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Isolation of *Pseudomonas* sp. S-47 and Its Degradation of 4-Chlorobenzoic Acid

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The strain of S-47 degrading 4-chlorobenzoic acid (4CBA) was isolated from Ulsan chemical industrial complex by enrichment cultivation with 1 mM 4CBA. The strain was Gram-negative rod and grew optimally at 30°C and pH 7 under aerobic condition, so that the organism was identified as a species of *Pseudomonas*. *Pseudomonas* sp. S-47 degraded 4-chlorobenzoic acid to produce a yellow-colored *meta*-cleavage product, which was confirmed to be 5-chloro-2-hydroxymuconic semialdehyde (5C-2HMS) by UV-visible spectrophotometry. 5C-2HMS was proved to be further transformed to 5-chloro-2-hydroxymuconic acid (5C-2HMA) by GC-mass spectrometry. This means that *Pseudomonas* sp. S-47 degraded 4CBA via 4-chlorocatechol to 5C-2HMS by *meta*-cleavage reaction and then to 5C-2HMA by 5C-2HMS dehydrogenase.

Key words: 4-Chlorobenzoic acid, degradation, *Pseudomonas* sp. S-47

Mono-chlorinated and poly-chlorinated benzoic acids can be produced from many kinds of herbicides, pesticides, and polychlorinated biphenyls (PCBs). Such common metabolites are still reported to act as toxic pollutants in the environments (8, 12, 15). However, these metabolites could be degraded by a variety of microorganisms. Therefore, biodegradation of chlorinated benzoic acids has been extensively studied for the purpose of microbial clean-up of polluted environments (5, 7, 8, 20). 3-Chlorobenzoic acid has often been reported to be a dead-end product (3) and to inhibit transformation of chlorinated biphenyls (23).

On the other hand, 4-chlorobenzoic acid (4CBA) is known to be readily degraded via several degradative pathways. As shown in Fig. 1, 4CBA is transformed to 4-hydroxybenzoic acid (4HBA) by dehalogenation (step A) in *Pseudomonas* sp. CBS3 (4), *Arthrobacter* sp. 4CB1 (1), *Arthrobacter* sp. SU1 (22), and *Alcaligenes* sp. A5 (14). 4HBA can be utilized as carbon and energy source via aromatic ring-fission processes (4, 25). It is also transformed to 4-chlorocatechol (4CC) by benzoate dioxygenation (step B) in *Pseudomonas* strain B13 (21). 4CC can be further degraded via *ortho*-cleavage (step C) or *meta*-cleavage (step D) of the aromatic ring (11, 18, 21). The *meta*-cleavage product of 4CC, 5-chloro-2-hydroxymuconic semialdehyde (5C-2HMS), has been reported to be transformed to 5-chloro-2-hydroxymuconic acid (5C-2HMA) by 5C-2HMS dehydrogenase (step E) in *Pseudomonas cepacia* P166, and then to chloroacetic acid, which is ultimately utilized as carbon and energy source through TCA cycle (3, 17).

In this study, a strain of S-47 was isolated as a 4CBA degrader and identified as a species of *Pseudomonas*. The strain degraded 4CBA to 4CC which was dioxygenated to form 5C-2HMS via *meta*-cleavage and then to 5C-2HMA.

Materials and Methods

Isolation and identification of strain S-47

Wastewater samples taken from Ulsan chemical industry complex were incubated in LB medium (Bacto tryptone, 10 g/L; Bacto yeast extract, 5 g/L; NaCl, 5 g/L; pH 7.0). The enriched culture was reinoculated in MM2 broth supplemented with 1 mM 4CBA and cultivated in a shaking incubator at 30°C as described by Kiyohara *et al.* (13). Several strains were isolated by plating the enriched culture on MM2 agar containing 1 mM 4CBA. The isolated strains were selected by their capabilities for degradation of 4CBA and other aromatic hydrocarbons. Those strains were also examined for their morphological and biochemical properties with a scanning electron microscope and API 20 E (API System, France).

Scanning electron microscopy

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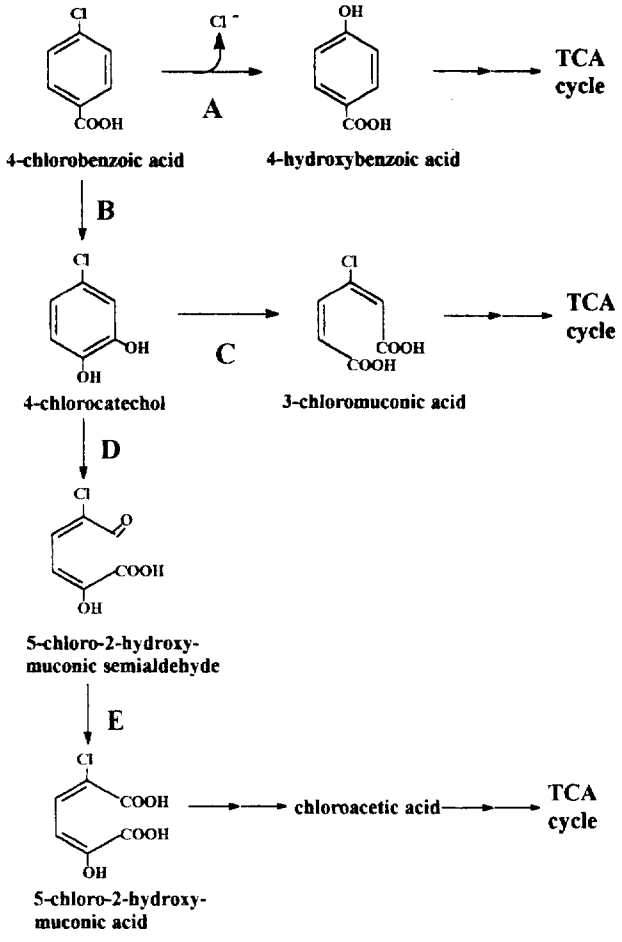

Fig. 1. Pathways of aerobic degradation of 4-cholorobenzoic acid. A, 4-chlorobenzoic acid dehalogenase; B, benzoic acid dioxygenase; C, catechol 1,2-dioxygenase; D, catechol 2,3-dioxygenase; E, 5C-2HMS dehydrogenase.

Intact colonies of strain S-47 grown on MM2 agar containing 1 mM 4CBA were pre-fixed with 2.5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 4 h, and then post-fixed with 1% osmium tetroxide in 0.2 M phosphate buffer for 12 hours as described by Ng *et al.* (19). The colonies were dehydrated through a series of ethanol concentration and isoamyl acetate and dried with a critical point dryer. The dried colonies were gold coated with a Sputter coater (Edwards, S150B), and then observed with a scanning electron microscope (Hitachi, S-507).

Growth and 4CBA degradation

The organism was grown in MM2 broth containing different concentrations of 4CBA at 30°C, and cell density of the culture was measured at 660 nm during cultivation. Degradation of 4CBA and other aromatic hydrocarbons was examined by growing cell assay as described by Arensdorf and Focht (2) and Harayama *et al.* (9). The cells grown in LB broth for 12 h at 30°C were harvested by centrifugation at 10,000×g for 10 minutes. The cells were usually washed twice with 10 mM phosphate buffer (pH 7.2) and then inoculated in MM2 broth. The cell suspensions with an added 1 mM 4CBA were incubated at 30°C. Small aliquots of the culture incubated for appropriate time were centrifuged to remove cells and then scanned for the metabolites at 200 to 500 nm with a UV-visible spectrophotometer (Pharmacia, Biochrom 4060, Sweden). In particular, 4CBA was examined at 234 nm and 5-chloro-2-hydroxymuconic semialdehyde (5C-2HMS) at 380 nm.

GC-MS

The culture of the organism grown in MM2 broth containing 1 mM 4CBA was centrifuged to remove cells and then acidified to pH 2.0 with H_2SO_4 as described by Dietrich *et al.* (6) and Marks *et al.* (16). The supernatants were extracted three times with 2 volume of diethyl ether. The organic phases were derivatized by adding an ethereal solution of diazomethane as described by Arensdorf and Focht (3). The methylated metabolites were analyzed with a GC-mass spectrometer (Hewlett Packard, 5890 series II, U.S.A.). The temperatures of injector and detector were 220°C and 200°C, respectively. The metabolites were separated on a DB-5 capillary column (30 m in length; 0.24 mm in inside diameter; 0.25 μm in film thickness; J & W Scientific, Folsom, Calif.) with a temperature program of 70°C (1 min. initial wait) to 200°C (5°C/min.) as described by Arensdorf and Focht (3), Sondossi *et al.* (23), and Spiess *et al.* (24).

Results and Discussion

Characteristics of strain S-47

The morphological and biochemical characteristics of strain S-47 are shown in Table 1. The strain was an aerobic Gram-negative rod and its optimal conditions for growth were 30°C and pH 7.0. The organism exhibited potential ability to degrade 4-chlorobenzoic acid, 4-hydroxybenzoic acid, benzoic acid, catechol, and protocatetuate. A scanning electron micrograph of the organism is also shown in Fig. 2. The strain was identified as a species of *Pseudomonas* on the basis of its characteristics, including the API results.

Degradation of 4CBA

Pseudomonas sp. strain S-47 showed maximum growth at 1 mM 4CBA in MM2 medium and slower

Table 1. Morphological and biochemical characteristics of isolate S-47

Parameters	Results
Shape	rod
Gram stain	
Oxygen requirement	aerobic
Optimum temperature	30°C
Optimum pH	pH 8
Oxidase	+
VP test	+
Motility	-
Indole production	
H_2S production	
Urea hydrolysis	
Citrate utilization	+
NO_2 production	+
Ampicillin (200 μg/ml)	resistant
Tetracycline (200 μg/ml)	resistant
Utilization of amino acids	
arginine	+
lysine	
ornithine	
tryptophane	
Fermentation of carborhydrates	
glucose	
mannitol	
inositol	
sorbitol	
rhamnose	
sucrose	
melibiose	
arabinose	
Degradation of aromatic hydrocarbons	
4-chlorobenzoic acid	+++
3-chlorobenzoic acid	++
4-hydroxybenzoic acid	+++
benzoic acid	+++
4-chlorobipheyl	+
catechol	+++
protocatechuate	+++
2,4-dichlorobenzoic acid	+

growth at higher concentrations as shown in Fig. 3. During cultivation of the organism in MM2 broth, the remaining 4CBA and its metabolites in the culture were quantified by the growing cell assay. As shown in Fig. 4, 4CBA (A) detected at 234 nm was decreased and *meta*-cleavage product (B), 5C-2HMS, detected at 380 nm was increased upto 9 h post-incubation. After 9 h, another metabolite (C) was detected at 265 nm, while 5C-2HMS was decreased. There have been several reports that 4CBA was converted to 4CC in *Pseudomonas cepacia* P166 (2) and *Pseudomonas acidovorans* M3GY (17), which was further metabolized to produce 5C-2HMS via *meta*-cleavage pathway. The same *meta*-cleavage product of 4CC, 5C-2HMS, was produced from 4CBA as well as 4CC by strain S-47 in this study when incubated

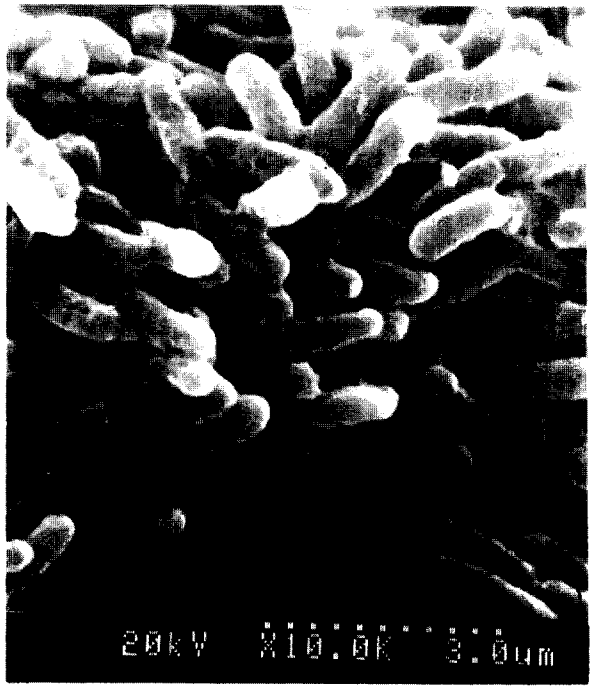

Fig. 2. A scanning electron micrograph of *Pseudomonas* sp. S-47.

for 12 h (Fig. 5). The metabolite produced from 4CBA by catechol 2,3-dioxygenase in *Pseudomonase* sp. WR912 was also reported to be 5C-2HMS detected at 380 nm (10).

The 5C-2HMS was further transformed to form 5-chloro-2-hydroxymuconic acid (5C-2HMA) by strain S-47 in this study. The structure of 5C-2HMA was confirmed by gas chromatography and mass spectrometry (Fig. 6). Fig. 6A shows the gas chromatogram of the methylated 5C-2HMA (retention

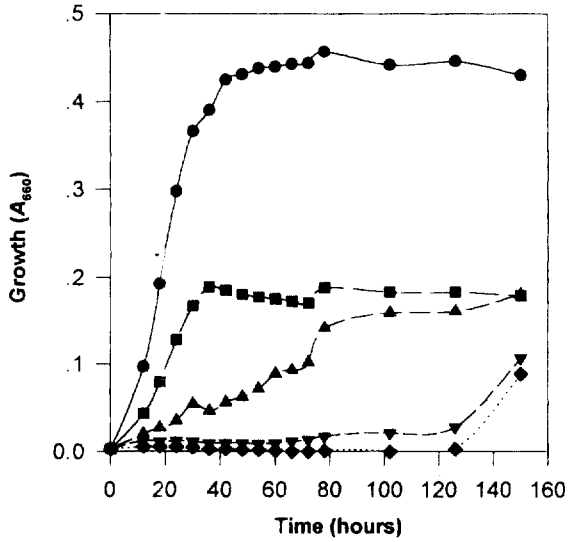

Fig. 3. Growth of *Pseudomonas* sp. S-47 on 4-chlorobenzoic acid. ●, 1 mM; ■, 2 mM; ▲, 3 mM; ▼, 4 mM; ◆, 5 mM.

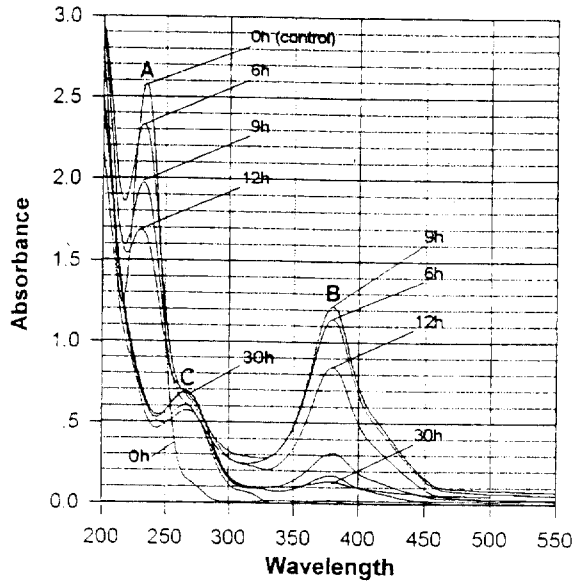

Fig. 4. Degradation of 4-chlorobenzoic acid and production of metabolites by *Pseudomonas* sp. S-47. A, 4-chlorobenzoic acid (4CBA); B, 5-chloro-2-hydroxymuconic semialdehyde (5C-2HMS); C, 2-hydroxypenta-2, 4-dienoic acid (2HP-2, 4DA).

time, 18.154 min) produced from 5C-2HMS. The mass spectrum of the methylated 5C-2HMA is

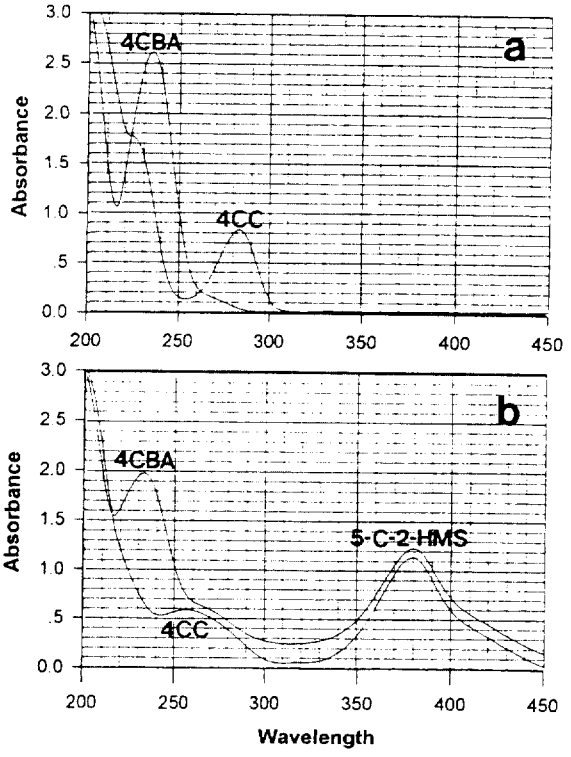

Fig. 5. UV-visible spectra of 4-chlorobenzoic acid and 4-chlorocatechol used as substrates (a) and their catabolites (b) produced by *Pseudomonas* sp. S-47 after 12 h incubation. Abbreviation: 4CBA, 4-chlorobenzoic acid; 4CC, 4-chlorocatechol; 5C-2HMS, 5-chloro-2-hydroxymuconic semialdehyde.

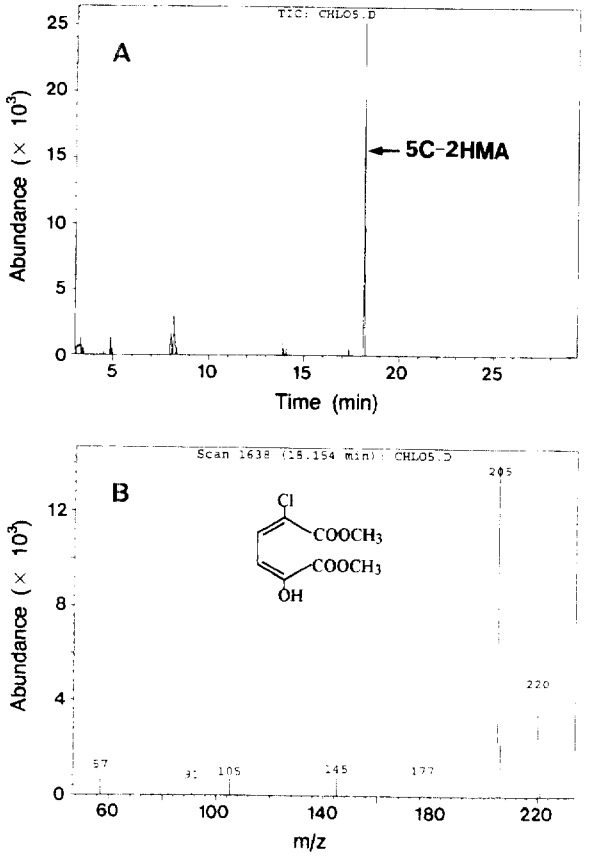

Fig. 6. GC (A) and MS (B) spectra of 5C-2HMA produced from 4-chlorobenzoic acid by *Pseudomonas* sp. S-47. abbreviation: 5C-2HMA, dimethylated 5-chloro-2-hydroxymuconic acid.

shown in Fig. 6B. Therefore, it was confirmed that *Pseudomonas* sp. S-47 degraded 4CBA to form 4CC and that the organism subsequently converted 4CC to 5C-2HMS by catechol 2, 3-dioxygenase and then to 5C-2HMA by 5C-2HMS dehydrogenase. However, the catabolic pathways after 5C-2HMA formation by this strain, including formation of the metabolite detected at 265 nm, should be further studied.

Acknowledgments

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