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NOTE

A Pathway for 4-Chlorobenzoate Degradation by *Pseudomonas* sp. S-47

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Abstract *Pseudomonas* sp. S-47 degraded 4-chlorobenzoate (4CBA) to 4-chlorocatechol (4CC) that was subsequently ring-cleaved to form 5-chloro-2-hydroxymuconic semialdehyde. These intermediate compounds were identified by GC-mass spectrometry and UV-visible spectrophotometry. 5-chloro-2-hydroxymuconic acid converted from 5-chloro-2-hydroxymuconic semialdehyde (5C-2HMS) was dechlorinated to produce 2-hydroxypenta-2,4-dienoic acid (2HP-2,4DA) by the strain. These results indicate that *Pseudomonas* sp. S-47 degrades 4CBA to 2HP-2,4DA via a novel pathway including the *meta*-cleavage of 4CC and dechlorination of 5C-2HMS.

Key words: 4-Chlorobenzoate, degradation pathway, *Pseudomonas* sp. S-47

Chlorinated benzoates are produced as common intermediates in the microbial degradation of chlorinated aromatic hydrocarbons including polychlorinated biphenyls [6]. It has often been reported that chlorobenzoates are accumulated as dead-end products of the microbial degradation of polychlorinated biphenyls [3] and that the compound inhibits the degradation of biphenyl and chlorobiphenyls by *Pseudomonas testosteroni* B-356 [18]. However, chlorobenzoates are not always dead-end metabolites of the chlorinated aromatics. 4-Chlorobenzoate (4CBA) was reported to be easily transformed to 4-hydroxybenzoate by dehalogenation in *Pseudomonas* sp. CBS3 [4], *Arthrobacter* sp. 4CB1 [1], *Arthrobacter* sp. SU1 [14], and *Alcaligenes* sp. A5 [10] as shown in step A of Fig. 1. The protocatechuate pathway beginning from 4-hydroxybenzoate has also been studied for corresponding enzymes and genes [19].

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There have been other reports on chlorobenzoate transformation by biphenyl-degrading bacteria. 4CBA can be transformed to 4-chlorocatechol (4CC) by benzoate dioxygenase, which can be further degraded by *ortho*-cleavage (step D of Fig. 1) or *meta*-cleavage (step F) of the aromatic ring [13]. The *ortho*-cleavage product, 3-chloromuconate, was reported to be transformed to *cis*-dienelactone by a cycloisomerase (step E) in *Pseudomonas* sp. B13 and *Pseudomonas putida* [7, 12]. The 5-chloro-2-hydroxymuconic semialdehyde (5C-2HMS), the *meta*-cleavage product of 4CC, was reported to be transformed to 5-chloro-2-hydroxymuconic acid by 5C-2HMS dehydrogenase (step G) in *Pseudomonas cepacia* P166, and then to chloroacetic acid which is ultimately dechlorinated to be utilized as a carbon and energy source [3, 11]. On the other hand, 2-hydroxypenta-2,4-dienoic acid (2HP-2,4DA) was reported to be produced as an intermediate metabolite during degradations of biphenyl [9], catechol [17] and *p*-cumate [5]. The 2HP-2,4DA can be hydrolyzed to form 2-oxo-4-hydroxyvaleric acid (step K) which is completely oxidized through the TCA cycle. Until now, nobody has reported that 2HP-2,4DA is produced from 4CC via a *meta*-cleavage (step F) and then dechlorination (step I) as seen in Fig. 1.

Growth of Strain S-47

Wastewater samples taken from a chemical industry complex of Ulsan, Korea were enriched in LB medium (Tryptone, 10 g/l; Yeast extract, 5 g/l; NaCl, 5 g/l; pH 7.0), and then inoculated in MM2 broth supplemented with 1 mM 4CBA. The strain S-47 was isolated via further enrichment in dilution series followed by plating on MM2 agar medium containing 1 mM 4CBA as the sole carbon and energy source, and identified as reported by Seo *et al.* [15]. The organisms were cultivated in MM2 broth containing various concentrations of 4CBA at 30°C. *Pseudomonas* sp. S-47 showed maximum growth at 1 mM of 4CBA, and retarded growth at higher concentrations.

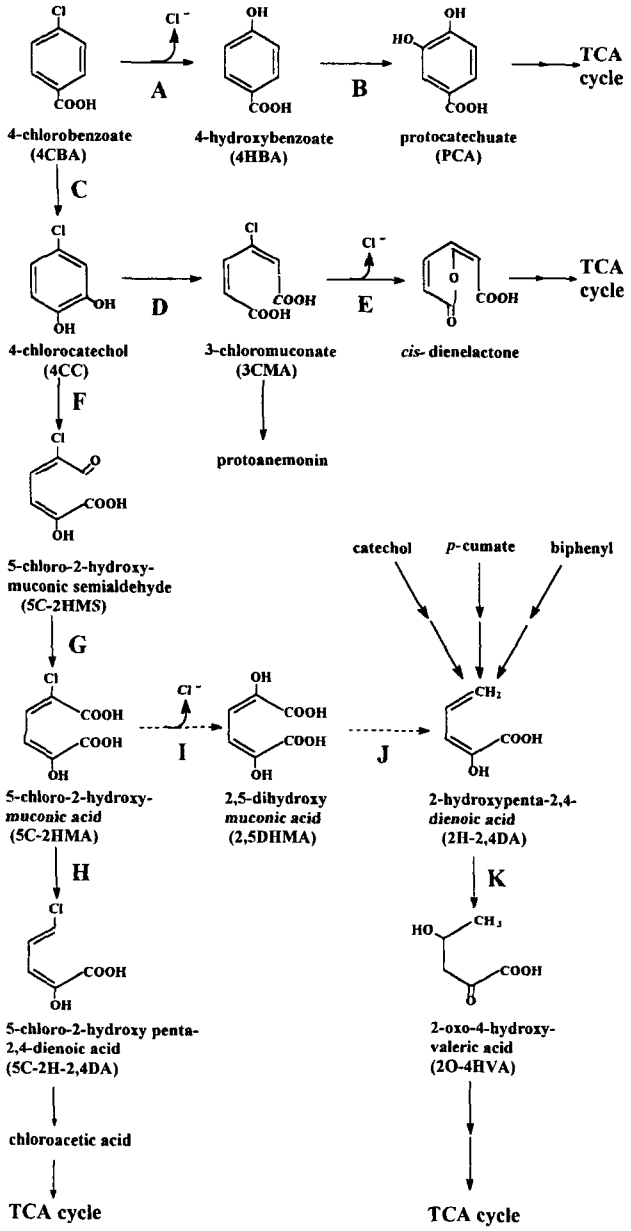


Fig. 1. Pathways for aerobic degradation of 4-cholorobenzoate which were reported in references.

Enzymes are 4-chlorobenzoate dehalogenase (A), 4-hydroxybenzoate hydroxylase (B), benzoate dioxygenase (C), catechol 1,2-dioxygenase (D), 3-chloromuconate cycloisomerase (E), catechol 2,3-dioxygenase (F), 5-chloro-2-hydroxymuconic semialdehyde dehydrogenase (G), 5-chloro-2-hydroxymuconate decarboxylase (H), 5-chloro-2-hydroxymuconate dehalogenase (I), 2,5-dihydroxymuconate decarboxylase (J), and 2-hydroxypenta-2,4-dienoate hydratase (K).

The strain was also grown in benzoate, 4HBA, catechol, and protocatetuate as the sole carbon and energy sources.

Degradation of 4CBA by Strain S-47

The degradation of 4CBA and other aromatic hydrocarbons by the strain S-47 was examined by growing cell and resting cell assays. The cells grown in LB broth for 12 h were inoculated in MM2 broth containing 1 mM 4CBA and incubated at 30°C for appropriate periods of time. Metabolites produced from 4CBA were then identified by scanning the absorbance at 200 to 500 nm according to the method described by Shimao *et al.* [16].

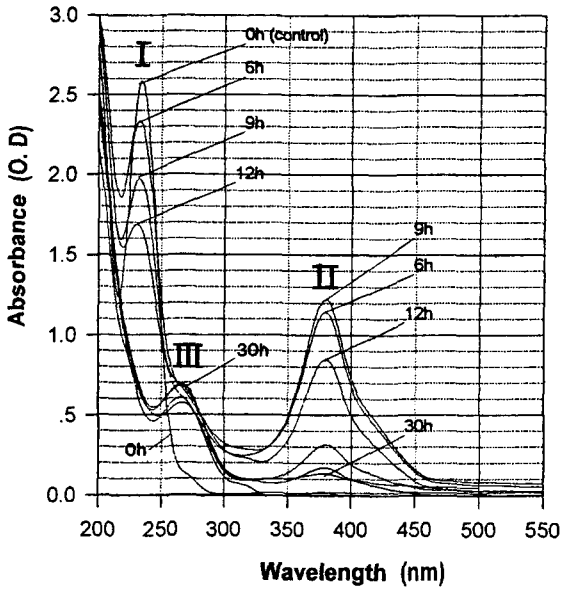


Fig. 2. Degradation of 4-chlorobenzoate and production of its metabolites by *Pseudomonas* sp. S-47.

Compounds are 4-chlorobenzoate (I), 5-chloro-2-hydroxymuconic semialdehyde (II), and 2-hydroxypenta-2,4-dienoic acid (III).

For gas chromatography and mass spectrometry of the metabolites, a culture of the strain S-47 grown in MM2 broth containing 1 mM 4CBA was centrifuged to remove cells and acidified to pH 2.0 with H_2SO_4. Metabolites were then extracted with diethyl ether. After derivatization with trimethylsilylimidazole (TMSIM), the metabolites were analyzed with a GC-mass spectrometer (5890 series II, Hewlett Packard, USA). The temperatures of injector and detector were 220°C and 200°C, respectively. The metabolites were separated on a HP-Innowax capillary column (30 m in length, 0.32 mm in inside diameter, 0.25 mm in film thickness) with a temperature program of 120°C to 280°C [3].

The degradation of 4CBA by *Pseudomonas* sp. S-47 in MM2 broth and the resulting metabolites were examined by the growing cell assay as shown in Fig. 2. When 4CBA (I) detected at 234 nm was metabolized by *Pseudomonas* sp. S-47, a metabolite (II) detected at 380 nm was produced as a function of time up to 9-h post-incubation. Afterwards, the metabolite (II) diminished and another metabolite (III) detected at 265 nm was produced. The metabolite (II) was identified as 5C-2HMS by analysis of GC-mass spectral data as shown in Fig. 3. The 5C-2HMS derivatized with TMSIM showed 11.12 min of

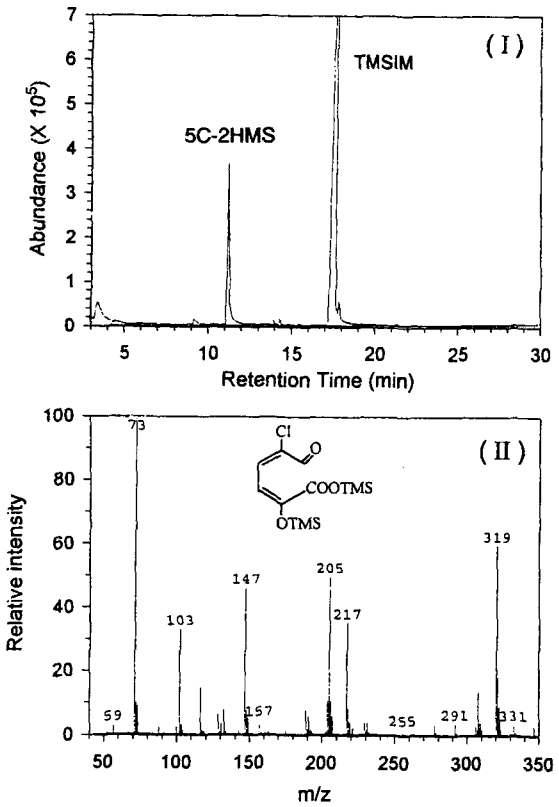


Fig. 3. GC (I) and MS (II) spectra of 5-chloro-2-hydroxymuconic semialdehyde produced from 4-chlorobenzoate by *Pseudomonas* sp. S-47.

5-Chloro-2-hydroxymuconic semialdehyde (5C-2HMS) was derivatized with trimethylsilylimidazole (TMSIM).

retention time. The mass spectrum of the compound had a molecular ion at *m/z* 319. The metabolite (III) detected at 265 nm was also spectrophotometrically identified to be 2HP-2,4DA which was shown to be produced from biphenyl by *Pseudomonas* sp. strain KKS102 as an intermediate metabolite [8].

The results of the growing cell assay for 4CBA degradation by *Pseudomonas* sp. S-47 are presented in Fig. 4. As 4CBA was degraded, 5C-2HMS was produced during the first 10 h and then diminished gradually. Chloride ions and 2HP-2,4DA began to be produced simultaneouly right after the start of 5C-2HMS production. Chloride ion and 2HP-2,4DA levels were markedly increased during 5- to 10-h post-incubation when large amounts of 5C-2HMS were produced. Chloride ions produced from 4CBA by the strain S-47 were colorimetrically determined according to the procedures described by Shimao *et al.* [16]. The organisms were cultivated in a chloride-free minimal medium containing 0.2% glucose, 0.2% maltose, and 1 mM 4CBA. One ml of the culture was reacted with 400 µl of 2.17 mM murcuric thiocyanate and 400 µl of 0.25 M ferric ammonium sulfate for 30 min. The ferric

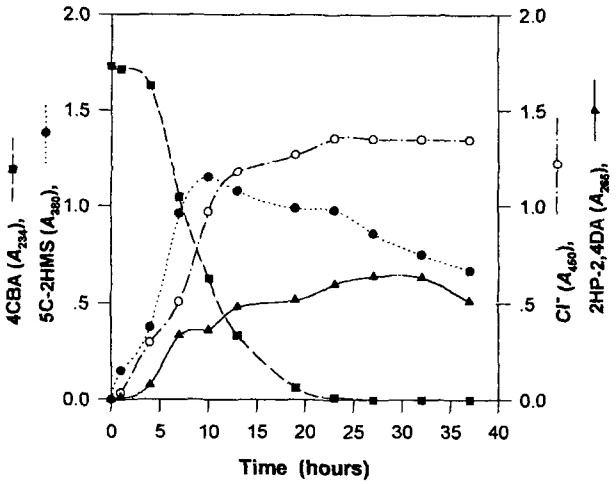


Fig. 4. Degradation of 4-chlorobenzoate to form 5-chloro-2-hydroxymuconic semialdehyde and 2-hydroxypenta-2,4-dienoate by *Pseudomonas* sp. S-47.

Abbreviation: 4CBA, 4-chlorobenzoate; 5C-2HMS, 5-chloro-2-hydroxymuconic semialdehyde; 2HP-2,4DA, 2-hydroxypenta-2,4-dienoate; Cl^-, chloride ion.

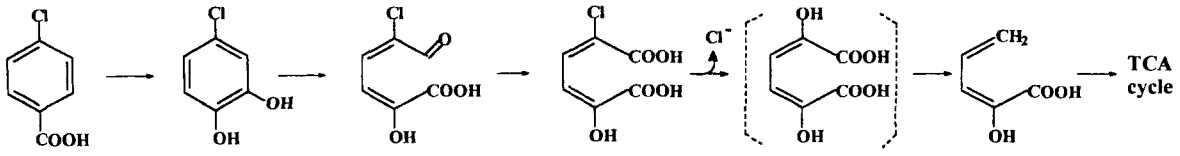
thiocyanate formed by the reaction was detected by measuring the absorbance at 450 nm.

Pathway for 4CBA Degradation

There have been several reports that 4CBA is converted to 4CC which can be further metabolized via *meta*-cleavage pathways leading to dehalogenation by *P. cepacia* P166 and *P. acidovorans* M3GY [3, 11]. They emphasized that 4CC is the central intermediate in the pathway, but did not mention the production of 2HP-2,4DA by dehalogenation. A catabolic pathway of biphenyl to 2-hydroxymuconic semialdehyde via benzoate was already reported by Arensdorf and Focht [3] and Kikuchi *et al.* [8]. The catabolic pathway of 4CC leading to chloroacetic acid as shown in steps F, G, and H of Fig. 1 was also studied by Arensdorf and Focht [3] and McCullar *et al.* [11]. In particular, the conversion of 5C-2HMS to 5C-2HMA by this S-47 strain was already reported in our previous paper [15].

The facts that 4CBA is transformed to 4CC by benzoate dioxygenase (TodA,B,C,D, XylX,Y, and BphA) and that 5C-2HMS is produced from 4CC by catechol 2, 3-dioxygenase (TodE, NahH, and DmpB) are well recognized by several reports [2, 11, 13, 15]. On the other hand, 2HP-2,4DA was reported to be produced as an intermediate during degradations of catechol by *Pseudomonas* sp. CF600 [17], *p*-cumate by *P. putida* F1 [5], and biphenyl by *P. pseudoalcaligenes* KF707 and *P. cepacia* LB400 [9]. The 2HP-2,4DA is then transformed to 2-oxo-4-hydroxyvaleric acid which could ultimately be catabolized through the TCA cycle.

However, there has been no report that 2HP-2,4DA is



4-chlorobenzoate (4CBA) — **4-chlorocatechol (4CC)** — **5-chloro-2-hydroxy-muconic semialdehyde (5C-2HMS)** — **5-chloro-2-hydroxy-muconic acid (5C-2HM)** — **2,5-dihydroxy-muconic acid (2,5DHMA)** — **2-hydroxypenta-2,4-dienoic acid (2HP-2,4DA)**

Fig. 5. A proposed pathway for degradation of 4-chlorobenzoate by *Pseudomonas* sp. S-47.

transformed from 4CC by *meta*-cleavage followed by dehalogenation. The production of 2HP-2,4DA and release of chloride ions from 5C-2HMS found in this study indicate that *Pseudomonas* sp. S-47 transforms 5C-2HMS to 5C-2HMA and then dechlorinates 5C-2HMA to produce 2HP-2,4DA via 2,5-dihydroxymuconic acid, as proposed in Fig. 5. This means that *Pseudomonas* sp. S-47 degrades 4CBA to 2HP-2,4DA via a novel catabolic pathway of 4CC by *meta*-cleavage and then dechlorination.

Acknowledgments

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