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Catabolic Degradation of 4-Chlorobiphenyl by *Pseudomonas* sp. DJ-12 via Consecutive Reaction of *meta*-Cleavage and Hydrolytic Dechlorination

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Abstract *Pseudomonas* sp. strain DJ-12 is a bacterial isolate capable of degrading 4-chlorobiphenyl (4CBP) as a carbon and energy source. The catabolic degradation of 4CBP by the strain DJ-12 was studied along with the genetic organization of the genes responsible for the crucial steps of the catabolic degradation. The catabolic pathway was characterized as being conducted by consecutive reactions of the *meta*-cleavage of 4CBP, hydrolytic dechlorination of 4-chlorobenzoate (4CBA), hydroxylation of 4-hydroxybenzoate, and *meta*-cleavage of protocatechuate. The *pcbC* gene responsible for the *meta*-cleavage of 4CBP only showed a 30 to 40% homology in its deduced amino acid sequence compared to those of the corresponding genes from other strains. The amino acid sequence of 4CBA-CoA dechlorinase showed an 86% homology with that of *Pseudomonas* sp. CBS3, yet only a 50% homology with that of *Arthrobacter* spp. However, the *fcb* genes for the hydrolytic dechlorination of 4CBA in *Pseudomonas* sp. DJ-12 showed an uniquely different organization from those of CBS3 and other reported strains. Accordingly, these results indicate that strain DJ-12 can degrade 4CBP completely via *meta*-cleavage and hydrolytic dechlorination using enzymes that are uniquely different in their amino acid sequences from those of other bacterial strains with the same degradation activities.

Keywords: 4-chlorobiphenyl, *meta*-cleavage, hydrolytic dechlorination, catabolic pathway, PcbC, FcbB, *Pseudomonas* sp. DJ-12

INTRODUCTION

Chlorinated aromatic compounds are some of the most serious environmental pollutants. The recalcitrant characteristics of these compounds, particularly their toxicity to living organisms, are due to their aromatic ring-structure along with chlorine substitution on the aromatic rings [1,2]. Polychlorinated biphenyls (PCBs) have been recognized as the priority pollutants contaminating the environment. Numerous studies have been performed on the microbial degradation of biphenyl and PCBs in both laboratory and natural environments [3-6].

Most of the microorganisms residing in nature are reported to metabolize only a limited group of aromatic pollutants. Furthermore, these organisms show a stringent specificity for particular steps of the degradative pathways [7]. Therefore, attempts to develop a strain capable of degrading a wide variety of pollutants and increasing the efficiency of degradation have been made using techniques of molecular recombination with preexisting genes specifying the biodegradation of the pollutants [8,9]. These recombinant strains were often good at degrading the tested compounds under laboratory conditions, yet failed under natural conditions [1]. This means there is still a need to isolate more potential microorganisms capable of degrading pollutant chemicals and cleaning up contaminated environments.

Pseudomonas sp. DJ-12 is a natural isolate, obtained by the current authors from contaminated soil, which can grow in biphenyl and 4-chlorobiphenyl (4CBP) as the sole carbon and energy source under aerobic conditions [10]. The gene cluster, designated as *pcbABCD*, encoding the four enzymes responsible for the degradation of 4CBP to 4-chlorobenzoate (4CBA) was previously cloned from the chromosome of the strain [11,12]. The DJ-12 strain was also found to degrade 4CBA to 4-hydroxybenzoate (4HBA) by hydrolytic dechlorination [11,13].

Accordingly, this study investigated the catabolic degradation of 4CBP by *Pseudomonas* sp. DJ-12 via consecutive reactions of *meta*-cleavage and hydrolytic dechlorination. The genetic organization of the genes responsible for the crucial steps in the ring-fission of

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4CBP and dechlorination of 4CBA was further analyzed.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

A previous report was already made on the degradation characteristics of *Pseudomonas* sp. DJ-12 as regards chlorinated aromatic hydrocarbons [12]. *E. coli* XL1-Blue and pBluescript SK(+) phagemid were used as the host strain and vector, respectively. The *Pseudomonas* sp. DJ-12 was cultivated at 37°C in a Luria-Bertani (LB) medium under aerobic conditions. The recombinant *E. coli* cells were cultivated in an MM2 medium supplemented with respective aromatic substrates or a modified Cl^--free medium supplemented with 1 mM 4CBA, as described by Tsoi *et al.* [14]. When necessary, ampicillin (100 μg/mL) and tetracycline (15 μg/mL) were added to the medium.

Resting Cell Assays for Degradation of Aromatics

The degradation of the aromatic compounds was examined using a resting cell assay as described by Harayama and Rekik [15] and Kim and Zylstra [16]. The cells grown in the LB broth for 12 h were harvested and then incubated in a 10 mM phosphate buffer containing the respective substrates at 37°C for an appropriate period of time. The degradation of each aromatic compound was examined by scanning the metabolites with a UV-visible spectrophotometer. In some cases, the degradation of the aromatic compounds by the strain DJ-12 and cloned cells was examined by growing cell assays. The cells in the LB broth containing each respective aromatic compound were examined for their degradation activity of the substrates and metabolites which were produced by the organism during cultivation at 30°C under aerobic conditions.

Enzyme Assays

Batch cultures of *Pseudomonas* sp. DJ-12 were carried out in a 5-L jar fermentor (Korea Fermentation Co., Korea) with a working volume of 3 L. The culture medium and culture conditions were the same as described by Lee *et al.* [17]. After incubation for 40 h, the bacterial cells were harvested by centrifugation and washed twice with a 10 mM phosphate buffer (pH 7.0). The washed cells were resuspended in the same buffer and the cell suspension was disrupted by sonication (Sonoprep 150) at 40 volts for 20 sec continuously for 15 min with 40 sec intervals. The sonicated cell suspension was then centrifuged (12,000 rpm for 30 min) at 4°C. The clear supernatant was used as an enzyme source for all the enzyme assays.

The 4-hydroxybenzoate 3-hydroxylase activity was assayed by the decrease in absorbance at 340 nm due to the oxidation of NADH [18]. Protocatechuate 2,3-dioxygenase was assayed by observing the accumulation of 2-hydroxymuconic semialdehyde at 375 nm [19]. The specific activities of the enzymes were defined as unit(s) per mg of protein. The protein concentration was determined by the Lowry method [20].

HPLC and GC- Mass Spectrophotometry

The metabolites produced from 4CBP or 4CBA were identified by HPLC using a reverse-phase C18 column (Waters, Milford, MA, USA). 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 detected at a wavelength of 254 nm. The metabolite, 4HBA, was extracted from the culture with diethyl ether and methylate with diazomethane, as described by Arensdorf and Focht [22], and then analyzed with a GC-mass spectrophotometer (Hewlett-Packard Co., CA, USA) using a DB-5 capillary column (length, 30 m; diameter, 0.24 mm; film thickness, 0.25 μm) supplied from J & W Scientific Co. (Foisom, CA, USA). The temperature was programmed to rise from 70°C (2 min initial wait) to 210°C at a rate of 20°C /min.

Cloning of Genes

The genes responsible for the *meta*-cleavage of 4CBP and hydrolytic dechlorination of 4CBA were cloned from the chromosomal DNA of *Pseudomonas* sp. DJ-12. The genomic DNA was extracted as described previously [23] and the DNA manipulation methods, including isolation of the plasmid, enzyme digestion, and electrophoresis, were performed as described by Sambrook *et al.* [24]. The genomic DNA of *Pseudomonas* sp. DJ-12 and the pBluescript SK(+) vector were digested with *Eco*RI and then ligated at 16°C for 16 h. The transformation of the recombinant plasmids into *E. coli* XL1-Blue was carried out according to the method of Sambrook *et al.* [24]. The transformants were selected on an LB medium containing ampicillin (100 μg/mL) and tetracycline (15 μg/mL), X-gal (20 mg/mL), and isopropyl-thio-β-D-galactoside (IPTG; 20 mg/mL). The *in vitro* packaged ligation mixtures were transfected into *E. coli* LE392 according to the standard protocol provided by Promega Co. (Madison, WI, USA). The cloned cells exhibiting dechlorination activity were selected from the transfectants by the method described in a previous paper [23]. Several subclones were constructed by the deletion method described by Sambrook *et al.* [24] using various endonucleases.

Electrophoresis and Southern Hybridization

The genomic DNA of *Pseudomonas* sp. DJ-12 and the recombinant plasmids were digested with various endonucleases and electrophoresed in a 0.7% agarose gel with a TAE buffer (pH 8.0). The DNA fragments were eluted from the gel using a GENECLEAN II Kit (Bio 101 Inc., La Jolla, CA, USA). The inserted DNA fragment (9.5 kb) isolated from pCJ1 was used as a probe by labeling it with biotin-14-dATP (BRL, Gaitherberg,

MD, USA) using a nick translation kit (Promega Co., Madison, WI, USA).

Southern hybridization was performed according to the procedures described by Koetsier *et al.* [25]. The electrophoresis gel suspended in 0.25 M HCl was reacted in a denaturation solution (1.5 M NaCl, 0.5 N NaOH) for 5 min, and then in a neutralization solution for 1 min. The DNAs were transferred to a nylon membrane (Hybond-N; Amersham International plc., Amersham, UK) and baked at 80°C for one and half hours. The DNAs adsorbed on the membrane were hybridized for 18 h in a hybridization solution (50% formamide, 6× SSC, 5× Denhardt's reagent, 25 mM sodium phosphat, salmon sperm DNA, 0.5% SDS, 5% dextran sulfate) containing the gene probe. The detection of the hybridized DNA bands was carried out using the BluGENE nonradioactive nucleic acid detection system (BRL, Gaitherberg, MD, USA).

Sequence Analysis

The nucleotide and deduced amino acid sequences were analyzed using DNASIS and PROSIS software (Hitachi version 7.0, Japan). The amino acid sequences were compared with the GenBank database using programs based on the BLAST algorithm [26]. Database searches for conserved segments were performed using the MOTIF program (Institute of Chemical Research, Kyoto University, Japan) against the PROSITE Pattern Library. Multiple alignments were generated using the Clustal X algorithm and used for a phylogenetic analysis. The data set was subjected to a UPGMA analysis of PHYLIP. The display program, TreeView (version 1.5.2), was used to visualize the phylogenies of the PHYLIP tree file.

RESULTS AND DISCUSSION

Degradation of 4-Chlorobiphenyl and Intermediates

Among the 4-chlorobiphenyl (4CBP) degraders previously isolated in the current authors' laboratory [10], The *Pseudomonas* sp. DJ-12 strain was selected to examine its degradative characteristics, as compared to those of *Pseudomonas* sp. P20 and *Pseudomonas pseudoalcaligenes* KF707, and the genetic structures of the major genes responsible for the major steps of degradation were analyzed. *Pseudomonas* sp. DJ-12 showed a good degradability of 4CBP and biphenyl (BP) to produce 4CBA and BA, respectively, as shown in Table 1. The P20 strain can degrade 4CBP and BP very effectively, however the resulting 4CBA and BA can not be degraded any further [10]. *P. pseudoalcaligenes* KF707 [27] used as a reference strain for BP degradation can not degrade 4CBP.

The *Pseudomonas* sp. DJ-12 exhibited a degradability of 4HBA as well as PCA. When the degradative characteristics of strain DJ-12 for these aromatic compounds were examined as a function of incubation time in an

Table 1. Degradation of aromatic hydrocarbons by bacterial strains

Strain	Degradation of aromatics						
	4CBP	4CBA	4HBA	BP	2,3-DHBP	BA	PCA
Pseudomonas sp. DJ-12	++	+++	+++	++	+++	++++++	
Pseudomonas sp. P20	+++	–	++	+++	++	–	–
Pseudomonas psuedoalcaligenes KF707	–	–	+	++	+++	+++	NT

4CBP, 4-chlorobiphenyl; 4CBA, 4-chlorobenzoate; 4HBA, 4-hydroxybenzoate; BP, biphenyl; 2,3-DHBP, 2,3-dihydroxybiphenyl; BA, benzoate; PCA, protocatechuate; +++, complete degradation after 12 h incubation; ++, 70% degradation after 12 h incubation; +, 40% degradation after 12 h incubation; –, no degradation after 12 h incubation; NT, not tested.

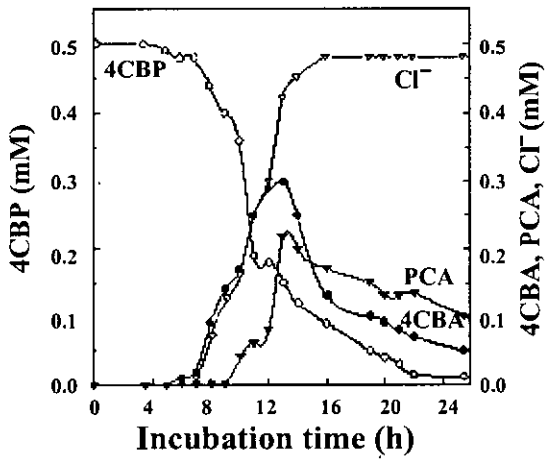


Fig. 1. Degradation of 4-chlorobiphenyl and production of metabolites by *Pseudomonas* sp. DJ-12. 4CBP, 4-chlorobiphenyl; 4CBA, 4-chlorobenzoate; PCA, protocatechuate; Cl^-, chloride ion.

MM2 medium containing 10 mM 4CBP, 4CBP began to be degraded about 4 h after incubation and 4CBA was produced at the same time, as shown in Fig. 1. Chloride ions and protocatechuate (PCA) were detected at about 6 and 10 h after incubation, respectively. However, the amount of 4CBA and PCA began to decrease about 13 h after incubation. These results show that 4CBP was converted to 4CBA by the strain DJ-12 and that 4CBA was further degraded to produce PCA by releasing chloride ions. The resulting PCA was continuously catabolized. These results suggest that strain DJ-12 can continuously degrade 4CBP and utilize the intermediate compounds as the carbon and energy sources. Therefore, the catabolic pathway for 4CBP degradation by *Pseudomonas* sp. DJ-12 was examined and the genetic structures of the genes responsible for the crucial degradative

reactions analyzed.

Genetic Analysis of *pcb* Genes for *meta*-Cleavage of 4CBP

The gene cluster, designated as *pcbABCD*, encoding the four enzymes responsible for the conversion of 4CBP and BP to 4CBA and BA, respectively, was cloned from the chromosomal DNA of strain DJ-12, as shown in Fig. 2 [12]. The biochemical properties of the PcbC,

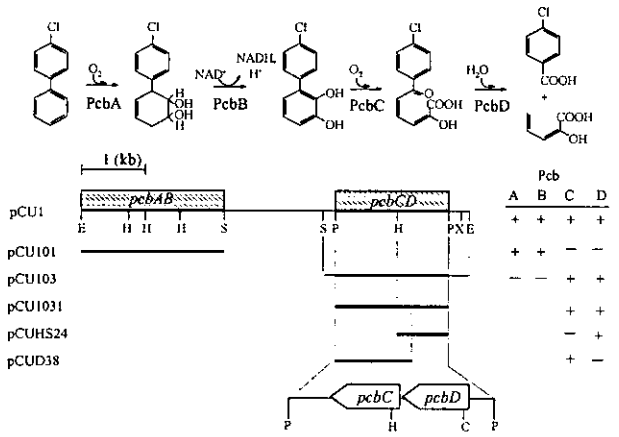


Fig. 2. Upper pathway for 4-chlorobiphenyl degradation by *Pseudomonas* sp. DJ-12 via *meta*-cleavage, and derivative clones harboring *pcbABCD* genes with their enzymatic activities. The functional region was localized by the subcloning and deletion mapping of pCU1031. PcbA, 4-chlorobiphenyl dioxygenase; PcbB, dihydrodiol dehydrogenase; PcbC, 2,3-dihydroxybiphenyl 1,2-dioxygenase; PcbD, 2-hydroxy-6-oxo-6-phenylhexa-2,4-dieonate hydrolase; E, *Eco*RI; H, *Hind*III; S, *Sal*I; P, *Pst*I; X, *Xho*I; C, *Cla*I.

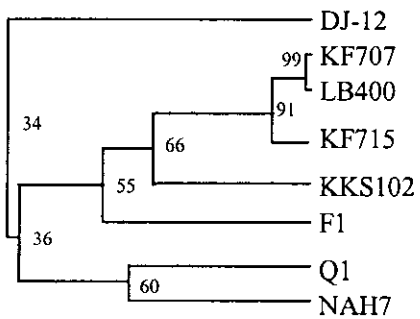


Fig. 3. Phylogenetic relationship of PcbC from *Pseudomonas* sp. DJ-12 with other corresponding enzymes. The numbers indicate the percentage of amino acid homology between each pair. The GenBank numbers of the *pcbC* and corresponding genes of the bacterial species are as follows in parenthesis: DJ-12, *Pseudomonas* sp. DJ-12 (D44550); KF707, *Pseudomonas pseudoalcaligenes* KF707 (M15333); LB400, *Pseudomonas* sp. strain LB400 (X66122); KF715, *Pseudomonas putida* KF715 (M33813); KKS102, *Pseudomonas* sp. strain KKS102 (M264333); F1, *Pseudomonas putida* F1 (J04996); Q1, *Pseudomonas paucimobilis* Q1 (M20640), NAH7, *Pseudomonas putida* (J04994).

2,3-dihydroxybiphenyl 1,2-dioxygenase (2,3-DHBP), were different from those of corresponding enzymes from other strains [28,29]. The *pcbAB* genes in the pCU101 were separated from the *pcbCD* gene by a 1.2 kb intergenic region [11]. The nucleotides of the *pcbC* and *pcbD* genes of *Pseudomonas* sp. DJ-12 were then sequenced and found to be composed of 960 and 849 bp, respectively.

When the PcbC amino acid sequence was compared with those of the *meta*-cleavage dioxygenases from other strains, the overall homology was 30 to 40%, as shown in Fig. 3. The PcbC from the *Pseudomonas* sp. DJ-12 was less closely related to the TodE from *Pseudomonas putida* F1 [30] than to the four BphCs from *Pseudomonas pseudoalcaligenes* KF707 [31], *Pseudomonas* sp. strain LB400 [5], *Pseudomonas putida* KF715 [3, 28], and *Pseudomonas* sp. strain KKS102 [32]. Lee *et al.* [28] reported that the PcbC of strain DJ-12 was immunochemically different from other 2,3-DHBP dioxygenases. This supports the current result of a low sequence homology between the PcbC of strain of DJ-12 and other corresponding dioxygenases for *meta*-cleavage of the aromatic compounds.

Genetic Analysis of *fcb* Genes for Hydrolytic Dechlorination of 4CBA

The 4CBA produced by the *meta*-cleavage of 4CBP in *Pseudomonas* sp. DJ-12 was hydrolytically dechlorinated to produce 4HBA and chloride ions under aerobic conditions [12,23]. 4CBA is known to be hydrolytically dechlorinated by the consecutive activities of 4CBA-CoA ligase, 4CBA-CoA dechlorinase, and 4HBA-CoA thioesterase in several bacteria [33-35]. The *fcb* genes responsible for the hydrolytic dechlorination of 4CBA were cloned from the chromosomal DNA of *Pseudomonas* sp. DJ-12 to obtain pKC1 [13]. Clones pKC15, pKC157, and pKC158, all exhibiting dechlorination activity on 4CBA, were constructed from pKC1 using a deletion method. The physical maps and dechlorination

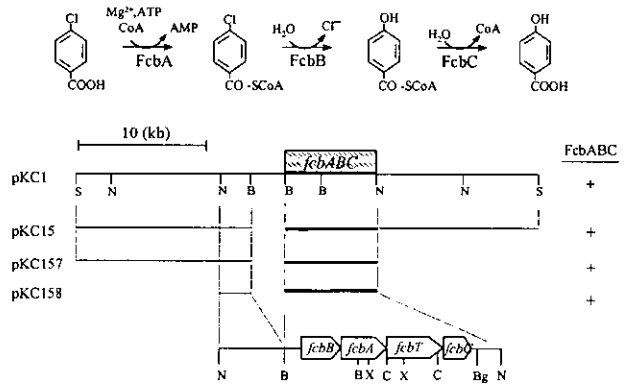


Fig. 4. Hydrolytic dechlorination of 4-chlorobenzoate by *Pseudomonas* sp. DJ-12 and genetic organization of *fcb* genes. FcbA, 4CBA-CoA ligase; FcbB, 4CBA-CoA dechlorinase; FcbC, 4HBA-CoA thioesterase; FcbT, 4CBA transporter; S, *Sal*I; N, *Not*I; B, *Bam*HI, X, *Xho*I; C, *Cla*I; Bg. *Bgl* II.

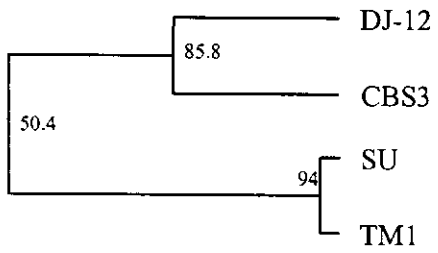


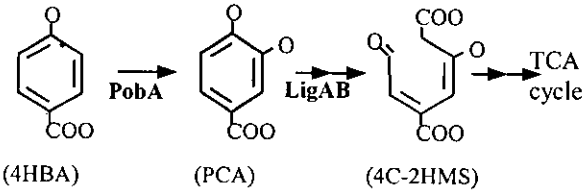
Fig. 5. Phylogenetic relationship of FcbB from *Pseudomonas* sp. DJ-12 with other corresponding enzymes. The numbers on the phylogenetic tree indicate the percentage of amino acid homology between each pair. The GenBank numbers of the *fcbB* and corresponding genes of the bacterial species are as follows in parenthesis: DJ-12, *Pseudomonas* sp. DJ-12 (AF051771); CBS3, *Pseudomonas* sp. CBS3 (A42560); SU, *Arthrobacter* sp. SU (A48956); TM1, *Arthrobacter* sp. TM1 (AF042490).

activities of these clones are shown in Fig. 4. The genes involved in the hydrolytic dechlorination of 4CBA were organized in the order of *fcbB-fcbA-fcbC* as a cluster, between *fcbA* and *fcbC* where *fcbT* was located as shown in Fig. 4. This organization of the *fcb* genes in strain DJ-12 was uniquely different from those of other reported strains, *Pseudomonas* sp. CBS3 [36], *Arthrobacer* sp. SU [35], and *Arthrobacer globiformis* KZT1 [14].

The *fcbB* gene encoding the 4CBA-CoA dechlorinase was composed of 810 bp. The FcbB polypeptide of strain DJ-12 was about 30 kDa in molecular mass with a sequence of 269 amino acids, just like other dehalogenases as a homotrimer of 30 kDa subunits [35,37]. The amino acid sequence of FcbB from strain DJ-12 showed an 85.8, 50.4, and 50.4% homology with those of the corresponding enzymes from *Pseudomonas* sp. CBS3 [36], *Arthrobacter* spp. SU [35] and TM1 strains (GenBank No. AF042490), respectively, as shown in Fig. 5.

Table 2. Specific enzyme activities of *Pseudomonas* sp. DJ-12 grown on 4-hydroxybenzoate

Enzyme	Gene	Specific activity
4HBA 3-hydroxylase	*PobA*	44.30
PCA 4,5-dioxygenase	*ligAB*	71.68
PCA 3,4-dioxygenase	*pcaGH*	< 1.0
PCA 2,3-dioxygenase	–	< 1.0
Catechol 2,3-dioxygenase	*xylE*	17.08
Catechol 1,2-dioxygenase	*CatA*	< 1.0



Hydroxylation of 4HBA and *meta*-Cleavage of PCA

The 4HBA produced from 4CBA by hydrolytic dechlorination was readily catabolized by *Pseudomonas* sp. DJ-12. The utilization of 4HBA and PCA by strain DJ-12 was examined in an MM2 medium using a resting cell assay. Several enzymatic assays were also performed using cell free extracts of the organisms [38]. The results of the enzyme activities are given in Table 2. The cell free extracts exhibited high activities of 4HBA 3-hydroxylase and PCA 4,5-dioxygenase, yet low activities of PCA 2,3-dioxygenase, PCA 3,4-dioxygenase, and catechol 1,2-dioxygenase. These results indicate that 4HBA is catabolized to produce PCA. The PCA was converted into a yellow-colored product that exhibited a strong absorbance at 410 nm, which is indicative of the *meta*-cleavage product from PCA, 4-carboxy-2-hydroxymuconic semialdehyde (4C-2HMS), as shown at the bottom of Table 2.

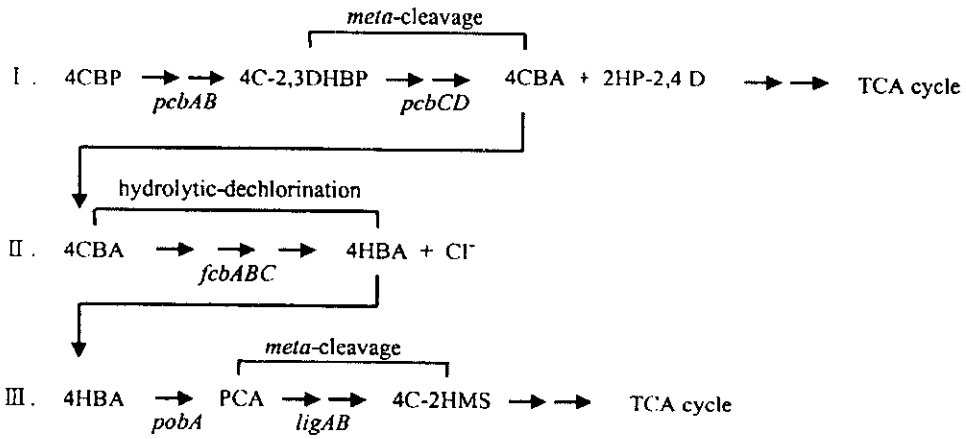


Fig. 6. Overall pathway for 4-chlorobiphenyl degradation by *Pseudomonas* sp. DJ-12 via consecutive reactions of *meta*-cleavage and hydrolytic dechlorination. The linear structured compounds, 2HP-2,4DA and 4C-2HMS, which are produced from the degradation of 4CBP are readily utilized as carbon and energy sources throughout the TCA cycle.

Therefore, the overall 4CBP degradation by *Pseudomonas* sp. DJ-12 can be formulated as a catabolic pathway, as shown in Fig. 6. The catabolic pathway is characterized by consecutive reactions of *meta*-cleavage and hydrolytic dechlorination occurring under aerobic conditions. The pathway can be divided into three major steps. The first is the dioxygenated degradation of 4CBP to produce 4CBA via *meta*-cleavage. The second is the hydrolytic dechlorination of 4CBA to 4HBA. The last includes the hydroxylation of 4HBA to PCA and *meta*-cleavage of PCA to produce 4C-2HMS. Accordingly, these results indicate that *Pseudomonas* sp. DJ-12 can degrade 4CBP completely utilizing the metabolites as the sole carbon and energy source.

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REFERENCES

[1] Chaudhry, G. R. and S. Chapalamadugu (1991) Biodegradation of halogenated organic compounds. *Microbiol. Rev.* 55: 59-79.

[2] Hardman, D. J. (1991) Biotransformation of halogenated compounds. *Crit. Rev. Biotech.* 11: 1-40.

[3] Hayase, N., K. Taira, and K. Frukawa (1990) *Pseudomonas putida* KF715 *bphABCD* operon encoding biphenyl and polychlorinated biphenyl degradation: cloning, analysis, and expression in soil bacteria. *J. Bacteriol.* 172: 1160-1164.

[4] Hofer, B., S. Backhaus, and K. N. Timmis (1994) The biphenyl/polychlorinated biphenyl-degradation locus (*bph*) of *Pseudomonas* sp. LB400 encodes four additional metabolic enzymes. *Gene* 130: 47-55.

[5] Seeger, M., K. N. Timmis, and B. Hofer (1995) Conversion of chlorobiphenyls into phenylhexadienoates and benzoates by the enzymes of the upper pathway for polychlorobiphenyl degradation encoded by the *bph* locus of *Pseudomonas* sp. strain LB400. *Appl. Environ. Microbiol.* 61: 2654-2658.

[6] Kim, E., Y. Kim, and C. K. Kim (1996) Genetic structures of the genes encoding 2,3-dihydroxybiphenyl 1,2-dioxygenase and 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid hydrolase from biphenyl- and 4-chlorobiphenyl-degrading *Pseudomonas* sp. DJ-12. *Appl. Environ. Microbiol.* 62: 262-265.

[7] Weightman, A. J., R. H. Don, P. R. Lehrbach, and K. N. Timmis (1984) The identification and cloning of genes encoding haloaromatic catabolic enzymes and the construction of hybrid pathways for substrate mineralization. *Basic Life Sci.* 28: 47-80.

[8] Adams, R. H., C. M. Huang, F. K. Higson, V. Brenner, and D. D. Focht (1992) Construction of a 3-chlorobiphenyl-utilizing recombinant from an intergenic mating. *Appl. Environ. Microbiol.* 58: 647-654.

[9] Hirose, J., N. Kimura, A. Suyama, A. Kobayashi, S. Hayashida, and K. Furukawa (1994) Functional and structural relationship of various extradiol aromatic ring-cleavage dioxygenase of *Pseudomonas* origin. *FEMS Microb. Lett.* 118: 273-278.

[10] Yun, D. J., J. J. Han, C. K. Kim, and Y. Kim (1992) Divergence of the *cbp* genes in 4-chlorobiphenyl catabolizing bacteria. *Kor. J. Microbiol.* 30: 53-59.

[11] Han, J. J., T. K. Sung, and C. K. Kim (1993) Cloning and expression of *pcbAB* genes from *Pseudomonas* sp. DJ-12 in *Escherichia coli*. *Kor. J. Microbiol.* 31: 129-135.

[12] Kim, C. K., T. K. Sung, J. H. Nam, Y. C. Kim, and J. K. Lee (1994) Cloning and expression of *pcbCD* genes in *Escherichia coli* from *Pseudomonas* sp. DJ-12. *Kor. J. Microbiol.* 32: 40-46.

[13] Chae, J. C., K. J. Ahn, and C. K. Kim (1998) Hydrolytic dechlorination of 4-chlorobenzoate specified by *fcbABC* of *Pseudomonas* sp. DJ-12. *J. Microbiol. Biotechnol.* 8: 692-695.

[14] Tsoi, T. V., G. M. Zaitsev, E. G. Plotnikova, I. A. Kosheleva, and A. M. Boronin (1991) Cloning and expression of the *Arthrobacter globiformis fcbA* gene encoding dehalogenase (4-chlorobenzoat-4-hydrolase) in *Escherichia coli*. *FEMS Microbiol. Lett.* 81: 165-170.

[15] Harayama, S. and M. Rekik (1989) Bacterial aromatic ring-cleavage enzymes are classified into two different gene families. *J. Biol. Chem.* 264: 15328-15333.

[16] Kim, E. and G. J. Zylstra (1995) Molecular and biochemical characterization of two *meta*-cleavage dioxygenases involved in biphenyl and *m*-xylene degradation by *Beijerinckia* sp. strain B1. *J. Bacteriol.*177: 3095-3103.

[17] Lee, J. Y., Y. S. Kim, K. S. Lee, K. H. Min, Y. C. Kim, C. K. Kim, and J. Y. Lim (1998) Culture conditions of *Escherichia coli* CK1092 for production of 2,3-dihydroxybiphenyl dioxygenase. *Kor. J. Microbiol.* 34: 20-25.

[18] Durham, D. R., C. G. McNamee, and D. B. Stewart (1984) Dissimilation of aromatic compounds in *Rhodotorula graminis*: Biochemical characterization of pleitiropically negative mutants. *J. Bacteriol.* 160: 771-774.

[19] Crawford, R. L. (1975) Novel pathway for degradation of protocatechuic acid in *Bacillus* sp. *J. Bacteriol.* 121: 531-536.

[20] Lowry, O. H., N. J. Rosebrough, A. C. Farr, and R. J. Randall (1951) Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.* 193: 265-275.

[21] van den Tweel, W. J. J., J. B. Kok, and J. A. M. de Bont (1987) Reductive dechlorination of 2,4-dichlorobenzoate to 4-chlorobenzoate and hydrolytic dehalogenation of 4-chloro-, 4-bromo-, and 4-iodobenzoate by *Alcaligenes denitrificans* NTB-1. *Appl. Environ. Microbiol.* 53: 810-815.

[22] Arensdorf, J. J. and D. D. Focht (1995) A *meta* cleavage pathway for 4-chlorobenzoate, an intermediate in the metabolism of 4-chlorobiphenyl by *Pseudomonas cepecia* P166. *Appl. Environ. Microbiol.* 61: 443-447.

[23] Chae, J. C. and C. K. Kim (1997) Dechlorination of 4-chlorobenzoate by *Pseudomonas* sp. DJ-12. *J. Microbiol.* 35: 290-294.

[24] Sambrook, J., E. F. Fritsch, and T. Maniatis (1989) *Molecular cloning: A Laboratory Manual*. 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

[25] Koetsier, P. A., J. Schorr, and W. Doerfler (1993) A rapid optimized protocol for downward alkaline Southern

blotting of DNA. *Biotechniques* 15: 260-262.
[26] Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman (1997) Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* 25: 3389-3402.
[27] Furukawa, K. and T. Miyazaki (1986) Cloning gene cluster encoding biphenyl and chlorobiphenyl degradation in *Pseudomonas pseudoalcaligenese. J. Bacteriol.* 166: 392-398.
[28] Lee, J., T. K. Sung, J. Moon, K. R. Min, C. K. Kim, and Y. Kim (1994) Comparison of enzymatic and immunochemical properties of 2,3-dihydroxybiphenyl 1,2-dioxygenase from four *Pseudomonas* strains. *FEMS Microbiol. Lett.* 120: 355-362.
[29] Nam, J. H., C. K. Kim, J. K. Lee, and K. J. Lee (1994) Homology analysis among the biphenyl and 4-chlorobiphenyl degrading genes by Sourthern hybridization. *Kor. J. Appl. Microbiol. Biotechnol.* 22: 37-44.
[30] Zylstra, G. J. and D. T. Gibson (1989) Toluene degradation by *Pseudomonas putida* F1. *J. Biol. Chem.* 264: 14940-14946.
[31] Taira, K., J. Hirose, S. Hayashida, and K. Furukaya (1992) Analysis of *bph* opron from the polychlorinated biphenyl-degrading strain of *Pseudomonas pseudoalcaligenes* KF707. *J. Biol. Chem.* 267: 4844-4853.
[32] Kimbara K., T. Hashimoto, M. Fukuda, T. Koana, M. Takagi, M. Oishi, and K. Yano (1989) Cloning and sequencing of two tandem genes involved in degradation of 2,3-dihydroxybiphenyl to benzoic acid in the polychlorinated biphenyl-degrading soil bacterium *Pseudomonas* sp. strain KKS102. *J. Bacteriol.* 171: 2740-2747.
[33] Copley, S. D. and G. P. Crooks (1992) Enzymic dehalogenation of 4-chlorobenzoyl coenzyme A in *Acinetobacter* sp. strain 4-CB1. *Appl. Environ. Microbiol.* 58: 1385-1387.
[34] Löffler, F., R. Müller, and F. Lingens (1991) Dehalogenation of 4-chlorobenzoate by 4-chlorobenzoate dehalogenase from *Pseudomonas* sp. CBS3: an ATP/Coenzyme A dependent reaction. *Biochem. Biophys. Res. Commun.* 176: 1106-1111.
[35] Schmitz, A., K.-H. Gartemann, J. Fiedler, E. Grund, and R. Eichenlaub (1992) Cloning and sequence of genes for dehalogenation of 4-chlorobenzoate from *Arthrobacter* sp. strain SU. *Appl. Environ. Microbiol.* 58: 4068-4071.
[36] Babbitt, P. C., G. L. Kenyon, B. M. Martin, H. Charest, M. Sylvestre, J. D. Scholten, K. H. Chang, P. H. Liang, and D. Dunaway-Mariano (1992) Ancestry of the 4-chlorobenzoate dehalogenase: Anaysis of amino acid sequence identities among families of acyl:adenyl ligase, enoyl-CoA hydratases/isomerase, and acyl-CoA thioesterases. *Biochemistry* 31: 5594-5604.
[37] Benning, M. M., K. L. Taylor, R.-Q. Liu, G. Yang, H. Xiang, G. Wesenberg, D. Dunaway-Mariano, and H. M. Holden (1996) Structure of 4-chlorobenzoyl coenzyme A dehalogenase determined to 1.8 Å resolution: An enzyme catalyst generated via adaptive mutation. *Biochemistry* 35: 8103-8109.
[38] Karegoudar, T. B., J. C. Chae, and C. K. Kim (1999) Catabolism of 4-hydroxybenzoic acid by *Pseudomonas* sp. DJ-12. *J. Microbiol.* 37: 123-127.

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