehyde (5C-2HMS) as shown in Fig. 1. The C23O enzyme for 4CC degradation has been isolated and compared to other isofunctional dioxygenases for its substrate specificity (10). However, the genes of *Pseudomonas* sp. S-47 responsible for the degradation of 4CBA/benzoate and 4CC/catechol have not been examined for comparison with isofunctional genes reported in other strains. In this study, therefore, the *benABCD* and *xylE* genes were cloned from the chromosomal DNA of *Pseudomonas* sp. S-47 in order to elucidate the genetic organization and nucleotide sequences of the genes and were examined for their expression in *E. coli* XL1-Blue.

Materials and Methods

Strains, plasmids, and culture media

The bacterial strains and plasmids used in this study are summarized in Table 2. *Pseudomonas* sp. S-47 is a natural isolate capable of degrading 4CBA and 4CC under aerobic conditions (17). *E. coli* LE392 and *E. coli* XL1-Blue were used as host strains for the cloning of the genes encoding 4CBA and 4CC dioxygenases. Other *E. coli* strains were the transformants carrying corresponding recombinant plasmids. Cosmid pWE15 and plasmid pUC18 were purchased from Stratagene Ltd (La Jolla, CA, USA) for use as cloning vectors.

Pseudomonas sp. S-47 and the cloned cells were cultivated at 37°C in Luria-Bertani (LB) broth

Table 1. Enzymes and gene designation for the *meta*-pathway operons for catabolism of benzoate and related aromatic compounds

Gene designation	<i>ben</i>	<i>xyl</i>	<i>bph</i>	<i>nah</i>	<i>dmp</i>	<i>tod</i>	<i>bnz</i>
Benzoate 1,2-dioxygenase (B12O)	<i>benABC</i>	<i>xylXYZ</i>	<i>bphXYZ</i>	<i>nahAcAdAbAa</i>		<i>todC1C2AB</i>	<i>bnzABC</i>
Benzoate <i>cis</i> -diol dehydrogenase (BDH)	<i>benD</i>	<i>xylL</i>	<i>bphL</i>	<i>nahB</i>		<i>todD</i>	<i>bnzE</i>
Catechol 2,3-dioxygenase (C23O)		<i>xylE</i>	<i>bphE</i>	<i>nahH</i>	<i>dmpB</i>	<i>tdE</i>	
2-Hydroxymuconic semialdehyde dehydrogenase (HMSD)		<i>xylG</i>	<i>bphG</i>	<i>nahI</i>	<i>dmpC</i>		
4-Oxalocrotonate isomerase (4OI)		<i>xylH</i>	<i>bphH</i>	<i>nahK</i>	<i>dmpI</i>		
4-Oxalocrotonate decarboxylase (4OD)		<i>HxylI</i>	<i>bphI</i>	<i>nahJ</i>	<i>dmpH</i>		
Sources: Strain	<i>Acinetobacter calcoaceticus</i>	<i>Pseudomonas putida</i> mt2	<i>Pseudomonas pseudoalcaligenes</i>	<i>Pseudomonas putida</i> G7	<i>Pseudomonas</i> sp. strain CF600	<i>Pseudomonas putida</i> F1	<i>Pseudomonas putida</i> 136-R3
Substrate	benzoate	xylene	biphenyl	naphthalene	methylphenol	toluene	benzene
Reference	(12, 13)	(4, 7)	(19)	(5)	(18)	(21, 22, 23)	(8, 20)

Table 2. Bacterial strains and plasmids used in this study

Strain and plasmid	Relevant marker	Source and Reference
Strain		
<i>Pseudomonas</i> sp. S-47	4CBA ⁺ , BA ⁺ , Cat ⁺	Seo <i>et al</i> (17)
<i>E. coli</i> CS1	<i>E. coli</i> LE392 carrying pCS1, Ap ^r , 4CBA ⁺ , BA ⁺ , Cat ⁺	This study
<i>E. coli</i> CS101	<i>E. coli</i> XL1-Blue carrying pCS101, Ap ^r , 4CBA ⁺ , BA ⁺ , Cat ⁺	"
<i>E. coli</i> CS201	<i>E. coli</i> XL1-Blue carrying pCS201, Ap ^r , 4CBA ⁺ , BA ⁺ , Cat ⁺	"
<i>E. coli</i> CS202	<i>E. coli</i> XL1-Blue carrying pCS202, Ap ^r , 4CBA ⁺ , BA ⁺ , Cat ⁺	"
<i>E. coli</i> XL1-Blue	<i>supE44 hsdR17 recA1 endA1 thi F'</i> [<i>proAB⁺ lacI^s lacZ</i> △M15 Tn10(<i>tet^r</i>)]	Stratagene Ltd.
<i>E. coli</i> LE392	F- <i>hsdR514</i> (<i>r_k⁻ m_k⁻</i>) <i>supE44 supR58 lacY1</i> or △(<i>lacIZY</i>)6 <i>galK2 galT22 metB1 trpR55 λ</i>	"
Plasmid		
pCS1	Ap ^r , insert of 40 kb <i>Sau3AI</i> fragment carrying 4CBA-degrading gene from chromosomal DNA of <i>Pseudomonas</i> sp. S-47 in <i>Bam</i> HI site of pWE15	This study
pCS101	Ap ^r , insert of 16 kb <i>Bam</i> HI fragment from pCS1 in <i>Bam</i> HI site of pWE15	"
pCS201	Ap ^r , insert of 15 kb <i>Bam</i> HI fragment from pCS101 in <i>Bam</i> HI site of pUC18	"
pCS202	Ap ^r , insert of 4 kb <i>Pst</i> I fragment from pCS201 in <i>Pst</i> I site of pUC18.	"
pUC18	Ap ^r , contains <i>lacZ</i> promoter for fusion protein induction	Stratagene Ltd.
pWE15	Ap ^r , cosmid vector	"

Abbreviation : 4CBA, 4-chlorobenzoate; BA, benzoate; Cat, catechol; Ap, ampicillin

medium or Minimal medium supplemented with 0.2 mM 4CBA or 4CC, 0.2% glucose, and 0.2% maltose according to the method described by Savard *et al.* (16). When required, the medium was solidified with 1.5% agar and added with 100 µg/ml ampicillin for selection of the cloned cells.

DNA manipulation and electrophoresis

Restriction enzymes, T4 ligase, and other enzymes were obtained from Promega Co. Ltd., (Madison, WI, USA). The enzymes were used for DNA manipulation according to the procedures described by Sambrook *et al.* (15). The recombinant plasmids and their products cleaved by restriction endonucleases were analyzed on 0.8% agarose gels, along with *Hind*III- or *Hind*III-*Eco*RI-fragments of lambda phage as size markers. Electrophoresis was performed in TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0) at 5 v/cm for 1 hour.

Cloning of dioxygenase genes

The genomic DNA of *Pseudomonas* sp. S-47 and plasmid DNA were prepared by the methods described by Birnboim and Doly (2). The total DNA of *Pseudomonas* sp. S-47 was digested with *Sau*3AI to generate predominantly 40.7 kb fragments. The *in vitro* manipulations for insertion of the *Sau*3AI fragment into the *Bam*HI-digested cosmid pWE15, *in vitro* packaging, and transduction into *E. coli* LE392 were carried out by the methods of Savard *et al.* (16). *E. coli* LE392 containing the hybrid cosmids were cultivated on solid minimal medium containing 1 mM 4CBA, 0.2% glucose, 0.2% maltose, and 100 µg/ml ampicillin

for screening of the cloned cells. A colony showing yellow color after the spraying of 1 mM 4CC solution onto the plate was selected and designated as *E. coli* CS1. The hybrid plasmid, pCS1, was isolated from *E. coli* CS1 by alkaline lysis, and then digested with *Bam*HI for construction of pCS101 by self-ligation. The 15-kb fragment obtained by digestion of pCS101 with *Bam*HI was inserted into the *Bam*HI-digested pUC18 plasmid by an *in vitro* ligation method. The resulting recombinant plasmid, pCS201, was digested again with *Pst*I and then self-ligated to construct pCS202 by the methods described above.

Those recombinant plasmids were transformed into *E. coli* XL1-Blue according to the method of Sambrook *et al.* (15). The transformant strains were selected on LB medium containing 100 µg/ml ampicillin, 15 µg/ml tetracycline, 40 µl X-gal, and 4 µl isopropylthio-β-D-galactoside (IPTG). The cloned cells carrying the *xylE* gene were identified by yellow coloring when 1 mM 4CC solution was sprayed over the colonies.

Resting cell assay for 4CBA and 4CC degradation

The degradation of 4CBA and 4CC by the cloned cells was examined by a resting cell assay as described by Harayama and Rekik (5) and Kim and Zylstra (11). The cloned cells grown in LB broth for 12 hours were harvested by centrifugation at 2,000×g. The collected cells were inoculated in 10 mM phosphate buffer containing 0.2 mM 4CBA or 4CC, and then incubated at 37°C for an appropriate period of time. Degradation of 4CBA and

4CC was examined by scanning the metabolite at wavelengths between 200 to 450 nm with a UV-visible spectrophotometer. The metabolites 5C-2HMS and 2H-2,4DA, which were produced from the substrates by the cells, were detected at 380 nm and 265 nm, respectively.

Results and Discussion

Cloning of *benABCD* and *xylE* genes

The genes responsible for the degradation of 4-chlorobenzoate (4CBA) to produce 2-hydroxypent-2,4-dienoate (2H-2,4DA) were cloned from the chromosome of *Pseudomonas* sp. S-47 using the *Bam*HI-digested pWE15 cosmid vector. The resulting recombinant plasmid was designated as pCS1 (48.7 kb) and contained a 40.7 kb *Sau*3AI fragment carrying the *benABCD*, *xylE*, and *xylGHI* genes for degradation of 4CBA. pCS1 was digested with *Bam*HI and then self-ligated to obtain pCS101, a subclone (24.4 kb) that contained a 16.4 kb insert harboring the *benABCD* and *xylE* genes. The pCS201 and pCS202 subclones which contained 15 kb and 4 kb fragments, respectively, were constructed by the subcloning strategy using pUC18 vector described in Materials and Methods.

The electrophoresis patterns of pCS1, pCS101,

and pCS201 digested with *Bam*HI are shown in Fig. 2. pCS1 (lane E) showed five *Bam*HI sites, four of which were found on the 40.7 kb insert. The 24.4 kb pCS101 (lane D) subcloned from pCS1 exhibited only 2 *Bam*HI sites. This indicates that the 16.4 kb *Sau*3AI-*Bam*HI fragment was inserted as part of pCS101 when the *Bam*HI digests of pCS1 were self-ligated. Two *Bam*HI sites were observed in the 17.7 kb pCS201 (lane C), which indicates that there is no *Bam*HI site on the DNA insert (15 kb) carrying the *benABCD* and *xylE* genes.

When the pCS201 digested with *Pst*I enzyme was self-ligated, the pCS202 subclone (6.7 kb) containing a 4.0 kb insert was obtained. pCS202 exhibited 4CBA and 4CC dioxygenase activities encoded by the *benABCD* and *xylE* genes. The electrophoresis patterns of pCS202 (6.7 kb) digested with various restriction enzymes are shown in Fig. 3. The *Pst*I fragment (4.0 kb) containing the *benABCD* and *xylE* genes was analyzed for restriction mapping. Two *Eco*RV, one *Sph*I, and three *Sac*II sites were observed on the DNA insert carrying the *benABCD* and *xylE* genes (Fig. 4).

The *benABCD* and corresponding isofunctional genes have been cloned from *Acinetobacter calcoaceticus* (12, 13), and *Pseudomonas putida* (9). The nucleotide sequences of the *benABC* and *benD* genes were also reported in *A. calcoaceticus* (13) and *Pseudomonas pseudoalcaligenes* KF707 (19). Furthermore, the *xylE* genes cloned from *Pseu-*

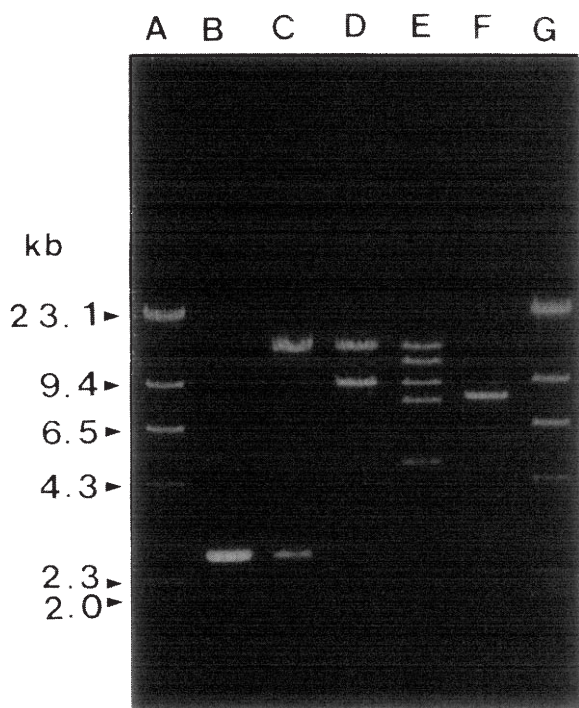


Fig. 2. Electrophoresis pattern of the plasmids digested with *Bam*HI. Lanes: A and G, size marker of *Hind*III-digested lambda DNA; B, pUC18; C, pCS201; D, pCS101; E, pCS1; F, pWE15.

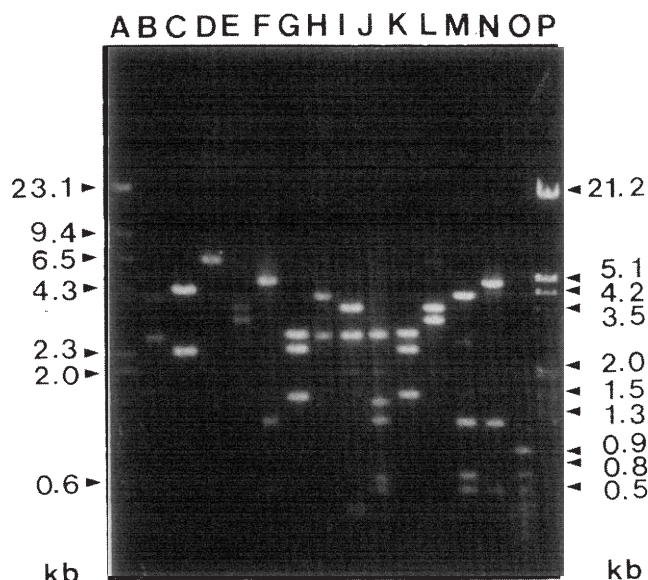


Fig. 3. Restriction analysis of recombinant plasmid pCS202 digested with various enzymes. Lanes: A, λ DNA-*Hind*III; B, *Bam*HI; C, *Eco*RV; D, *Hind*III; E, *Sph*I; F, *Sac*II; G, *Bam*HI-*Eco*RV; H, *Bam*HI-*Hind*III; I, *Bam*HI-*Sph*I; J, *Bam*HI-*Sac*II; K, *Hind*III-*Eco*RV; L, *Hind*III-*Sph*I; M, *Hind*III-*Sac*II; N, *Sph*I-*Sac*II; O, *Sau*3AI; P, λ DNA-*Hind*III-*Eco*RI.

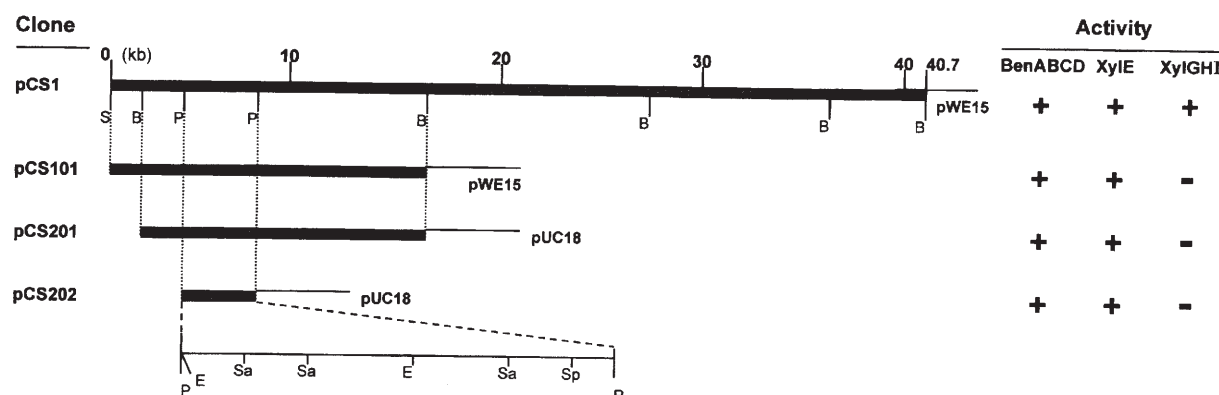


Fig. 4. Physical maps and degradation activities of the pCS1 recombinant plasmid and its subclones. Abbreviation: S, *Sau* 3AI; B, *Bam*HI; P, *Pst*I; Sa, *Sac*II; E, *Eco*RV; Sp, *Sph*I.

Pseudomonas cepacia G4 (14) and *Pseudomonas* sp. strain IC (3) were analyzed for their nucleotide sequences, which are composed of 945 and 1,241 base pairs, respectively. Thereby, the genes specifying for B12O and C23O dioxygenases in *Pseudomonas* sp. S-47 were proven to be localized in the 4.0 kb DNA insert of the pCS202 clone.

Expression of *benABCD* and *xylE*

The recombinant plasmids pCS1, pCS101, pCS

201, and pCS202 containing *benABCD* and *xylE* genes were transformed into *E. coli* XL1-Blue to obtain *E. coli* transformants CS1, CS101, CS201, and CS202, respectively. These transformant cells as well as the *Pseudomonas* sp. S-47 wild type strain were examined for their dioxygenase activities on 4CBA and 4CC by resting cell assay as shown in Fig. 5 and 6. During 24 hours of incubation, *E. coli* CS1 (Fig. 5A) degraded 4CBA (peak I) to produce 5-chloro-2-hydroxymuconic semialdehyde (5C-2HMS) (peak III), which was then transformed to produce 2H-2,4,4DA (peak IV) in a process of reaction incubation. *E. coli* CS1 degraded 4CC (peak II) more rapidly to produce 5C-2HMS (peak III), which was then transformed to 2H-2,4,4DA (peak IV) as incubation time was extended (Fig. 5B). The metabolite 5C-2HMS which was produced from 4CC by the C23O enzyme encoded by *xylE* of *E. coli* CS1 was continuously degraded to 2H-2,4,4DA by the *xylGHI* products. The metabolite 2H-2,4,4DA was detected at 265 nm in *E. coli* CS1 and *Pseudomonas* sp. S-47, as reported by Harayama *et al.* (6). This result indicates that the DNA insert (40.7 kb) of pCS1 carries *xylGHI* as well as the dioxygenase genes (*benABC* and *xylE*) and 1,2DH-4CBA dehydrogenase (*benD*) responsible for 4CBA and 4CC degradation.

However, the *E. coli* CS202 strain harboring pCS 202 which carried the *benABCD* and *xylE* genes degraded 4CBA (Fig. 6A) as well as 4CC (Fig. 6B) to produce 5C-2HMS (peak III), which accumulated without further degradation even after extended incubation. Such accumulation of 5C-2HMS was also observed in the experiments with *E. coli* CS101 or CS201 strains. These results indicated that pCS202 (4.0 kb) as well as pCS101 and pCS201 retained the *benABCD* and *xylE* genes – *xylGHI* genes were deleted from the pCS1 in the process of subcloning. Neidle *et al.* (13) cloned the *benABCD* genes from

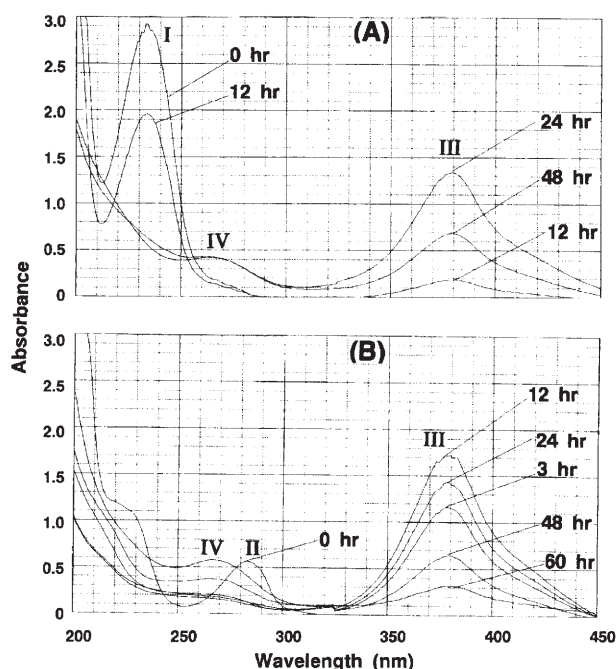


Fig. 5. Biodegradation of 4-chlorobenzoate (A) and 4-chlorocatechol (B) by the recombinant strain *E. coli* CS1. The cells were incubated in 10 mM potassium phosphate buffer containing 0.2 mM 4-chlorobenzoate or 0.2 mM 4-chlorocatechol. Peaks: I, 4-chlorobenzoate (4CBA); II, 4-chlorocatechol (4CC); III, 5-chloro-2-hydroxymuconic semialdehyde (5C-2HMS); IV, 2-hydroxypent-2,4-dienoic acid (2H-2,4DA)

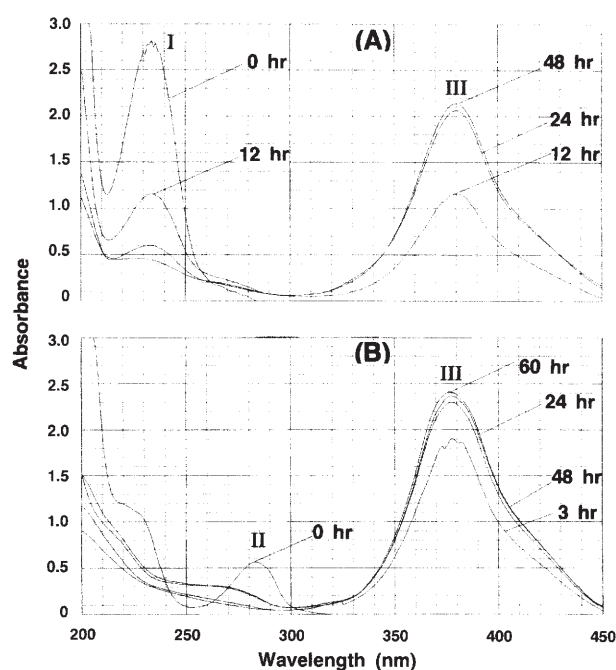


Fig. 6. Biodegradation of 4-chlorobenzoate (A) and 4-chlorocatechol (B) by the recombinant strain *E. coli* CS202. The cells were incubated in 10 mM potassium phosphate buffer containing 0.2 mM 4-chlorobenzoate or 0.2 mM 4-chlorocatechol. Peaks: I, 4-chlorobenzoate (4CBA); II, 4-chlorocatechol (4CC); III, 5-chloro-2-hydroxymuconic semialdehyde (5C-2HMS).

A. calcoaceticus and expressed the cloned *benABCD* dioxygenase system and *benD* together at high levels in *E. coli*. The *benABCD* genes of *P. putida* were reported to be regulated by *benR* for their expression (9). Therefore, the expression in *E. coli* of *benABCD* and *xylE* genes cloned from *Pseudomonas* sp. S-47 in this study suggests that the regulatory *benR* gene is also included in the insert (4.0 kb) of the pCS202.

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