J. Microbiol. Biotechnol. (1998), **8**(6), 692–695

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

NOTE

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

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Received: March 18, 1998

Abstract *Pseudomonas* sp. DJ-12 was able to degrade 4-chlorobenzoate by hydrolytic dechlorination to produce 4-hydroxybenzoate and chloride ion. The *fcbABC* genes responsible for the hydrolytic dechlorination were cloned from the chromosomal DNA of the organism. The genes were found to be organized in the order *fcbB-fcbA-fcbC*, but there was an intergenic space between the *fcbA* and *fcbC* genes.

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

Chlorinated aromatics have attracted public and scientific concern, as one of the largest groups of environmental pollutants. The concern is because of their worldwide distribution, persistence in the environment, and toxicity to living organisms. The recalcitrance of the compounds is related to the number and position of the halogen substituents [3]. As a general rule, the cleavage of the carbon-halogen bond has been recognized as the most critical step in the microbial degradation of these compounds [10]. There have been a number of review articles dealing with the mechanism of microbial dehalogenation of the haloaromatics [6, 9, 10]. One of the most intensively studied mechanisms is reductive dehalogenation occurring under anaerobic conditions. Under aerobic conditions, hydrolytic dehalogenation of the haloaromatics has also been reported in several bacterial strains whereby the halogen substituent is replaced by a hydroxyl group from water [15, 19], as seen in Fig. 1.

Pseudomonas sp. strain DJ-12 was isolated from contaminated waste from a Taejon industrial complex in Korea. This strain is able to transform 4-chlorobiphenyl to 4-chlorobenzoate (4CBA) by *meta*-cleavage of the benzene ring under aerobic conditions [12]. The nucleotide sequence of the gene encoding this aromatic ring-fission dioxygenase was reported previously [11]. The 4CBA was observed to be subsequently degraded by the organism to 4-hydroxybenzoate (4HBA). The strain DJ-12 could also degrade 4-bromobenzoate and 4-iodobenzoate by dehalogenation, but not 2-chlorobenzoate, 3-chlorobenzoate, and 2,4-dichlorobenzoate [5]. The present study reports that *Pseudomonas* sp. DJ-12 degrades 4CBA by hydrolytic dechlorination and that *fcbABC* genes on the chromosomal DNA of the strain specify the dechlorination of 4CBA.

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Dechlorination of 4-Chlorobenzoate

Pseudomonas sp. strain DJ-12 was cultivated in a chloride-free medium [20] containing 1 mM 4CBA under aerobic conditions. *Pseudomonas* sp. DJ-12 and the transfectants cultivated for 1 day were examined for production of chloride ions in 96-well microtiter plates. Chloride ions produced from 4CBA were examined by the colorimetric method as described by Bergmann and Sanik [4].

In order to reconfirm the dechlorination activity of the organism, the genes responsible for dechlorination were cloned from its chromosomal DNA. The chromosomal DNA isolated from the organism was digested with *Sau*3AI and ligated with cosmid pWE15 digested with *Bam*HI at 16°C for 16 h. The *in vitro* packaged ligation mixtures were transfected into *E. coli* LE392 according to the standard protocol provided by Promega Co. (Madison, U.S.A.). A cloned plasmid, in which about a 40-kb *Sau*3AI fragment of the DNA was inserted, showed strong dechlorination activity. This was designated as pKC1. The dechlorination genes in the pKC1 plasmid were further subcloned to construct pKC15 (36-kb), pKC152 (30-kb), pKC157 (22-kb), and pKC158 (12-kb) by the deletion method described by Sambrook *et al.* [16] using various endonucleases.

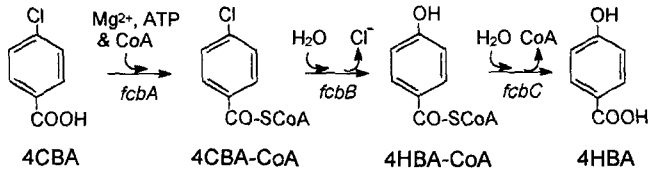

Fig. 1. Pathway for hydrolytic dechlorination of 4-chlorobenzoate.

fcbA, *B*, and *C* encode 4CBA-CoA ligase, 4CBA-CoA dehalogenase, and 4HBA-CoA thioesterase, respectively. Abbreviation: 4CBA, 4-chlorobenzoate; 4CBA-CoA, 4-chlorobenzoate-coenzyme A; 4HBA-CoA, 4-hydroxybenzoate-coenzyme A; 4HBA, 4-hydroxybenzoate.

Dechlorination of 4CBA by each cloned cell was also examined by the resting cell assay according to the procedures described by Arensdorf and Focht [1]. The cells collected from the Luria-Bertani (LB) culture were washed three times with 50 mM potassium phosphate buffer (pH 7.5) and then incubated in the same buffer which contained 1 mM 4CBA. Chloride ions released by the dechlorination of 4CBA were measured by the colorimetric method and the resulting metabolite, 4HBA, was examined by reverse-phase high pressure liquid chromatography using a μBondapak™ C_{18} column (Waters, Milford, U.S.A.). A mobile phase of methanol-H_2O-acetic acid (60:40:1) was used with a flow rate of 0.4 ml/min [21]. 4CBA and 4HBA were quantified by detection at 254 nm. A representative result of dechlorination of 4CBA by the cloned cell is shown in Fig. 2. The production of 4HBA and chloride ions increased while 4CBA was degraded as a function of reaction time. This result verifies that *Pseudomonas* sp. DJ-12 degraded 4CBA to produce 4HBA and chloride ion by the mechanism of hydrolytic dechlorination.

Hydrolytic dechlorination of chlorinated aromatics was studied in *Pseudomonas* sp. CBS3 [14] and *Arthrobacter*

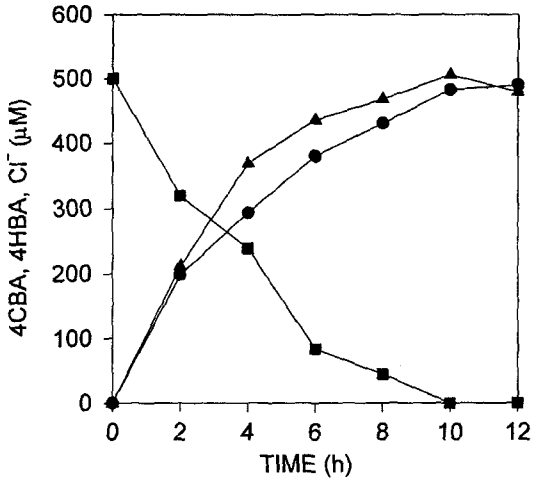

Fig. 2. Dechlorination of 4-chlorobenzoate by *E. coli* KC152 to produce 4-hydroxybenzoate and chloride ion.

■, 4-chlorobenzoate; ●, 4-hydroxybenzoate; ▲, chloride ion.

sp. 4-CB1 [7]. In those studies, it was reported that the chlorine substituent of the chloroaromatics was replaced by a hydroxyl group derived from water by consecutive reactions with 4CBA-CoA ligase, 4CBA-CoA dehalogenase, and 4HBA-CoA thioesterase encoded by *fcbA*, *fcbB*, and *fcbC*, respectively. Cloned cells constructed in this study exhibited hydrolytic dechlorination activity to 4CBA, as shown in the right portion of Fig. 3. However, the dechlorination activity was not expressed by pKC14, where the 3.5-kb *Bam*HI fragment was deleted.

Localization of *fcbABC* Genes

The *fcbABC* genes involved in the hydrolytic dechlorination of 4CBA were examined for their location and organization in the chromosome of *Pseudomonas* sp. DJ-12. The

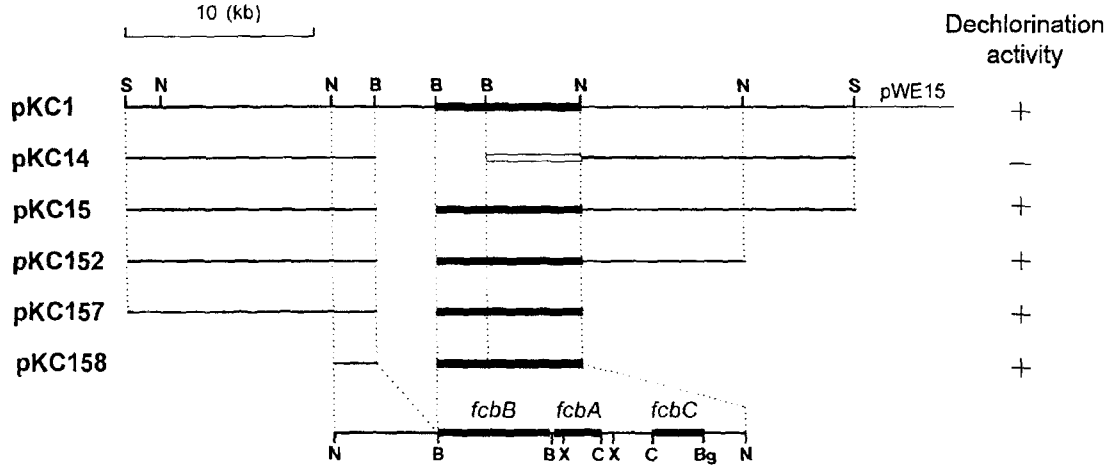

Fig. 3. Physical maps of recombinant plasmids and their dechlorination activities to 4-chlorobenzoate.

fcbA, *B*, and *C* indicate the loci of the genes which were hybridized with 4CBA-CoA ligase, 4CBA-CoA dehalogenase, and 4HBA-CoA thioesterase, respectively. Abbreviation: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; N, *Not*I; S, *Sau*3AI; X, *Xho*I.

clone of pKC158 exhibiting the dechlorination activity of 4CBA was hybridized with each of the three genes isolated from *Pseudomonas* sp. CBS3 (Fig. 4). The fragments of pKC158 digested with several endonucleases were blotted from the agarose gel onto a nylon membrane by the procedures described by Koetsier *et al.* [13]. Southern hybridization was performed with an ECL direct nucleic acid labeling and detection system (Amersham, Buckinghamshire, UK). The probes used for this study were the DNA fragments carrying *fcbA* (*Nde*I fragment, 1463-bp), *fcbB* (*Hin*dIII-*Bst*XI fragment, 701-bp), and *fcbC* (*Eco*RI-*Hin*dIII fragment, 236-bp), which were isolated from pCBSII cloned from *Pseudomonas* sp. CBS3 [8].

The *Bam*HI fragments (3.5-kb) were hybridized with *fcbB* and are shown in lanes 2, 5, 6, and 7 in Fig. 4B. Other DNA segments containing the *Bam*HI fragment (lane 3 and 4) also showed hybridization with *fcbB*. The *Xho*I-*Cla*I fragments (1.1-kb) showed hybridization with *fcbA* as shown in lanes 3, 5, and 6 in Fig. 4C. The hybridization bands also appear in all lanes (Fig. 4C) loaded with the DNA segments containing the *Bam*HI-*Cla*I fragment (1.2-kb). In addition, the faint signals on the top of all lanes (indicated by arrows) were identified as a pWE15 vector. Several regions of the vector were homologous to the *fcbA* gene when their base sequences were compared by using the software of DNASIS (Hitachi version 7.06). In Fig. 4D, all the DNA segments including the *Cla*I-*Bgl*II fragment (1.5-kb) were clearly hybridized with the *fcbC* gene. Therefore, the three genes of *fcbA*, *fcbB*, and *fcbC* cloned from chromosomal DNA of *Pseudomonas* sp. DJ-12 appeared to specify the hydrolytic dechlorination of 4CBA. The genes were organized as a cluster in the *Not*I fragment (12-kb) of pKC158 and arranged in the order of *fcbB-fcbA-fcbC*, as shown in Fig. 3.

The genes for hydrolytic dechlorination of 4-chlorobenzoate were cloned in *Arthrobacter* [18, 20] and *Pseudomonas* [17]. The three genes, *fcbA*, *fcbB*, and *fcbC* in *Pseudomonas* sp. CBS3 [2] and *Arthrobacter* sp. SU [18] were reported to be organized as an operon. However, the gene order (*fcbB-fcbA-fcbC*) of *Pseudomonas* sp. CBS3 was different from that (*fcbA-fcbB-fcbC*) of *Arthrobacter* sp. SU. Schmitz *et al.* [18] reported that there was no identity in nucleotide sequences of *fcbC* gene between *Pseudomonas* sp. CBS3 and *Arthrobacter* sp. SU.

The order of the three genes in *Pseudomonas* sp. DJ-12 in the present study, *fcbB-fcbA-fcbC*, was the same as that of *Pseudomonas* sp. CBS3. However, the three genes of *Pseudomonas* sp. CBS3 were organized in tandem without intergenic space between the genes, whereas, in *Pseudomonas* sp. DJ-12, there was an intergenic region (at least 1.6-kb of *Cla*I fragment in size) between the *fcbA* and the *fcbC* genes. These results suggest that *fcbABC* genes which specify dechlorination of 4CBA have been diversely evolved by the processes of rearrangement among different genera of the 4CBA-dechlorinating bacteria and even in different strains of the genus *Pseudomonas* with dechlorination activity.

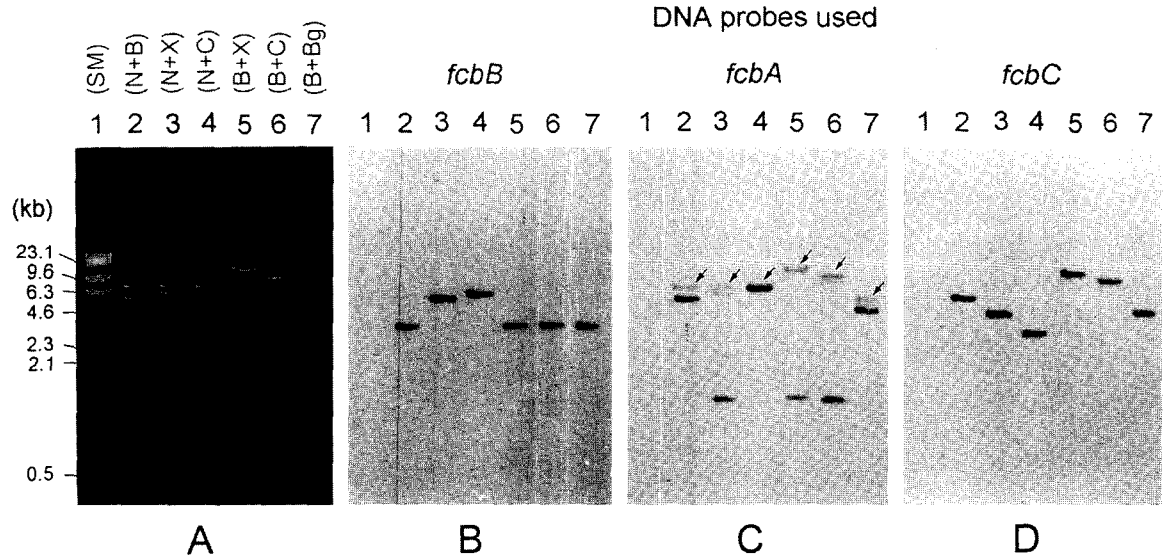

Fig. 4. Electrophoretic (A) and Southern hybridization (B, C, and D) patterns of pKC158 digested with several restriction enzymes.

The genes of 4CBA-CoA dehalogenase (*fcbB*), 4CBA-CoA ligase (*fcbA*), and 4HBA-CoA thioesterase (*fcbC*) isolated from *Pseudomonas* sp. CBS3 were used as DNA probes. Arrows indicate the DNA fragments containing a pWE15 vector. Abbreviation: SM, size marker; B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; N, *Not*I; X, *Xho*I.

Acknowledgments

We thank Dr. R. Müller for providing plasmid pCBSII. This work was supported by grants from KOSEF through the Research Center for Molecular Microbiology at Seoul National University and in part from the Ministry of Education (project No. BSRI-97-4432), Korea.

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