Utilization of Aromatic Carboxylic Acids by *Pseudomonas ochraceae*

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The metabolic ability of *Pseudomonas ochraceae* was examined with various aromatic carboxylic acids. The bacterial growth was supported with phthalic acid, tele-phthalic acid, m-hydroxybenzoic acid and p-hydroxybenzoic acid. Iso-phthalic acid sustained the bacterial growth to lesser extent. The bacteria scarcely grew on benzoic acid, salicylic acid, gentisic acid and gallic acid. The cellular respiration was enhanced with several compounds such as succinic acid, protocatechuic acid and the corresponding carbon sources used in the growth medium. Protocatechuate 4, 5-dioxygenase activity was demonstrated in the cell-free extract, although its activity changed considerably depending on the carbon sources in the growth medium. The highest specific activity was found in the extract from p-hydroxybenzoic acid-grown bacteria. The preliminary spectral analyses suggested that p-and m-hydroxybenzoic acid-grown bacteria contained a red pigment whose quantity appeared to correlate to the activity of protocatechuate 4, 5 -dioxygenase.

Phthalic acid, one of the environmental pollutants, is degraded by various microorganisms (1-4). Phthalic acid is converted to protocatechuic acid, possibly, by several oxygenations and subsequent decarboxylation (2-4). Then, the benzene ring of protocatechuic acid is ruptured either at 3, 4-position or at 4, 5-position. The former cleavage was catalyzed by protocatechuate 3, 4-dioxygenase and the latter by protocatechuate 4, 5-dioxygenase. The ring fission products are further metabolized via to β-ketoadipate pathway (5, 6) for 3, 4-fission and α-hydroxy-γ-carboxymuconic acid (HCMA) pathway (7, 8) for 4,5-fission, respectively.

As described in previous paper (8), *Pseudomonas ochraceae* grown on phthalic acid possessed protocatechuate 4, 5-dioxygenase. In this paper, it will be reported that the same bacteria metabolize several aromatic carboxylic acids as well as phthalic acid via to HCMA pathway.

Materials and Methods

Chemicals — All chemicals were reagent grade commercial materials and used without further purification.

Growth Experiments — *P. ochraceae* was isolated by enrichment culture technique as described previously (8, 9). The bacteria grown on phthalic acid-agar slants was

transferred into 250 ml of phthalic acid medium, pH 7. 5, containing lg of $(NH_4)_2SO_4$, 0.75g of phthalic acid, 375mg of K_2HPO_4,125mg of KH_2PO_4, 0.2g of yeast extract and 50mg of $MgCl_2$. $6H_2O$. The mixture was precultured at 28℃ in 500 ml Sakaguchi flask for 22h with reciprocal shaking (95 rev/min). Then, an aliquot of 2 ml was transferred into each 250 ml of medium where 0.75g of various carbon sources replaced phthalic acid. The cultures were carried out as above. The bacterial growth was estimated in terms of turbidity by measuring the absorbance of diluted cell suspensions at 600nm, using a Hitachi recording spectrophotometer 200-10, in cuvettes with 1 cm light path.

Determination of Cellular Respiration —The respiratory activity of the bacteria was polarographically estimated by measuring the oxygen consumption activity at 26℃ with Union Giken Oxygen Meter equipped with a recorder. The reaction mixture (1.92ml) contained 200μmol of potassium phosphate buffer, pH 7.0, the bacterial cells (dry weight, 0.58-1.39mg) which was suspended in 0.02 M potassium phosphate buffer, pH 7.0, and 15μmol of protocatechuic acid or 60μmol of various compounds which were neutralized with NaOH. The reactions were started by the addition of the cell suspension.

Preparation of Cell-free Extract —The bacteria was harvested by centrifugation, washed with 0.02 M potassium phosphate buffer, pH 7.0, and suspended into 3-fold volumes of the same buffer. The cell-free extract was prepared as described previously(9).

Measurement of Enzyme Activities — The activity of protocatechuate 4, 5-dioxygenase was spectrophotometrically determined as described previously (8, 9). The activity of fumarase was determined at 24℃ by the method of Massey (10). The activity of inorganic pyrophosphatase was determined by essentially similar method of Heppel (11). The reaction mixture (1.0 ml) containing 70μmol of barbital-NaOH buffer, pH 7.2, 2μmol of sodium pyrophosphate, 2μmol of $MgCl_2$ and the cell-free extract (0.034-0.052mg protein) was incubated at 31℃ for 0, 5 and 10 min. Then, an amount of inorganic ortho-phosphate formed was determined by the method of Fiske-SubbaRow (12). Protein concentration was determined by the method of Lowry *et al.* using bovine serum albumin as a standard (13).

Analysis of Difference Spectrum — Both the sample and reference cuvettes (light path 1 cm) contained 2.5 ml of the cell-free extract and were preincubated at 24℃ for 5 min. Then, 10mg of sodium dithionite was slowly added to the sample cuvette. After 1 min, the difference spectrum of the dithionite-reduced cell-free extract versus the oxidized one was measured with Shimadzu recording spectrophotometer UV-300.

Results

Growth Experiments — The growth ability of *P. ochraceae* on various carbon sources was examined by measuring the increase of absorbance at 600nm. As shown in Fig. 1, the bacteria grew well on tele-phthalic acid, m-hydroxybenzoic acid, p-hydroxybenzoic acid and succinic acid as well as phthalic acid. The almost same generation time (about 1.0 h) was obtained with these carbon sources. On glucose or iso-phthalic acid, the growth was slower (generation time, about 1.3 h) and incomplete. The bacteria scarcely grew on

salicylic acid, benzoic acid, gentisic acid and gallic acid.

Respiratory Activities of Bacterial Cells — Table I shows the respiratory activities of the bacterial cells grown on various carbon sources. The bacterial cells showed the appreciable respiratory activity in the presence of the same compound as the carbon source used in the growth medium. The typical examples were observed in p-hydroxybenzoic acid-, m-hydroxybenzoic acid -, and tele-phthalic acid-grown bacteria. However, several compounds besides the carbon sources used in the growth medium also enhanced the cellular respiration. Thus, succinic acid, tele-phthalic acid and, particularly, protocatechuic acid

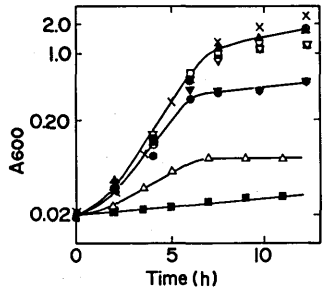

Fig. 1. Bacterial growth on various carbon sources. The bacteria precultured in phthalic acid medium was transferred into a new medium containing phthalic acid (▲), iso-phthalic acid (▼), tele-phthalic acid (▽), m-hydroxybenzoic acid (○), p-hydroxybenzoic acid (□), succinic acid (×), benzoic acid (△), salicylic acid (■) and glucose (●). Other conditions were described under "Methods."

Table I

Respiratory activities of bacterial cells. The cellular respiration in the presence of indicated compounds was determined by measuring oxygen uptake of the bacterial cells grown on various carbon sources. The respiratory activities were calculated by assuming the concentration of oxygen in the assay mixture to be 0.25 mM at 26℃. The other conditions were described under "Methods." Abbreviations: Gl, glucose; Su, succinic acid; Ph, phthalic acid; i-Ph, iso-phthalic acid; t-Ph, tele-phthalic acid; Sa, salicylic acid; m-HBA, m-hydroxybenzoic acid; p-HBA, p-hydroxybenzoic acid; BA, benzoic acid; PCA, protocatechuic acid.

Additions	Bacteria grown on						
	Gl	Su	Ph	i-Ph	t-Ph	m-HBA	p-HBA
	μmol O_2 consumed/min/mg dry weight of cells						
Gl	22	15	15	17	15	18	26
Su	37	30	28	28	36	61	49
Ph	32	15	36	21	31	12	36
i-Ph	16	12	14	13	16	18	21
t-Ph	29	15	23	16	75	58	91
Sa	2	4	4	3	4	5	7
m-HBA	23	11	22	20	24	131	33
p-HBA	6	7	6	4	9	6	232
BA	5	7	8	7	8	6	8
PCA	64	14	55	40	85	63	71

effectively enhanced the cellular respiration.

Effects of Carbon Sources on Enzyme Activities — Three enzyme activities of the cell-free extracts from the bacteria grown on various carbon sources were examined. The activities both of fumarase and inorganic pyrophosphatase were almost unaltered with carbon sources (Fig. 2A and B). However, the activity of protocatechuate 4,

5-dioxygenase changed significantly depending on the carbon sources, suggesting that protocatechuate 4, 5-dioxygenase was induced in the bacteria adapted with several aromatic carboxylic acids (Fig. 2C). A small activity observed in glucose-and succinic acid-grown bacteria may be ascribed to the residual enzyme activity of the bacteria precultured on phthalic acid medium. Among three isomers of phthalic acid, phthalic acid most actively induced the enzyme. The enzyme activities from tele-and iso-phthalic acids -grown bacteria were slightly less than that from phthalic acid-grown bacteria. p-Hydroxybenzoic acid was better inducer than m-isomer and was the best inducer among the carbon sources examined. Thus, the cell-free extract prepared from p--hydroxybenzoic acid-grown bacteria showed 5.5-times higher activity compared with that from phthalic acid -grown bacteria. This fact is important for the isolation and purification of protocatechuate 4, 5-dioxygenase along with the stabilizing effect of polyhydroxyl compounds on the enzyme reported in the previous paper (9). In contrast to p-and m-isomers, o-isomer (salicylic acid) was not used by the bacteria.

All the cell-free extracts prepared in this study showed no activity of protocatechuate 3, 4-dioxygenase which was measured as described by Fujisawa and Hayaishi (14).

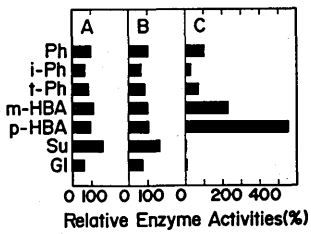

Fig. 2. Effect of carbon sources on enzyme activities. The cell-free extract was prepared from bacterial cells grown on various carbon sources. Then, the activities of fumarase (A), inorganic pyrophosphatase (B) and protocatechuate 4,5-dioxygenase (C) were measured as described under "Methods." One hundred per cent activity was 3.6 units/mg for fumarase, 42 μmol Pi/min/mg for inorganic pyrophosphatase and 0.73 unit/mg for protocatechuate 4,5-dioxygenase, respectively. Abbreviations were described in Table I.

Difference Spectra — The bacterial cells grown on p-hydroxybenzoic acid were reddish brown, whereas those grown on succinic acid were yellowish white. Based on this observation, the redox difference spectra of the cell-free extracts were examined. The cell-free extract from succinic acid-grown bacteria showed a spectrum which was composed of several overlapped spectra of cytochromes (Fig. 3A). The essentially similar spectrum was observed with the cell-free extracts from glucose-, iso-phthalic acid-, tele-phthalic acid-, and

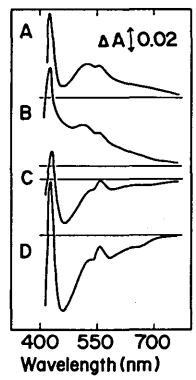

Fig. 3. Redox difference spectra of cell-free extracts. The redox difference spectra of the cell-free extracts from (A) succinic acid-, (B) phthalic acid-, (C) m-hydroxybenzoic acid-, and (D) p-hydroxybenzoic acid-grown bacteria were measured as described under "Methods." Protein concentrations of the cell-free extracts were 20.0 mg/ml for A, 16.2 mg/ml for B, 19.0 mg/ml for C, and 21.6 mg/ml for D, respectively.

phthalic acid-grown bacteria (Fig. 3B). With the cell-free extract from p-hydroxybenzoic acid-grown bacteria, the spectrum was markedly different from those obtained with above bacteria (Fig. 3D) and showed absorption minima at 460nm and 580nm. This spectral species was also found in the cell-free extract from m-hydroxybenzoic acid-grown bacteria, although its content was less compared with p-hydroxybenzoic acid-grown bacteria (Fig. 3C).

Discussion

P. ochraceae, which was originally isolated by its growth ability on phthalic acid, strongly adapted to metabolize the several other aromatic and non-aromatic carboxylic acids such as succinic acid, tele-phthalic acid, p-and m-hydroxybenzoic acids. These compounds fully supported the bacterial growth and appreciably enhanced the cellular respiration (Fig. 1, Table I). Iso-phthalic acid and glucose were relatively poor carbon sources. The reason for this was not clear. Benzoic acid, salicylic acid, gallic acid and gentisic acid stimulated neither the bacterial growth nor the cellular respiration and were not utilized by the bacteria.

Protocatechuic acid effectively stimulated the cellular respiration of the bacteria grown on phthalic acid, iso-phthalic acid, tele-phthalic acid, p-and m-hydroxybenzoic acids (Table I).Furthermore, the cell-free extracts prepared from the above bacteria showed the significant activity of protocatechuate 4,5-dioxygenase and, in contrast, did not show the activity of protocatechuate 3,4-dioxygenase. Although the detailed biochemical investigations were premature, these results suggested that these carbon sources would be converted to protocatechuic acid in the cell and further metabolized via to HCMA pathway.

The spectral shapes shown in Fig. 3 indicated that p-and m-hydroxybenzoic acid-grown bacteria contained some red pigment. The cell-free extract from p-hydroxybenzoic acid-grown bacteria exhibited the highest activity of protocatechuate 4,5-dioxygenase and the highest content of the pigment (Fig. 2 and 3). With m-hydroxybenzoic acid-grown bacteria, both the activity of protocatechuate 4,5-dioxygenase and the content of the the pigment were somewhat lower than those with p-hydroxybenzoic acid-grown bacteria. With phthalic acid-grown bacteria which showed the relatively low activity of protocatechuate 4.5-dioxygenase, the content of the pigment in the cell-free extract was apparently too low to judge by spectral shape. Recently, during the purification process of protocatechuate 4,5-dioxygenase, a small amount of the pigment was also observed in phthalic acid-grown bacteria. Thus, the content of the pigment in the bacterial cells appeared to correlate to the activity of protocatechuate 4,5-dioxygenase. The preliminary study on the pigment suggested that the pigment was high molecular weight (about 10^5 dalton) and the dithionite-reduced pigment was not auto-oxidizable. Therefore, it seems likely that the pigment participates through its redox cycle in the bacterial metabolism of protocatechuic acid. A more detailed investigations on the purification and physiological function of the pigment are in progress.

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