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Hydrolytic Dechlorination of 4-Chlorobenzoate by *Pseudomonas* sp. Strain DJ-12, and Organization of Its *fcb* Gene Cluster

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The recalcitrance of the chlorinated aromatic hydrocarbons is characterized by the aromatic structure as well as chlorination of the compounds. That means degradation of such aromatics by microorganisms should be preceded by benzene ring-cleavage and dechlorination. Microbial dechlorination of the recalcitrant xenobiotics has been known to occur by several types of mechanisms. *Pseudomonas* sp. strain DJ-12 was found to degrade 4-chlorobenzoate via hydrolytic dechlorination to produce 4-hydroxybenzoate and chloride ion. The *fcb* genes responsible for the hydrolytic dechlorination of 4CBA were cloned from chromosome of DJ-12 strain. The *fcbA, B,* and *C* genes for dechlorination of 4-chlorobenzoate were found to be in order of *fcbB-A-C* as an operon. In the region between *fcbA* and *C*, there were *fcbT1, T2,* and *T3* genes involved in transportation of the compound. Therefore, the organization of the *fcb* genes responsible for hydrolytic dechlorination of 4-chlorobenzoate in *Pseudomonas* sp. DJ-12 is uniquely different from those of other reported organisms.

1. Introduction

Chlorinated aromatic compounds are one of the largest groups of environmental pollutants which have a contaminated in the nature during uses as herbicides, insecticides, fungicides, solvents, hydraulic and heat transfer fluids, plasticizers, and intermediates for chemical synthesis. Because of their persistence, toxicity, bioaccumulation, and transformation into hazardous metabolites, public concerns have been attracted in terms of human health problems such as carcinogenicity, mutagenicity, and disturbance in endocrine systems (7, 12, 21).

Chlorinated aromatics are basically consisted of substituted chlorines (carbon-chlorine bond) and aromatic structure (carbon-carbon bond). Their biological recalcitrance is related to the number and position of chlorine substituents on the aromatic rings (4, 14). The carbon-chlorine bond is genellary considered to become more recalcitrant due to increased electronegativity of the substituent. Therefore, dechlorination has been focused as the most important step in the bioremediation of these compounds (9, 10, 15, 19).

4-Chlorobenzoate (4CBA) is introduced into the environment through its use as a precursor in the synthesis of dye stuffs, pigments, and pharmaceuticals. It is also generated as a by-product in the microbial breakdown of certain herbicides and the pollutants, such as polychlorinated biphenyls, polychlorinated benzoates, DDT, bidicin (1, 13).

Several microorganisms, such as *Pseudomonas* (2, 5), *Arthrobacter* (8, 24, 25), *Alcaligenes* (20), and *Corynebacterium* (22) have been reported to degrade 4CBA via dechlorination to produce 4-hydroxybenzoate (4HBA). Particularly, the hydrolytic dechlorination of 4CBA to 4HBA was recognized to be carried out by a sequencial reaction of 4CBA-CoA ligase, 4CBA-CoA dehalogenase, and 4HBA-CoA thioesterase requiring CoA, ATP, and Mg2+ as seen in Fig. 1 (3, 6, 24). The genes encoding these three enzymes were revealed in *Pseudomonas* sp. CBS3 (3), *Arthrobacter* sp. SU (23), and *Arthrobacter* sp. TM1 (Gartemann, GenBank accession number AF042490). The corresponding enzymes involved in the dechlorination of 4CBA have been characterized in *Pseudomonas* sp. CBS3 (6) and *Arthrobacter* sp. 4CB-1 (8).

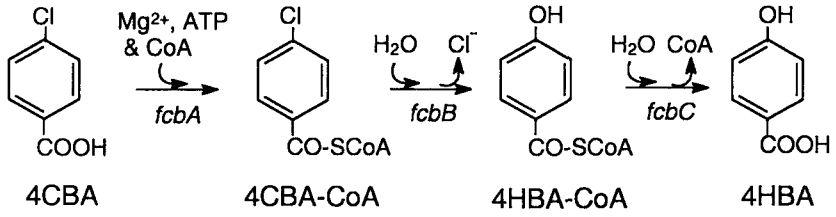

Fig. 1. Pathway for hydrolytic dechlorination of 4-chlorobenzoate. *fcbA, B,* and *C* encode 4CBA-CoA ligase, 4CBA-CoA dehalogenase, and 4HBA-CoA thioesterase, respectively.

Pseudomonas sp. DJ-12 is an isolate capable of utilizing biphenyl (BP) or 4-chlorobiphenyl (4CB) as the sole carbon and energy source (16). This strain was able to transform 4CB and BP to 4CBA and benzoate by *meta*-cleavage of the biphenyl ring under aerobic conditions (17). In previous study, the genes for biphenyl ring-fission were cloned and analyzed for nucleotide sequence (18). In this study, therefore, the genes for hydrolytic dechlorination of 4CBA in *Pseudomonas* sp. DJ-12 were investigated by sequence analysis.

2. Hydrolytic Dechlorination of 4CBA

Pseudomonas sp. DJ-12 can degrade completes 4-chlorobenzoate (4CBA), 4-iodobenzoate and 4-bromobenzoate. However, degradation of 4-fluorobenzoate by the organism was only 40% in comparison with other halogenated benzoates. The organism degraded 0.5 mM of 4CBA within 16 hours releasing corresponding amount of chloride ions. The supernatant of a sample incubated in phosphate buffer containing 0.5 mM of 4CBA for 3 hours was extracted with diethyl ether and methylated with diazomethane. The methylester of 4-hydroxybenzoate (4HBA) produced by dechlorination of 4CBA was detected at

32.1 min of running time and identified by mass spectrometry. The mass spectrum of methylester of 4HBA was matched with that of standard substance which had a molecular ion at *m/z* 152 and major fragment ion at *m/z* 121 ($M-OCH_3$).

The *fcb* genes encoding 4CBA-CoA ligase, 4CBA-CoA dehalogenase, and 4HBA-CoA thioesterase involved in hydrolytic dechlorination of 4CBA were cloned to understand the dechlorination reaction by *Pseudomonas* sp. DJ-12 at a molecular level. About 40-kb *Sau*3AI fragment containing the corresponding genes was cloned from the genomic DNA of strain DJ-12 and designated as pKC1. The dechlorination genes in the pKC1 plasmid were further subcloned to construct pKC14 (33-kb), pKC15 (36-kb), pKC16 (22-kb), pKC152 (30-kb), pKC157 (22-kb), and pKC158 (12-kb) by partial digestion with *Not*I and *Bam*HI.

The clone harboring pKC157 dechlorinated 0.5 mM 4CBA completely to 4HBA when incubated for 12 hours. The resulting 4HBA and chloride ion were accumulated in the buffer after complete degradation of 4CBA. This implies that the corresponding genes for further degradation of 4HBA do not existed in the cloned cells. The metabolite, 4HBA, produced from 4CBA via dechlorination was reconfirmed by gas chromatography using DB-5 capillary column and mass spectrometry. The resulting chromatogram and mass spectrum were shown in Fig. 2.

The metabolite 8.4 min was identified as a methylester of 4HBA (III). The methylester has a molecular ion at *m/z* 152 and major fragment ions at *m/z* 121 ($M-OCH_3$) and at *m/z* 93 ($M-COOCH_3$). These compounds were transformed from 4HBA in the process of methylation with diazomethane. These results confirm that the genes responsible for hydrolytic dechlorination of 4CBA are cloned and well expressed in *E. coli*.

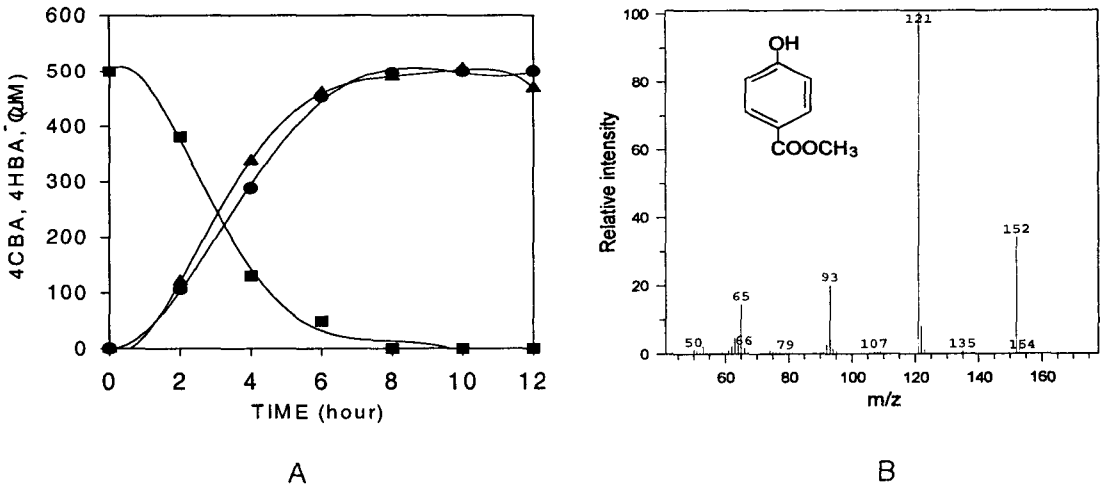

Fig. 2. Dechlorination of 4-chlorobenzoate by *E. coli* KC 157 to produce 4-hydroxybenzoate and chloride ion (A), and mass spectrum of the metabolite identified as the methylester of 4HBA produced (B). ■; 4-chlorobenzoate, ▲; chloride ion, ●; 4-hydroxybenzoate.

3. Organization of *fcb* genes

The organization of the *fcb* genes of *Pseudomonas* sp. DJ-12 was studied by analyzing the nucleotide sequences of 8,160 bp containing the *fcbABC* genes. The *fcb* gene cluster among them was summarized in Table 1. The six orfs containing *fcbA*, *fcbB*, and *fcbC* were consecutively organized in the order of *fcbB-fcbA-orf1-orf2-orf3-fcbC* with three orfs between the *fcbA* and *fcbC* which are unknown in 4CBA metabolism previously . Henceforth, the three orfs were designated *fcbT1*, *fcbT2*, and *fcbT3* in this study.

Table 1. Open reading frames in *fcb* gene cluster

Gene	Nucleotide position in sequence (bp)	G+C (%)	Deduced amino acid residue	Molecular weight (kDa)	Gene product
fcbB	2148-2957	61.9	269	30	4CBA-CoA dehalogenase
fcbA	2969-4487	61.9	505	54	4CBA-CoA ligase
fcbT1	4531-5511	56.1	326	36.5	Periplasmic membrane protein
fcbT2	5520-6065	59.5	181	20	Integral membrane protein
fcbT3	6078-7397	57.7	439	46.5	Integral membrane protein
fcbC	7400-7828	58	142	16	4HBA-CoA thioesterase

The genes for hydrolytic dechlorination of 4CBA in this DJ-12 strain was uniquely organized in comparison with those of the following reported bacteria. The nucleotide sequences of the genes for hydrolytic dechlorination of 4CBA have been reported in *Pseudomonas* sp. CBS3 (3) and *Arthrobacter* sp. SU (23). In addition, two more dechlorinase gene clusters have been enrolled in GenBank database. One is a repetitive gene cluster located downstream of reported one in *Arthrobacter* sp. SU (GenBank No. AF030397). The other one is derived from *Arthrobacter* sp. TM1 (GenBank No. AF042490). The gene organization among these 4CBA-degrading bacteria is compared in Fig. 3.

The *fcbA* gene encoding 4CBA-CoA ligase of *Pseudomonas* sp. DJ-12 was consisted of 1518 nucleotides, which can encode a polypeptide of molecular weight 54 kDa containing 505 amino acid residues. A deduced amino acid sequence of the 4CBA-CoA ligase showed 57.7%, 44%, and 44.2% identities to those of corresponding enzymes from *Pseudomonas* sp. CBS3 (3), *Arthrobacter* sp. SU (23), and *Arthrobacter* sp. TM1, respectively.

The *fcbB* gene encoding 4CBA-CoA dehalogenase was composed of 810 nucleotides, which can encode a polypeptide of molecular weight 30 kDa containing 269 amino acid residues. The G+C content of the structural gene was 61.9%. A promoter-like sequence (-35 and -10 region) and a putative

ribosome-binding sequence (AAGGAG) were found to be located upstream from the start codon of the gene. A deduced amino acid sequence of the 4CBA-CoA dehalogenase showed 85.8%, 50.4%, and 50.4% identities to those of corresponding enzymes from *Pseudomonas* sp. CBS3 (3), *Arthrobacter* sp. SU (23), and *Arthrobacter* sp. TM1, respectively.

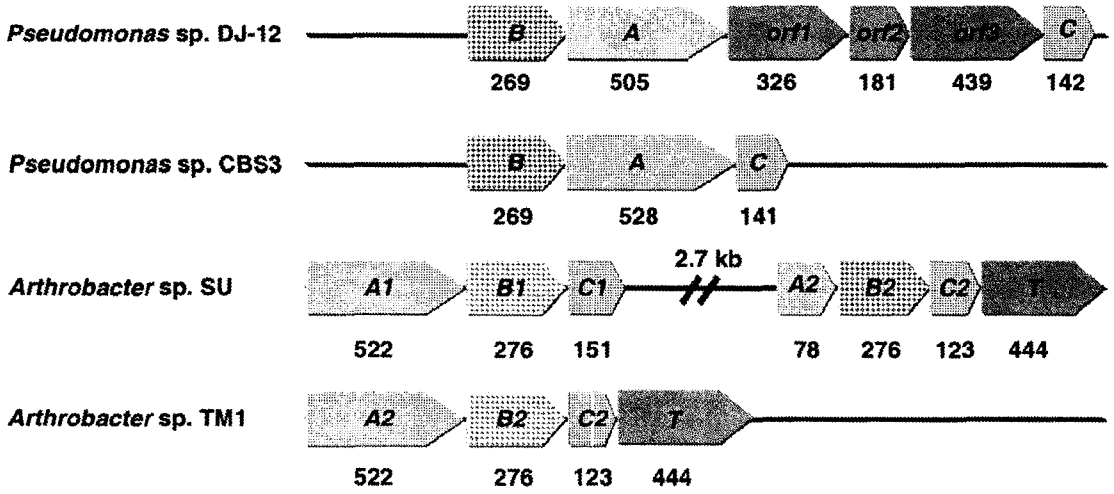

Fig. 3. Arrangement of the *fcb* gene clusters of *Pseudomonas* sp. DJ-12 and other 4CBA-degrading bacteria. *A*, *B*, and *C* indicate the genes encoding 4CBA-CoA ligase, 4CBA-CoA dehalogenase, and 4HBA-CoA thioesterase, respectively. The figures indicate the number of amino acid residues. *T1*, *T2*, and *T3* genes similar to C4-dicarboxylate transport system in *Rhodobacter capsulatus* are supposed to encode the periplasmic membrane proteins for transportation of 4CBA.

The *fcbC* gene encoding 4HBA-CoA thioesterase catalyzing the reaction from 4HBA-CoA to 4HBA was consisting of 429 nucleotides, which can encode a polypeptide of molecular weight 16 kDa containing 142 amino acid residues. A deduced amino acid sequence of the 4HBA-CoA thioesterase showed 64.8% identity to that of the corresponding enzymes from *Pseudomonas* sp. CBS3 (3), but did not show identity to those of corresponding enzymes from *Arthrobacter* sp. SU (23) and *Arthrobacter* sp. TM1.

The *fcbT1*, *fcbT2*, and *fcbT3* located between *fcbA* and *fcbC* genes exhibited homology with periplasmic solute transport systems (transporter). These genes have a similar structure to *dctPQM* genes in *Rhodobacter capsulatus* (11) encoding a TRAP (tripartic ATP-independent periplasmic) transporter. That means the genes are involved in uptake of 4CBA as substrate. Therefore, the organization of *fcb* genes responsible for hydrolytic dechlorination of 4CBA in *Pseudomonas* sp. DJ-12 is uniquely different from those of other reported bacteria.

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