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Dechlorination of 4-Chlorobenzoate by *Pseudomonas* sp. DJ-12

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4-Chlorobiphenyl-degrading *Pseudomonas* sp. DJ-12 was able to degrade 4-chlorobenzoate (4CBA), 4-iodobenzoate, and 4-bromobenzoate completely under aerobic conditions. During the degradation of 4CBA by *Pseudomonas* sp. DJ-12, chloride ions were released by dechlorination and 4-hydroxybenzoate was produced as an intermediate metabolite. The *Not*I-DNA fragments of pKC157 containing dechlorination genes hybridized with the gene encoding 4CBA : CoA dehalogenase of *Pseudomonas* sp. CBS3 which is responsible for the hydrolytic dechlorination of 4CBA. These results imply that *Pseudomonas* sp. DJ-12 degrades 4CBA to 4-hydroxybenzoate via dechlorination as the initial step of its degradative pathway. The genes responsible for dechlorination of 4CBA were found to be located on the chromosomal DNA of *Pseudomonas* sp. DJ-12.

Key words: 4-Chlorobenzoate, dechlorination, *Pseudomonas* sp. DJ-12

Chlorinated aromatic compounds are one group of most serious environmental pollutants, because they are highly resistant to biodegradation and toxic to living organisms in the environment (7, 15). Therefore, microbial degradation of these pollutant chemicals has been investigated under both laboratory and *in situ* conditions. In particular, the biochemical reactions of dechlorination and ring-cleavage of the compounds as well as expression of the genes for these reactions have been intensively studied, since they are the most crucial steps for biodegradation of the chloroaromatics (10, 11, 22). 4-Chlorobenzoate (4CBA) is a metabolite produced by the degradation of various chloroaromatics such as herbicides and polychlorinated biphenyls. 4CBA could be degraded by several microorganisms via dechlorination to produce protocatechuate or via ring-cleavage after it is converted to 4-chlorocatechol as shown in Fig. 1 (11, 15, 19).

Several microorganisms such as *Pseudomonas* spp. (2, 6) and *Arthrobacter* spp. (8, 18, 20) have been reported to degrade 4CBA by hydrolytic dechlorination and ring-cleaving dioxygenation. Particularly, the hydrolytic dechlorination of 4CBA to 4-hydroxybenzoate (4HBA) was recognized to be carried out by a sequencial reaction involving 4CBA : CoA ligase, 4CBA : CoA dehalogenase, and 4HBA : CoA thioesterase (4, 6). The genes encoding these three enzymes have been sequenced in *Pseudomonas* sp. CBS3 (4) and the 4CBA : CoA dehalogenase involved in the dechlorination has been partially characterized in those microorganisms (6, 8).

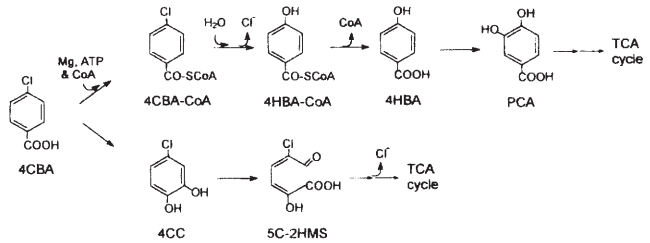

Fig. 1. Metabolic pathway of 4-chlorobenzoate. Abbreviation: 4CBA, 4-chlorobenzoate; 4HBA, 4-hydroxybenzoate; PCA, protocatechuate; 4CC, 4-chlorocatechol; 5C-2HMS, 5-chloro-2-hydroxymuconic semialdehyde; CoA, coenzyme A; TCA, tricarboxylic acid.

Pseudomonas sp. DJ-12, an aerobic isolate of a 4-chlorobiphenyl (4CB) and biphenyl degrader, has been studied in relation to the ring-cleavage reaction of 4CB (13) and genetic structure of the dioxygenase gene (12). 4CBA produced from 4CB could be readily degraded by this organism, but the mechanism for degradation of 4CBA has not been investigated. In this study, therefore, the mechanism of 4CBA degradation by *Pseudomonas* sp. DJ-12 and the localization of the genes responsible for degradation were investigated.

Materials and Methods

Bacterial strain and growth medium

Pseudomonas sp. DJ-12 was isolated from con-

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taminated wastewater (13). This organism was cultivated in Luria-Bertani (LB) medium supplemented with ampicillin (50 μg/ml) as a selective marker. The chloride-free minimal medium was used for detection of chloride ions produced from 4CBA by dechlorination as described by Tsoi *et al.* (21). The minimal medium consisted of 1 mM 4CBA, 4 g K_2HPO_4, 0.4 g $NaH_2PO_4 \cdot H_2O$, 0.5 g $(NH_4)_2SO_4$, 0.15 g $MgSO_4 \cdot H_2O$, 0.01 g $Ca(NO_3) \cdot H_2O$, 0.2 g trypton, and 0.1 g yeast extract in 1 L distilled water.

DNA manipulation

Total genomic DNA of *Pseudomonas* sp. DJ-12 was extracted as described by Ausubel *et al.* (3). DNA manipulation including the isolation of plasmid DNA, enzyme digestion, and electrophoresis was done as described by Sambrook *et al.* (16). The cosmid pWE15 and *E. coli* LE392 were used as vector and host strain, respectively. Genomic DNA of *Pseudomonas* sp. DJ-12 and pWE15 cosmid vector digested with *Sau*3AI and *Bam*HI, respectively, were ligated together for 16 hours at 16°C. After *in vitro* packaging of the ligation mixtures, the genomic library was constructed by transfection into *E. coli* LE392 according to the supplier's manual (Promega). Cloned cells showing dechlorinationa activity were selected from the transfectants by examination of chloride ion production after incubation for 1 day in 96-well microtiter plates.

Southern hybridization

DNA fragments were transferred from the electrophoresed gel to a nylon membrane using 1 N NaOH as described by Koetsier *et al.* (14). Hybridization was performed with the ECL direct nucleic acid labelling and detection systems (Amersham) according to the manufacturer's instruction. The DNA fragments used as probe were provided with the QIAEX kit (QIAGEN, Hilden, Germany).

UV-visible spectrophotometry

Resting cell assay was carried out for detection of the substrate and metabolite by the method described by Arensdorf and Focht (1). The samples prepared for analysis were scanned from 200 nm to 300 nm with a UV-visible spectrophotometer (LKB 4060, Pharmacia, Sweden). 4CBA and 4HBA were examined at 234 nm and 245 nm, respectively.

High pressure liquid chromatography

The haloaromatics were quantified by HPLC as described by van den Tweel *et al.* (22). The HPLC apparatus was equipped with a Waters 510 HPLC pump and Waters 486 tunable absorbance detector (Waters). Substrates were separated on a μBondapak™ C18 column (diameter, 3 mm; length, 300 mm) at a flow rate of 0.4 ml/min with mobile phase of methanol-water-acetic acid (60:40:1). The substrates were monitored by absorbance at 254 nm.

GC-mass spectrometry

The metabolite samples collected from the culture were methylated with diazomethane as described by Arensdorf and Focht (2). Mass spectra were produced with a Hewlett-Packard model 5890 GC-mass spectrometer equipped with mass selective detector 5972 Metabolites were separated on a HP-Innowax capillary column (length, 30 m; diameter, 0.32 mm; film thickness, 0.25 μm). The temperature was increased from 120°C (holding time, 2 min) to 280°C (holding time, 1 min) at a rate of 4°C/min. The temperature of the injector and detector were 220 and 200°C, respectively.

Chloride ion determination

Chloride ions released by the dechlorination of 4CBA were quantified colorimetrically by measuring the absorbance at 453 nm after reaction of the supernatant of *Pseudomonas* sp. DJ-12 culture with 0.069% $Hg(SCN)_2$ and 0.25 M ferric ammonium sulfate by the methods described by Bergman and Sanik (5).

Results and Discussion

Degradation of halobenzoates

The degradation of several halogenated benzoates by *Pseudomonas* sp. DJ-12 is shown in Table 1. 4-Iodobenzoate and 4-bromobenzoate were readily degraded by the organism as potentially as 4-chlorobenzoate. The degradation rate of 4-fluorobenzoate by this organism was only 40%. Howe-

Table 1. Degradation of several haloaromatics by *Pseudomonas* sp. DJ-12 via dehalogenation

Substrate	Initial conc. (mM)	Final conc. (mM)	Chloride ion (mM)	Ratio of degradation (%)
4-chlorobenzoate	0.5	0.01	0.5	100
3-chlorobenzoate	0.5	0.5	<0.01	0
2-chlorobenzoate	0.5	0.5	<0.01	0
2,4-dichlorobenzoate	0.5	0.5	<0.01	0
2,4-dichlorophenoxy-acetate	0.5	0.5	<0.01	0
4-fluorobenzoate	0.5	0.3	ND[a]	40
4-iodobenzoate	0.5	<0.01	ND	100
4-bromobenzoate	0.5	<0.01	ND	100

[a] ND, not determined.

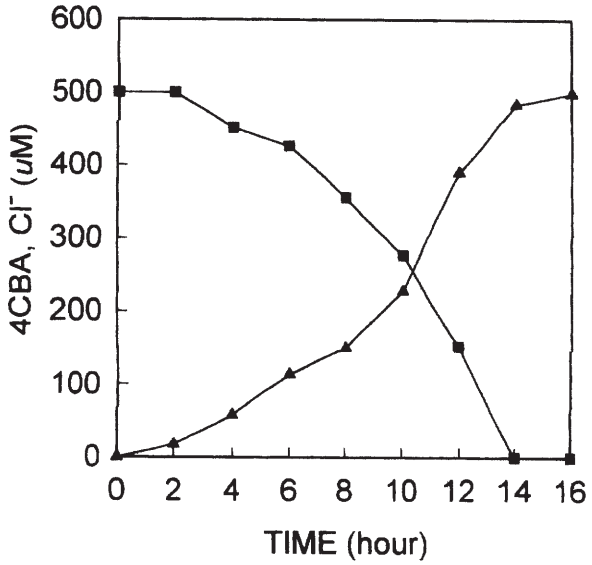

Fig. 2. Dechlorination of 4-chlorobenzoate by *Pseudomonas* sp. DJ-12 releasing chloride ions. ■, 4-chlorobenzoate; ▲, chloride ion.

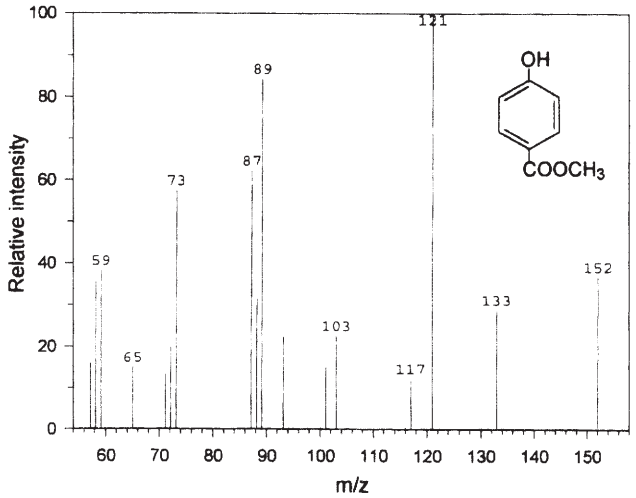

Fig. 3. Mass spectrum of the metabolite identified as the methylester of 4-hydroxybenzoate produced by dechlorination of 4-chlorobenzoate.

ver, the benzoates, which are substituted with chlorine at the second and/or third carbon positions, were not degraded by *Pseudomonas* sp. DJ-12 in this study. van den Tweel *et al.* (22) also reported that halobenzoates substituted with different halogens at the same position were equally degraded by *Alcaligenes denitrificans* NTB-1, except for fluorobenzoate. The dehalogenation of different 4-halobenzoates was reported to be specified by 4-halobenzoyl CoA dehalogenase in *Arthrobacter* sp. strain 4-CB1 (8). These results indicate that degradation specificity of the halogenated benzoates are mainly determined by the substituted position of halogen atoms.

Pseudomonas sp. DJ-12 completely degraded 0.5 mM of 4CBA within 16 hours, releasing a corresponding amount of chloride ions as shown in Fig. 2. That the degradation of 4CBA by *Pseudomonas* sp. DJ-12 occurs via dechlorination was shown by gas chromatography. 4-Hydroxybenzoate produced by the dechlorination of 4CBA was identified by mass spectrometry as shown in Fig. 3. The methylester of 4HBA was matched with an authentic standard substance which has a molecular ion at m/z 152 and major fragment ion at m/z 121 ($M\text{-}OCH_3$). These results imply that *Pseudomonas* sp. DJ-12 degrades 4CBA to 4HBA via hydrolytic dechlorination which releases chloride ions.

The genes for hydrolytic dechlorination of 4CBA

The genes responsible for the dechlorination of 4CBA were cloned from the chromosomal DNA of *Pseudomonas* sp. DJ-12 to obtain pKC1. The clones of pKC152, pKC157, and pKC16 exhibiting dechlorination activity on 4CBA were constructed from pKC1 by a deletion method using various endonucleases as described by Sambrook *et al.* (16). The physical maps and dechlorination activities of the clones are shown in Fig. 4. The clone of pKC157 dechlorinated 0.5 mM 4CBA completely to 4HBA when incubated together for 12 hours as shown in Fig. 5. The absorbance peak of 4CBA at 234 nm was shifted to 245 nm which absorbes 4HBA as a function of incubation time. The resulting 4HBA was accumulated in the culture medium after the complete degradation of 4CBA. This was due to lack of enzymes in the cloned cells for the further degradation of 4HBA. When the mixture of 4CBA and 4HBA was examined by UV-spectrophotometry, a single absorbance peak appeared between 234 nm and 245 nm depending on the ratio of two compounds, without showing two peaks at both 234 and 245 nm. These results indicate that the clones constructed in this study contain the genes for dechlorination of 4CBA.

The genes (*fcbA, B, C*) for hydrolytic dechlorination have been reported to encode 4CBA : CoA li-

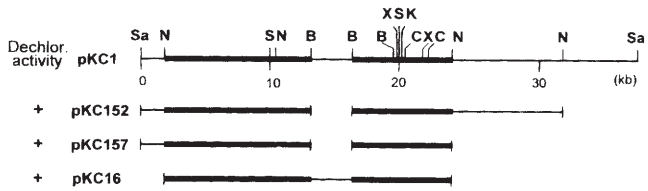

Fig. 4. Physical maps of the recombinant plasmids containing dechlorination gene and their dechlorination activities on 4-chlorobenzoate. Abbreviation: B, *Bam*HI; C, *Cla*I; K, *Kpn*I; N, *Not*I; S, *Sal*I; Sa, *Sau*3AI; X, *Xho*I.

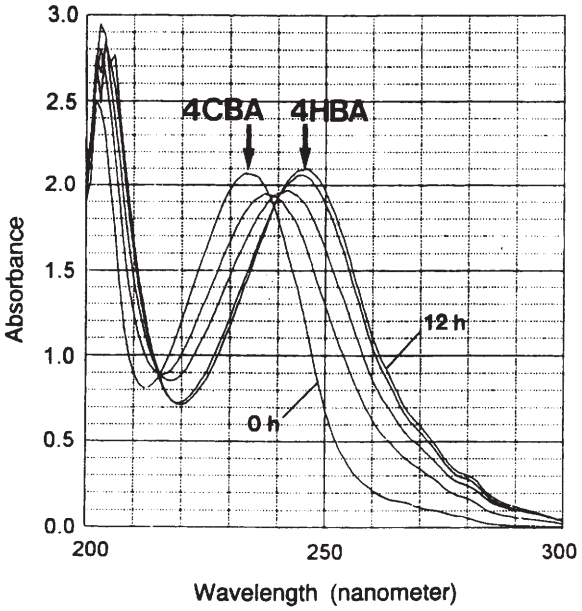

Fig. 5. Conversion of 4-chlorobenzoate to 4-hydroxybenzoate by *E. coli* KC157. The UV-spectra shows the shift of the absorbance peaks from 234 nm (4-chlorobenzoate) to 245 nm (4-hydroxybenzoate) during dechlorination of 4-chlorobenzoate.

gase, 4CBA : CoA dehalogenase, and 4HBA : CoA thioesterase, respectively, in *Pseudomonas* sp. CBS3 (4) and *Arthrobacter* spp. (18, 21). The clone of pKC 157 hybridized with the *fcbB* gene of pCBSII (9) cloned from *Pseudomonas* sp. CBS3 as DNA probe. The *Bam*HI DNA fragments specifically hybridized with the dehalogenase gene as shown in lanes 2, 3, and 4

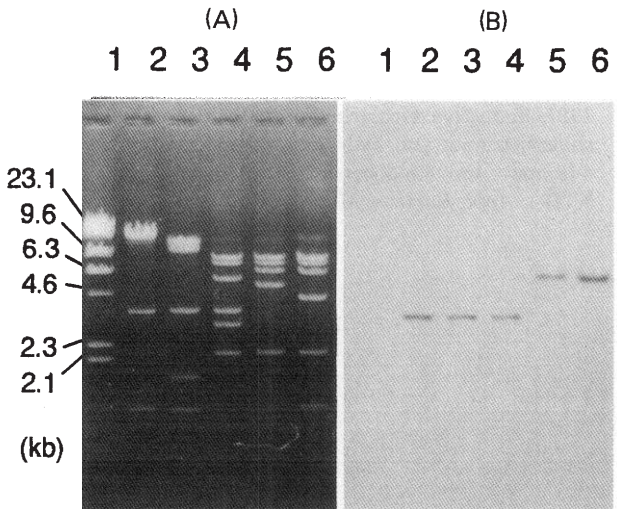

Fig. 6. Electrophoresis (A) and Southern hybridization (B) of pKC157 digested with restriction enzymes. *Hind*III-*Bst*XI fragment of pCBSII containing 4CBA:CoA dehalogenase gene was used as the DNA probe. Lanes: 1, size marker; 2, *Bam*HI-*Xho*I; 3, *Bam*HI-*Cla*I; 4, *Bam*HI-*Kpn*I; 5, *Not*I-*Xho*I; 6, *Not*I-*Cla*I.

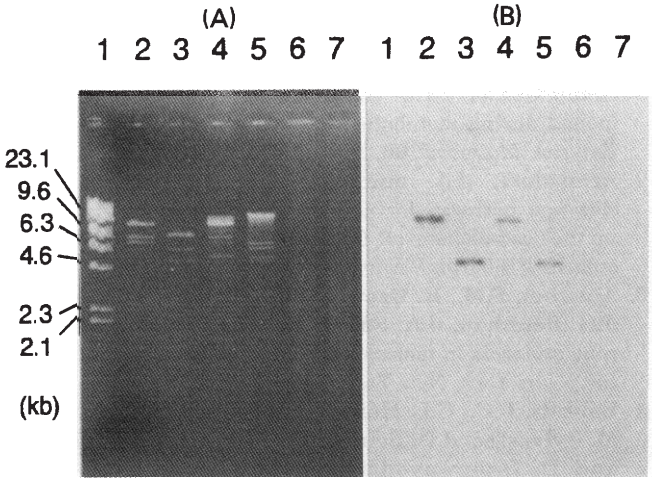

Fig. 7. Electrophoresis (A) and Southern hybridization (B) of pKC16, genomic DNA, and plasmid DNA of *Pseudomonas* sp. DJ-12 digested with restriction enzymes. *fcbB* of *Pseudomonas* sp. CBS3 was used as the DNA probe. Lanes: 1, size marker; 2, *Not*I-pKC16; 3, *Sal*I-pKC16; 4, *Not*I-chromosomal DNA; 5, *Sal*I-chromosomal DNA; 6, *Not*I-plasmid DNA; 7, *Sal*I-plasmid DNA.

of Fig. 6. The DNA segments containing the *Bam*HI fragment also showed hybridization signals as shown in lanes 5 and 6. These results indicate that *Pseudomonas* sp. DJ-12 degrades 4CBA to 4HBA via 4CBA : CoA dechlorinase which is homologous to that of *Pseudomonas* sp. CBS3.

Schmitz *et al.* (18) cloned and sequenced the genes for dehalogenation of 4-chlorobenzoate from the plasmid of *Arthrobacter* sp. strain SU1. Thereby, the dechlorination gene of *Pseudomonas* sp. DJ-12 was localized. The chromosomal and plasmid DNAs were isolated separately from the organism, digested with various endonucleases and then hybridized with the *fcbB* gene of *Pseudomonas* sp. CBS3 as shown in Fig. 7. Hybridization signals appeared in the 15 kb fragments of pKC16 (lane 2), the chromosomal DNA (lane 4) digested with *Not*I, the 6 kb fragments of pKC16 (lane 3) and chromosomal DNA (lane 5) digested with *Sal*I. However, no signal was observed in the plasmid DNAs (lane 6 and 7). Therefore, the dechlorination gene of *Pseudomonas* sp. DJ-12 was confirmed to be located in the chromosomal DNA, just as in *Pseudomonas* sp. CBS3 reported by Savard *et al.* (17).

Acknowledgments

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