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Catabolism of 4-Hydroxybenzoic Acid by *Pseudomonas* sp. DJ-12

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A *Pseudomonas* sp. strain DJ-12 isolated by 4-chlorobiphenyl enrichment culture technique is capable of utilizing 4-hydroxybenzoic acid as a sole source of carbon and energy. The bacterium catabolized 4-hydroxybenzoic acid through the intermediate formation of protocatechuic acid, which was further metabolized. The cell free extracts of *Pseudomonas* sp. DJ-12, grown on 4-hydroxybenzoic acid showed higher activities of 4-hydroxybenzoate 3-hydroxylase and protocatechuate 4,5-dioxygenase, but the activity of catechol 2,3-dioxygenase was lower. The results suggest that 4-hydroxybenzoic acid is catabolized via protocatechuic acid rather than catechol or gentisic acid in this bacterium and that the protocatechuic acid formed was metabolized through a *meta*-cleavage pathway by protocatechuate 4,5-dioxygenase.

Key words: Catabolism, 4-hydroxybenzoic acid, protocatechuic acid, protocatechuate 4,5-dioxygenase, *Pseudomonas* sp. DJ-12.

Several catabolic pathways have been known for the breakdown of aromatic compounds. The β-ketoadipate pathway is one common mechanism for the degradation of aromatic compounds (22, 23). 4-Hydroxybenzoic acid (4-HBA) is one such compound that is frequently used as a source of carbon through this pathway (7, 16, 18, 25). It is generally known that the aromatic ring of protocatechuic acid (PCA) is opened in reactions catalyzed by three kinds of dioxygenases: protocatechuate 3,4-dioxygenase (6, 28), protocatechuate 4,5-dioxygenase (19), and protocatechuate 2,3-dioxygenase (27). Among these dioxygenases, protocatechuate 3,4-dioxygenase is the most commonly characterized enzyme (20). Much of this knowledge was derived from studies of members of the genus *Pseudomonas,* because this genus is ubiquitous and possesses the ability to utilize a wide range of aromatic compounds as carbon and energy sources.

Pseudomonas sp. strain DJ-12 was originally isolated as a 4-chlorobiphenyl degrading bacterium from contaminated wastewater (12). This bacterium degrades 4-chlorobiphenyl to produce 4-chlorobenzoic acid (4-CBA) by cleaving the benzene ring at the *meta*-position (8). 4-CBA can be degraded to 4-HBA by a dechlorination reaction and the resulting 4-HBA is further degraded by this organism (1). However, the pathway for the degradation of 4-HBA by this bacterium has not been elucidated. In this paper we studied the catabolic pathway of 4-HBA by *Pseudomonas* sp. DJ-12.

Materials and Methods

Bacterial strain and growth conditions

Pseudomonas sp. DJ-12 was maintained on the slants of substrate-mineral salts medium and also cultivated in Luria-Bertani (LB) medium supplemented with ampicillin as reported by Chae et al. (1) and Han et al. (8). This bacterium was adapted to growth in mineral salts medium supplemented with 4-hydroxybenzoic acid as a sole source of carbon and energy on a rotary shaker at 150 rpm at 30°C. Growth of the bacterium was determined by measuring the optical density of the culture medium at 600 nm. The time course of 4-HBA utilization by this bacterium was studied by cultivating the bacterium in the mineral salts medium supplemented with 4-HBA. The residual 4-HBA in the medium was determined spectrophotometrically at 243 nm at different incubation periods. Resting cell studies (3) were performed by cultivating the organism in mineral salts medium supplemented with 4-HBA (2 mM). The cells were harvested and washed with 10 mM phosphate buffer (pH 7). The washed cells were suspended in the same buffer containing each substrate and incubated at different incubation periods. The samples were taken out and centrifuged at 5,000 rpm for 10 min. The pellet was

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used for the analysis of residual substrates spectrophotometrically at their respective wavelength maxima.

Preparation of cell free extract

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 used were the same as described (13). The medium was inoculated with 1.5% (v/v) inoculum of 40 h grown culture in the same medium. After incubation for 40 h, the bacterial cells were harvested by centrifugation (5,000 rpm for 15 min) and washed twice with 10 mM phosphate buffer (pH 7). 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 centrifuged (12,000 rpm for 30 min) at 4°C. The clear supernatant was used as an enzyme source for all enzyme assays.

Enzyme assays

4-Hydroxybenzoate 3-hydroxylase activity was assayed by the decrease in absorbance at 340 nm due to the oxidation of NADH (5). The assay system contained 1 ml of 10 mM Tris-HCl buffer (pH 8), 200 μM NADH, 1.5 μM 4-HBA, enzyme, and distilled water to make a final volume of 3 ml. The reaction was initiated by the addition of 4-HBA. Protocatechuate 3,4-dioxygease activity was assayed by measuring the decrease in the absorbance at 290 nm (15). The assay system contained 2 ml of 100 mM phosphate buffer (pH 7), enzyme, and distilled water to bring the volume to 2.5 ml. The reaction was initiated by the addition of 0.5 ml of 1 mM PCA. Protocatechuate 4,5-dioxygenase assay system (3 ml) contained 2.8 ml of tris-HCl buffer (pH 9.5) and 0.1 ml of enzyme in a cuvette having a light path of 1 cm. The reaction was initiated by the addition of 0.1 ml of 10 mM PCA (21). The enzyme activity was assayed by measuring the initial increase in the absorbance at 410 nm due to the formation of 4-carboxy 2-hydroxymuconic semi-aldehyde which showed a yellow colour.

Protocatechuate 2,3-dioxygenase was assayed by observing the accumulation of 2-hydroxymuconic semialdehyde at 375 nm (2). The assay mixture contained 0.1 M PCA in 3 ml of 100 mM phosphate buffer (pH 7.2) and the reaction was initiated by adding 0.1 ml of cell free extract. Catechol 1.2-dioxygenase assay consisted 2 ml of 100 mM phosphate buffer (pH 7), 0.4 ml of 10 mM EDTA, 0.1 ml of enzyme, and 0.3 ml of distilled water. The reaction was initiated by the addition of 0.3 ml of 1 mM catechol. Activity was measured spectrophotometrically by an increase in absorbance at 260 nm due to the formation of *cis, cis*-muconic acid (9). Catechol 2,3-dioxygenase assay system contained 2.8 ml of 10 mM phosphate buffer (pH 7) and 0.1 ml of enzyme in a cuvette. The reaction was initiated by the addition of 0.1 ml of 10 mM catechol. The enzyme activity was measured spectrophotometrically at 375 nm due to the formation of 2-hydroxymuconic semialdehyde (11). Protocatechuate non-oxidative decarboxylase activity was assayed by measuring the increase in absorbance at 270 nm (4). The assay system contained 2.5 ml of tris-acetate buffer (pH 7.6), enzyme, and distilled water in a total volume of 3 ml. The reaction was initiated by the addition of 0.1 ml of 10 mM PCA. Protein concentration of the enzyme solution was determined by the method of Lowry et al. (14) using bovine serum albumin as the standard. The specific activity of the enzyme is defined as μM of substrate converted or product formed per min per mg of protein.

Mode of ring cleavage of protocatechuate

Mode of ring cleavage of protocatechuate (PCA) was determined by using cell free extract as described by Stanier et al. (24). Cell free extract of 4-HBA-grown cells were prepared in Tris-HCl buffer (pH 8). The ring cleavage assay system contained 2 ml of cell free extract, 0.5 ml of toluene, and 0.2 ml of 20 M PCA. The tubes were shaken and the development of the bright yellow colouration within a few minutes indicated *meta*-cleavage of PCA. If the result was negative, the tubes were shaken for 1 h at 30. About 1 g of ammonium sulphate was added, followed by one drop of 1% freshly prepared sodium nitroprusside solution, and then by about 0.5 ml of ammonia. After mixing, the development of a deep purple colouration due to the presence of β-ketoadipate indicated *ortho*-cleavage of PCA.

Results and Discussion

The bacterium, *Pseudomonas* sp. DJ-12, was isolated by a selective enrichment technique with 4-chlorobiphenyl as a source of carbon and energy. This organism is capable of degrading 4-HBA in mineral salts medium supplemented with 4-HBA. The growth behaviour of *Pseudomonas* sp. DJ-12 and the utilization of 4-HBA were tested in mineral salts medium containing 4-HBA. The results on the growth and degradation of 4-HBA by the bacterium at different incubation periods are shown in Fig. 1. The maximum growth of the bacterium and complete degradation of 4-HBA (1.5 mM) were observed at about 80 h and 100 h of incubation, respectively. The utilization of

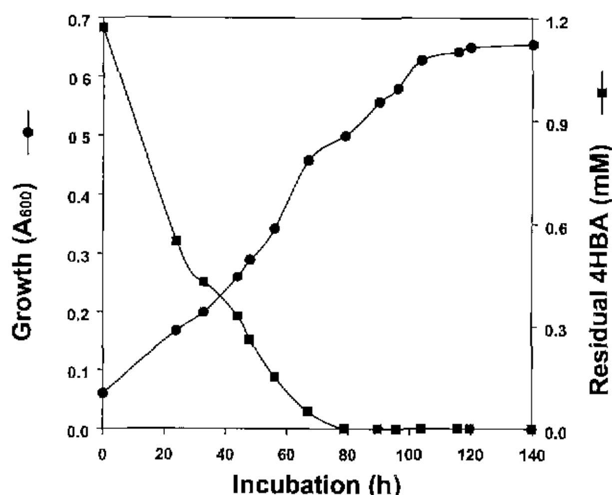

Fig. 1. Growth behaviour and catabolism of 4-hydroxybenzoic acid by *Pseudomonas* sp. DJ-12 in mineral salts medium containing 4-hydroxybenzoic acid. (●), growth and (■), residual 4-hydroxybenzoic acid.

4-HBA and PCA by the bacterium were also studied with resting cell experiments by cultivating the cells in 10 mM phosphate buffer (pH 7) containing 2 mM of each substrate at different incubations. The utilization of 4-HBA and PCA by *Pseudomonas* sp. DJ-12 is presented in Figs. 2 and 3, respectively. The cells of *Pseudomonas* sp. DJ-12 were capable of utilizing these substrates very rapidly. Further, when the resting cell experiments were performed by incubating the whole cells or cell free extract of *Pseudomonas* sp. DJ-12 grown on 4-hydroxybenzoate in phosphate buffer (pH 7) containing 4-HBA (2 mM) and dioxygenase inhibitors (1 mM) such as 2, 2'-dipyridyl or phenanthroline (3), a trace amount of metabolite accumulation was noticed. This metabolite showed wavelength maxima at 245 and 295 nm which corresponded well with that of authentic PCA.

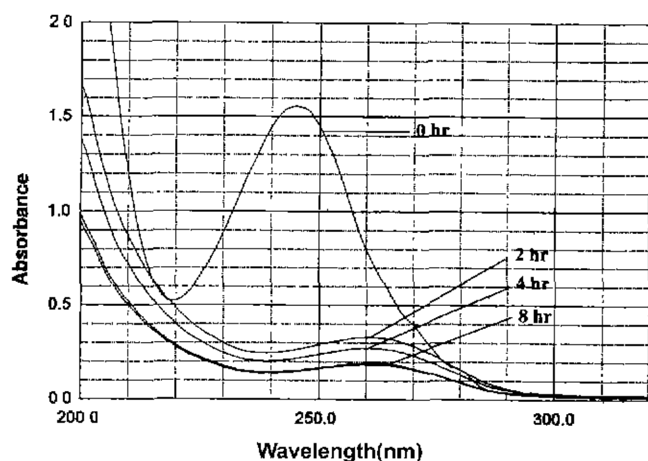

Fig. 2. Degradation of 4-hydroxybenzoic acid by cells of *Pseudomonas* sp. DJ-12. The cells were incubated in 10 mM phosphate buffer (pH 7) containing 4-hydroxybenzoic acid at different time intervals.

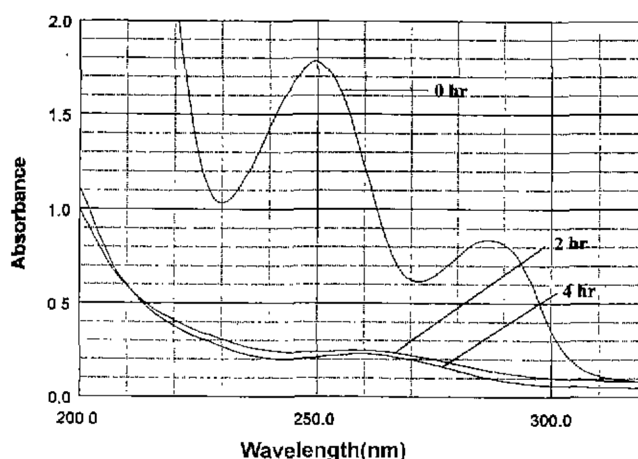

Fig. 3. Degradation of protocatechuic acid by cells of *Pseudomonas* sp. DJ-12. The cells were incubated in 10 mM phosphate buffer (pH 7) containing protocatechuic acid (2 mM) at different intervals.

Various enzymatic assays were performed by using the cell free extracts of *Pseudomonas* sp. DJ-12 grown in the mineral salts medium supplemented with 4-HBA. The results of the activities of various enzymes are given in Table 1. The cell free extract showed high activities of 4-hydroxybenzoate 3-hydroxylase, protocatechuate 4,5-dioxygenase and low activity of catechol 2,3-dioxygenase. However, the cell free extract showed negligible activities of protocatechuate 2,3-dioxygenase, catechol 1,2-dioxygenase and protocatechuate non-oxidative decarboxylase. These results indicate that 4-HBA may be catabolized via PCA. Furthermore, when the cell free extract was incubated with 4-HBA and 2, 2'-dipyridyl or phenanthroline overnight, the ether extract of this reaction mixture showed the accumulation of PCA. The cell free extract of 4-HBA-grown cells in Tris-HCl buffer (pH 8.5) converted PCA to a yellow coloured product, which showed strong absorbance at 410 nm, which is indicative of the formation of the *meta*-cleavage product of

Table 1. Activities of enzymes in cell-free extracts of *Pseudomonas* sp. DJ-12 grown on 4-hydroxybenzoic acid.

Enzyme	Specific activity
4-Hydroxybenzoate 3-hydroxylase	44.30
Protocatechuate 4,5-dioxygenase	71.68
Protocatechuate 3,4-dioxygenase	<1.0
Protocatechuate 2,3-dioxygenase	<1.0
Protocatechuate non oxidative decarboxylast	<1.0
Catechol 2,3-dioxygenase	17.08
Catechol 1,2-dioxygenase	<1.0

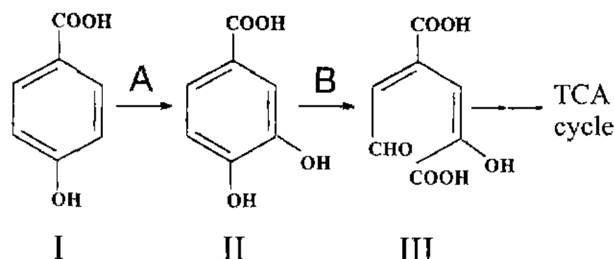

Fig. 4. Proposed catabolic pathway of 4-hydroxybenzoic acid by *Pseudomonas* sp. strain DJ-12. I, 4-hydroxybenzoic acid, II, protocatechuic acid, III, 4-carboxy 2 hydroxymuconic semialdehyde. A, 4-hydroxybenzoate 3-hydroxylase, B, protocatechuate 4,5-dioxygenase.

PCA, the 4-carboxy 2-hydroxymuconic semialdehyde. Our attempts to produce an accumulation of appreciable amounts of PCA by whole cells and cell free extracts using different inhibitors were not successful. This is mainly because the low conversion rate of 4-HBA to PCA, when compared with high protocatechuate 4,5-dioxygenase activity may explain the non accumulation of PCA in the culture medium. Further, the cell free extract showed the activity of catechol 2,3-dioxygenase. This is mainly because the enzyme exhibited broad specificity (26).

It is evident from the above results and from the knowledge of 4-HBA degradation to conclude that 4-HBA was initially hydroxylated to PCA by a NADH requiring hydroxylase. The formed PCA is further converted to 4-carboxy 2-hydroxymuconic semialdehyde via a *meta*-cleavage pathway by protocatechuate 4,5-dioxygenase. On the basis of the above results a proposed pathway for the catabolism of 4-HBA by *Pseudomonas* sp. DJ-12 is shown in Fig. 4. Kersten et al. (10) showed that PCA is subjected to ring cleavage by protocatechuate the 4,5-dioxygenase by *Sphingomonas paucimobilis* SYK-6. Noda et al. (19) reported the cleavage of PCA by 4,5-dioxygenase in *Pseudomonas paucimobilis,* which is capable of degrading vanillic acid. More recently Masai et al. (17) reported the complete degradation of PCA by *Spingomonas paucimobilis* SYK-6. The protocatechuate 4,5-dioxygenase catalyzes the 4,5-cleavage of PCA to form 4-carboxy 2-hydroxymuconic semialdehyde, which is nonenzymatically converted into an intramolecular hemihecital form and then dehydrogenated by carboxymuconic semialdehyde dehydrogenase. Therefore, above results provide an evidence that 4-HBA is catabolized by *Pseudomonas* sp. DJ-12 via PCA rather than catechol or gentisate.

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