

p-Hydroxyphenylacetic Acid Metabolism in *Pseudomonas putida* F6

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***Pseudomonas putida* F6 was found to metabolize *p*-hydroxyphenylacetic acid through 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxymandelic acid, and 3,4-dihydroxybenzaldehyde. Cell extracts of *P. putida* F6 catalyze the NAD(P)H-independent hydroxylation of *p*-hydroxyphenylacetic acid to 3,4-dihydroxyphenylacetic acid which is further oxidized to 3,4-dihydroxymandelic acid. Oxidation and decarboxylation of the latter yields 3,4-dihydroxybenzaldehyde. A red-brown color accompanies all of the above enzyme activities and is probably due to the polymerization of quinone-like compounds. 3,4-Dihydroxybenzaldehyde is further metabolized through extradiol ring cleavage.**

The microbial metabolism of hydroxylated aromatics such as biogenic amines (dopamine), amino acids (tyrosine), and carboxylic acids (*p*-hydroxyphenylacetic acid [P-OHPA]) has received a lot of attention (7, 13, 14, 21, 22, 27, 32–34). P-OHPA is an intermediate in the microbial degradation of the aromatic amino acids tyrosine and phenylalanine (4, 15, 22). The predominant route described for microbial P-OHPA degradation is through 3,4-dihydroxyphenylacetic acid, which is subsequently ring-cleaved by an extradiol dioxygenase to produce 2-hydroxy-5-carboxymethylmuconic semialdehyde (1, 3–5, 7, 16, 22–24, 26, 32). Kishore and coworkers reported the conversion of P-OHPA to *p*-hydroxymandelic acid in the phenylalanine-degrading *Aspergillus niger* (15). Hareland and coworkers showed that P-OHPA-1-hydroxylase from *Pseudomonas acidovorans* produced 2,5-dihydroxyphenylacetic acid (homotigistic acid) from P-OHPA through hydroxylation at the C-1 ring position and a subsequent NIH shift (11).

Here we present evidence for a novel bacterial pathway for P-OHPA degradation in *Pseudomonas putida* F6 through 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxymandelic acid, and 3,4-dihydroxybenzaldehyde.

MATERIALS AND METHODS

Bacterial strain. *P. putida* F6 was isolated from soil on a mineral medium as previously described (10), except that nutrient concentrations were decreased to 25% of the published concentration and P-OHPA was the sole source of carbon and energy. *P. putida* F6 was identified through partial 16S DNA sequences.

Media and buffers. The culture medium is 1/4 Evans medium as previously described, supplemented with a vitamin mix (1 mg/liter) (10). Cells were washed in ice-cold 50 mM phosphate buffer (pH 7.0). All whole-cell oxygen consumption experiments were carried out using 50 mM potassium phosphate buffer (pH 7.0). All enzyme assays were carried out in 50 mM Tris buffer (pH 7.0).

Growth and harvesting of cells. *P. putida* F6 was grown in batch culture with P-OHPA as the sole source of carbon and energy. Cells were harvested in the exponential phase of growth with an optical density at 540 nm of 0.4 to 0.6. Cells were immediately placed on ice, centrifuged at 15,000 × *g* for 10 min at 4°C, and washed once in ice-cold K₂HPO₄/KH₂PO₄ buffer (pH 7.0). Samples for whole-cell oxygen consumption experiments were resuspended in ice-cold K₂HPO₄/KH₂PO₄ buffer (pH 7.0) to a final cell density of 1.0 to 1.5 g/liter and placed on ice. Cell samples for enzyme assays were resuspended in 50 mM K₂HPO₄/KH₂PO₄ buffer (pH 7.0) containing 10% (wt/vol) glycerol and 1 mM dithiothre-

itol to a final cell dry weight between 10 and 15 g/liter. These cells were subjected to two passages through a precooled French press and then centrifuged at 38,720 × *g* for 30 min. The supernatant, henceforth referred to as the cell extract, was collected and stored on ice.

Oxygen consumption by whole cells. Oxygen consumption experiments were carried out at 30°C using a Rank Brothers oxygen electrode as previously described (20). The final cell density in the assay varied between 0.08 and 0.12 mg dry weight of cells per ml. Substrate solutions (10 mM in 50 mM K₂HPO₄/KH₂PO₄ buffer [pH 7.0]) were added to an initial concentration of 0.5 mM.

Enzyme assays. Cell extracts from P-OHPA and glucose-grown cells were subjected to enzyme assays in 50 mM air-saturated Tris buffer (pH 7.5) at 30°C. Cell extract was added to a final concentration of 0.1 to 0.2 mg of protein per ml. No cofactors were added to the assays unless otherwise stated. Enzyme activities were measured using the oxygen electrode and high-pressure liquid chromatography (HPLC).

Determination of enzyme activities by oxygen consumption methods. Oxygen electrode assays were monitored continuously at 30°C as described for whole-cell assays, but a cell extract replaced whole cells. Substrates were added last to the assays to an initial concentration of 1.0 mM.

Determination of enzyme activities by product formation. Cell extracts were incubated under aeration with various substrates supplied at an initial concentration of 1.0 mM. Fifty-microliter samples were taken 0, 0.5, 1, 3, 5, and 10 min after the addition of the substrate. Samples were acidified with 0.1 volume of 1 N phosphoric acid to stop the reaction. Samples were stored on ice for 10 to 20 min, centrifuged at 21,000 × *g* for 30 min in a bench-top centrifuge at 4°C and frozen at –20°C. Control assays were performed with boiled cell extracts (100°C for 15 min) or with Tris buffer. All samples were analyzed by HPLC using a C-18 Nucleosil 100-column HPLC column (125 × 3 mm) (Macherey-Nagel, Oensingen, Switzerland) and an HPLC instrument equipped with a diode array detector and a mass detector run in the atmospheric pressure chemical ionization (APCI) mode with positive ionization (1100 series; Hewlett-Packard Co., Palo Alto, Calif.). A 0.1% phosphoric acid and methanol (97.5:2.5) mix was used as the eluent at a flow rate of 1.0 ml/min. P-OHPA, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxymandelic acid, 3,4-dihydroxybenzaldehyde, and 3,4-dihydroxybenzoic acid had retention times of 6.8, 3.6, 1.1, 4.1, and 3.2 min, respectively. Standards were acidified with 0.1 N hydrochloric acid and treated in the same way as assay samples for HPLC analysis.

Cell extracts of *P. putida* F6 tested for the presence of noncovalently bound cofactors were centrifuged (21,000 × *g* for 30 min) through a 0.5 K Biomax centrifuge filter. The filtrate was placed on ice, and the retentate was washed in ice-cold 50 mM K₂HPO₄/KH₂PO₄ buffer (pH 7.0) and centrifuged as above. The retentate was resuspended in 50 mM K₂HPO₄/KH₂PO₄ buffer (pH 7.0) containing 10% (wt/vol) glycerol and 1 mM dithiothreitol, and placed on ice.

3,4-Dihydroxybenzaldehyde dehydrogenase assay. A potential 3,4-dihydroxybenzaldehyde dehydrogenase activity was assayed in 50 mM Tris buffer (pH 7.5) containing 1 mM NAD(P)⁺ and *P. putida* F6 cell extract (0.1 to 0.2 mg/ml). Enzyme activity was monitored using the HPLC system described above.

In the first of two modifications of this assay, sodium cyanide (NaCN) was added to a final concentration of 1 mM to inhibit any oxygenase or oxidase activity present in the cell extract, thus potentially enabling the detection of a dehydrogenase with a low affinity or activity for 3,4-dihydroxybenzaldehyde. A second modification consisted of the removal of oxygen in an assay through the

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addition of glucose oxidase (0.1 U), glucose (2.5 mM), and catalase (5 U) to 50 mM Tris buffer, cell extract, and NAD(P)⁺, in an oxygen electrode chamber. The oxidation of glucose results in the complete removal of oxygen in the chamber within 1 min as measured by an oxygen electrode experiment. After the oxygen concentration was reduced to zero, the substrate 3,4-dihydroxybenzaldehyde was added to the chamber to a final concentration of 1 mM. These modified assays were stopped through addition to the oxygen electrode of 0.1 volume of 1 N hydrochloric acid at 0, 15, and 30 min after the addition of substrate. Assay samples were treated as described above.

Anaerobic substrate consumption. All anaerobic experiments (except the 3,4-dihydroxybenzaldehyde dehydrogenase experiments described above) were carried out in sealed 2-ml HPLC vials. The headspace in the vial was flushed with nitrogen before the addition of 50 μ l of nitrogen-flushed substrate. In an additional experiment, nitrate (0.5 mM) was included in the anaerobic assay mix to determine whether it could act as an electron acceptor. Aerobic controls (500- μ l assay in a 2.5-ml assay vial) were incubated by shaking at 250 rpm in parallel, with glucose oxidase, glucose, catalase, cell extract, and one of the aromatic substrates.

Protein determination. Protein concentrations in cell extracts were determined using the method of Bradford (6).

Chemicals. All fine chemicals were supplied by Sigma Chemical Co., Buchs, Switzerland.

RESULTS

Strain isolation and substrate range. *P. putida* strain F6 was isolated from soil by selection on P-OHPA. This organism is capable of growth on P-OHPA, phenylacetic acid, or glucose as the sole carbon and energy source. It releases a strong red-brown pigment into both solid and liquid growth media when grown on P-OHPA. This pigment was not present on any other growth source. It grows poorly on *m*-hydroxyphenylacetic acid and not at all on *o*-hydroxyphenylacetic acid.

Oxygen consumption by whole cells. Washed suspensions of *P. putida* F6 cells grown on P-OHPA consumed oxygen at various rates when supplied with various carbon sources, as shown in Table 1. The rate of oxygen consumption was the highest for P-OHPA and 3,4-dihydroxyphenylacetic acid at 430 ± 14 and 236 ± 17 nmol min⁻¹ mg (dry weight) of cells, respectively. The rate of oxygen consumption by washed cell suspensions of P-OHPA-grown cells when fed *m*-hydroxyphenylacetic acid was less than 5% of the rate for P-OHPA (Table 1). P-OHPA-grown cells failed to consume oxygen when fed *o*-hydroxyphenylacetic acid. P-OHPA-grown cells consumed oxygen in the presence of 3,4-dihydroxybenzaldehyde and 3,4-dihydroxybenzoic acid. The rate of oxygen consumption in the presence of 3,4-dihydroxybenzaldehyde was 1.5 times higher than that of 3,4-dihydroxybenzoic acid. Glucose-grown cells failed to oxidize any of the carbon sources tested, indicating that the degradation pathway of P-OHPA is inducible (Table 1).

Enzyme assays. Addition of P-OHPA, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxymandelic acid, 3,4-dihydroxybenzaldehyde, and 3,4-dihydroxybenzoic acid to cell extracts resulted in an increased consumption of oxygen (Table 2). HPLC analysis of the corresponding samples showed substrate consumption and product formation (with the exception of 3,4-dihydroxybenzoic acid). A reproducible lag phase of between 60 and 90 s was observed for oxygen consumption by cell extracts of *P. putida* F6 after P-OHPA was added.

To determine whether the enzyme activities were dependent on noncovalently bound cofactors, the cell extract was ultrafiltered and washed with Tris buffer (pH 7.0). The retentate (washed-cell extract) consumed substrates at a slightly lower

TABLE 1. Oxygen consumption by washed cell suspensions of batch-grown cells of *P. putida* F6 grown on *p*-hydroxyphenylacetic acid

Substrate	Rate of oxygen consumption ^a (nmol min ⁻¹ mg dry weight ⁻¹ of cells)
<i>p</i> -Hydroxyphenylacetic acid.....	430 \pm 14
<i>m</i> -Hydroxyphenylacetic acid.....	14 \pm 5
3,4 Dihydroxyphenylacetic acid.....	236 \pm 17
2,5 Dihydroxyphenylacetic acid.....	12 \pm 2
3,4 Dihydroxybenzaldehyde.....	40 \pm 7
3,4 Dihydroxybenzoic acid.....	37 \pm 1

^a All experimental values are the averages of three independent determinations. No oxygen consumption was observed in washed-cell suspensions grown on glucose for any of the substrates listed in this table.

rate (90%) than unwashed cell extract. Incubation of the filtrate alone with substrates did not result in substrate consumption. Addition of the filtrate to the washed cell extract produced the same substrate consumption rates as the washed cell extract without the added filtrate.

***p*-Hydroxyphenylacetic acid hydroxylase assay.** In cell-extract assays, P-OHPA was consumed at a rate of 221 mU of enzyme per mg of protein (Table 2). Consumption of P-OHPA resulted in the formation of 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxymandelic acid, and 3,4-dihydroxybenzaldehyde (Fig. 1). The rate of consumption of P-OHPA remained linear over the first 3 min of the assay (Fig. 1). Stoichiometric conversion of substrate to product was observed for the first minute of the enzyme assay. The initial rate of P-OHPA consumption was proportional to the protein concentration in the range from 0.05 to 0.2 mg of protein ml⁻¹. 3,4-Dihydroxymandelic acid accumulated from P-OHPA before the appearance of 3,4-dihydroxyphenylacetic acid (Fig. 1). This is in keeping with the observed rates of oxygen and substrate consumption for 3,4-dihydroxymandelic acid and 3,4-dihydroxyphenylacetic acid (Table 2). The decrease in the rate of P-OHPA consump-

TABLE 2. Substrate and oxygen consumption rates by cell extracts of *P. putida* F6

Substrate	Parameter measured	Specific enzyme activity ^a (nmol min ⁻¹ mg of protein ⁻¹) of cells grown on:	
		Glucose	P-OHPA
<i>p</i> -Hydroxyphenylacetic acid	Oxygen	1.5 \pm 0.3	137 \pm 12
	Substrate	ND	221 \pm 18
<i>p</i> -Hydroxymandelic acid	Oxygen	ND	62 \pm 16
	Substrate	ND	—
3,4 Dihydroxyphenylacetic acid	Oxygen	2.1 \pm 0.2	124 \pm 17
	Substrate	ND	220 \pm 8
3,4 Dihydroxymandelic acid	Oxygen	0.7 \pm 0	39 \pm 5
	Substrate	ND	55 \pm 6
3,4 Dihydroxybenzaldehyde	Oxygen	1 \pm 0.5	220 \pm 16
	Substrate	ND	239 \pm 25
3,4 Dihydroxybenzoic acid	Oxygen	ND	31 \pm 5
	Substrate	ND	30 \pm 5

^a ND, no detectable activity; —, not determined. No detectable consumption of *m*-hydroxyphenylacetic acid, *o*-hydroxyphenylacetic acid, and 2,5-dihydroxyphenylacetic acid was observed with cell extracts of *p*-hydroxyphenylacetic acid-grown cells. All experimental data are the means of at least three independent determinations.

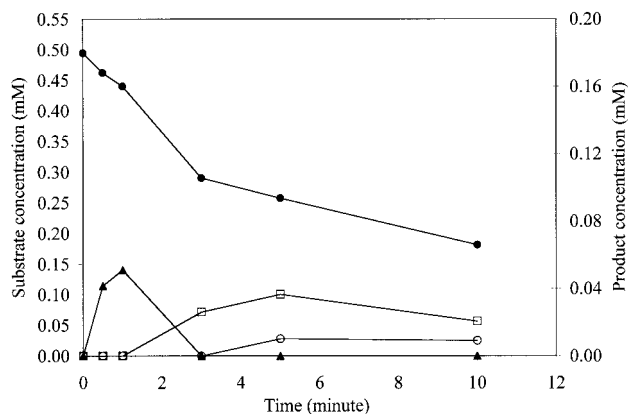


FIG. 1. *p*-Hydroxyphenylacetic acid (●) consumption and product formation by cell extracts of *P. putida* F6. 3,4-Dihydroxyphenylacetic acid (□), 3,4-dihydroxymandelic acid (▲), and 3,4-dihydroxybenzaldehyde (○).

tion and the accumulation of 3,4-dihydroxyphenylacetic acid (Fig. 1) coincided with the appearance of a red-brown color. 3,4-Dihydroxybenzaldehyde started to be detectable (>0.01 mM) after 5 min, only after 3,4-dihydroxymandelic acid was completely depleted (Fig. 1) and the formation rate of 3,4-dihydroxyphenylacetic acid had slowed. The concentration of 3,4-dihydroxybenzaldehyde was the same in samples taken at 5 and 10 min, respectively (Fig. 1).

The initial rate of substrate depletion and the appearance of the red-brown color was approximately proportional to the cell extract concentration in the assay. When acidified for analysis by HPLC, the assay samples lost some of the strong color that had formed in the assay. The pellet resulting from centrifugation of the acidified sample was red-brown in color, and the supernatant was a light red-brown color. A red-brown pellet was also observed in unacidified centrifuged samples. The enzyme assays with a red-brown color had an absorption maximum of 303 nm at pH 2.0, 330 nm at pH 7.0, and 356 nm at pH 12.0.

3,4-Dihydroxyphenylacetic acid consumption by cell extracts. The rate of oxygen consumption by cell extracts of strain F6 when supplied with 3,4-dihydroxyphenylacetic acid was 90% of that measured for P-OHPA (Table 2). The rate of 3,4-dihydroxyphenylacetic acid consumption as measured by HPLC was 220 μ M per mg of protein (Table 2). 3,4-Dihydroxyphenylacetic acid was converted to 3,4-dihydroxymandelic acid and 3,4-dihydroxybenzaldehyde (Fig. 2), albeit not in stoichiometric amounts; only 75% of the consumed 3,4-dihydroxyphenylacetic acid appeared as products. HPLC analysis showed that 3,4-dihydroxymandelic acid concentrations reached a maximum (0.036 mM) after 3 min and rapidly decreased over the next 2 min (Fig. 2). 3,4-Dihydroxybenzaldehyde started to accumulate (to a maximum of 0.025 mM) when the rate of 3,4-dihydroxymandelic acid accumulation had decreased to just over 4% of the initial accumulation rate (Fig. 2). A red-brown color also appeared in 3,4-dihydroxyphenylacetic acid depletion assays.

3,4-Dihydroxymandelic acid consumption by cell extracts. Addition of 3,4-dihydroxymandelic acid to cell extracts of *P. putida* F6 resulted in an oxygen consumption rate of 39.0 ± 5.0

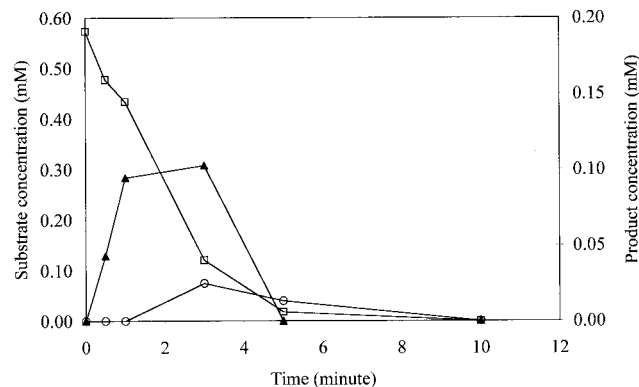


FIG. 2. 3,4-Dihydroxyphenylacetic acid (□) consumption and product formation by cell extracts of *P. putida* F6. 3,4-Dihydroxymandelic acid (▲), and 3,4-dihydroxybenzaldehyde (○).

$\text{nmol min}^{-1} \text{mg of protein}^{-1}$ and a substrate consumption rate of $55.0 \pm 6.0 \text{ nmol of substrate min}^{-1} \text{ per mg of protein}$ (analyzed by HPLC) (Table 2). These levels are more than 3 times lower than the corresponding rates seen for P-OHPA and 3,4-dihydroxyphenylacetic acid (Table 2). The 3,4-dihydroxybenzaldehyde concentration slowly increased over time from 0 to 0.06 mM in 30 min with the consumption of 0.17 mM 3,4-dihydroxymandelic acid (Fig. 3). No other products were detected by HPLC. The addition of thiamine pyrophosphate (cocarboxylase) had no effect on 3,4-dihydroxymandelic acid consumption by cell extracts of *P. putida* F6. The red-brown color seen in previous assays slowly appeared in assay mixtures. The intensity of the color was less than that seen for assays with P-OHPA and 3,4-dihydroxyphenylacetic acid.

3,4-Dihydroxybenzaldehyde consumption. A yellow color formed in the enzyme assays containing 3,4-dihydroxybenzaldehyde and the cell extract of *P. putida* F6. The yellow color disappeared upon acidification of the assay mix. Furthermore, HPLC analysis failed to show 3,4-dihydroxybenzoic acid formation. The consumption of the 3,4-dihydroxybenzaldehyde was independent of NAD(P)^+ . 3,4-Dihydroxybenzaldehyde was consumed at a rate of 239 μ M per mg of protein (Table 2). In parallel oxygen electrode assays, an oxygen consumption

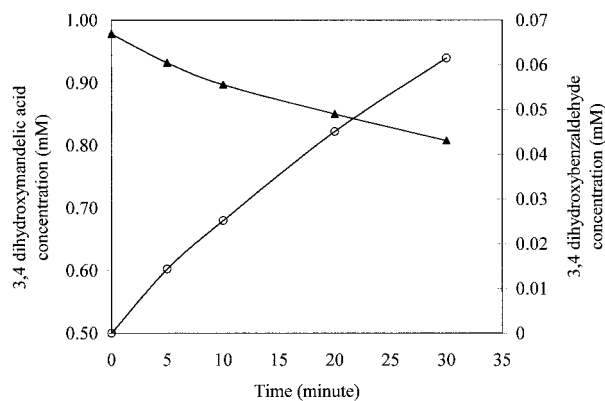


FIG. 3. 3,4-Dihydroxymandelic acid (▲) consumption by cell extracts of *P. putida* F6 with the resultant production of 3,4-dihydroxybenzaldehyde (○) over time by cell extracts of *P. putida* F6.

rate of 220 mU (nanomoles of oxygen per minute per milligram of protein) was measured (Table 2). The rate of consumption of 3,4-dihydroxybenzaldehyde, tested at a concentration of 0.05 mM, was 12 mU per mg of protein. During anaerobic experiments performed with 3,4-dihydroxybenzaldehyde in the presence of cell extract and NAD(P)⁺, no consumption of 3,4-dihydroxybenzaldehyde could be detected. The addition of the sodium cyanide to a 3,4-dihydroxybenzaldehyde assay containing NAD(P)⁺ failed to show any consumption of 3,4-dihydroxybenzaldehyde under aerobic conditions. A product of 3,4-dihydroxybenzaldehyde degradation was detected by HPLC and had a retention time of 1.4 min and an absorption maximum at 318 nm.

3,4-Dihydroxybenzoic acid consumption. The rate of 3,4-dihydroxybenzoic acid degradation was 30 mU/mg of protein, nearly eightfold lower than that of 3,4-dihydroxybenzaldehyde (Table 2). No products were detected by HPLC (data not shown).

Anaerobic substrate consumption assays. The role of oxygen in the degradation of P-OHPA and its metabolites was investigated by incubating substrates with cell extracts of *P. putida* F6 under anaerobic conditions. Under anaerobic conditions cell extracts failed to consume any substrate. The addition of nitrate as an electron acceptor to anaerobic assays did not result in the consumption of substrate or in the appearance of product, as measured by HPLC (data not shown).

Abiotic control. Based on a previous report on the chemically catalyzed decarboxylation of 3,4-dihydroxyphenylacetic acid and 3,4-dihydroxymandelic acid to 3,4-dihydroxybenzaldehyde (19), we decided to test the stability of our substrates. One millimolar solutions of substrates or a combination of substrates in an equimolar ratio (0.5 mM) were acidified with 0.1 volume of 1 N phosphoric acid, stored frozen (− 20°C) for 1 month, and tested for the depletion of substrates or formation of products. Neither a depletion of substrates nor product formation was observed as a result of the storage conditions employed (results not shown). Short-term storage (8 h) of a 1 mM 3,4-dihydroxyphenylacetic acid or 3,4-dihydroxymandelic acid solution at room temperature in a 50 mM Tris buffer (pH 7.5) did not reduce the levels of substrate or result in product formation. Under the assay and storage conditions employed the substrates were stable.

Stoichiometry of substrate to oxygen consumption. In stoichiometric studies performed with cell extracts of *P. putida* F6 incubated with either P-OHPA or 3,4-dihydroxyphenylacetic acid, a decrease was observed in the ratio of oxygen to substrate consumption upon addition of catalase. The addition of catalase and ethanol resulted in 1.4- and 1.5-fold greater oxygen consumption for assays containing P-OHPA and 3,4-dihydroxyphenylacetic acid, respectively, compared to enzyme assays with catalase but no ethanol added. The ratio of 3,4-dihydroxybenzaldehyde to oxygen consumption was 1:1 in the presence or absence of catalase. Furthermore, the subsequent addition of ethanol did not alter the ratio of substrate to oxygen consumption.

Enzyme assays in the presence of ¹⁸O water. Liquid chromatography/mass spectrometry analysis was performed on samples from enzyme assays containing cell extracts of *P. putida* F6 and 3,4-dihydroxyphenylacetic acid as substrate in the presence of ¹⁸O water for 2 min. Reactions were stopped

through the addition of hydrochloric acid as described for all enzyme assays. Liquid chromatography/mass spectrometry analysis of the assay showed a product with the same retention time as 3,4-dihydroxymandelic acid and a molecular mass equal to and two mass units heavier than the mass of the standard solution of 3,4-dihydroxymandelic acid. Assays with heat-treated cell extract (100°C for 30 min) did not result in any product formation. Analysis of standard solutions of substrates and products showed no exchange between ¹⁸O water and either 3,4-dihydroxymandelic acid or 3,4-dihydroxyphenylacetic acid within 2 min.

DISCUSSION

P-OHPA has received attention because it is an intermediate in the metabolism of tyrosine. *P. putida* F6 is capable of growth with P-OHPA as a sole carbon and energy source. Whole-cell oxygen electrode studies showed a mostly predictable profile with P-OHPA and 3,4-dihydroxyphenylacetic acid giving strong reactions. However, oxygen consumption by whole cells in the presence of 3,4-dihydroxybenzaldehyde has never been reported for P-OHPA-grown microorganisms (1, 7, 11, 32, 33).

Investigation of enzyme activities in crude cell extracts showed that *P. putida* F6 possesses a novel pathway for P-OHPA metabolism that involves 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxymandelic acid, and 3,4-dihydroxybenzaldehyde as intermediates (Fig. 4). Both of these compounds accumulated with P-OHPA as a substrate and consumed themselves in enzyme assays. The first step in the proposed pathway is the conversion of P-OHPA to 3,4-dihydroxyphenylacetic acid. The involvement of an oxidase rather than a hydroxylase in the first reaction was suggested by the NAD(P)H-independent nature of the reaction. The detection of 3,4-dihydroxymandelic acid and 3,4-dihydroxybenzaldehyde in assays with either P-OHPA or 3,4-dihydroxyphenylacetic acid as a substrate suggested that the next step involved oxygenation at the α -carbon rather than immediate ring cleavage by a dihydroxyphenylacetic acid dioxygenase (E.C.1.13.11.15). The observation that the oxygenation at the α -carbon was independent of NAD(P)H strongly suggests that this step is also catalyzed by an oxidase. This sequence of two subsequent oxidase reactions bears a resemblance to the fate of L-tyrosine in the presence of a phenol oxidase isolated from insects (*Sacrophaga bullata*), plants (*Haas avocado*), and fungi (mushroom tyrosinase), respectively (9, 17, 30, 36). Phenol oxidase has both a monophenol oxidase (monophenolase) and diphenol oxidase (diphenolase) activity (12, 21, 22, 31). The monophenolase activity was shown to be responsible for the formation of a catechol (dihydroxyphenylalanine) from a phenol (tyrosine) through direct interaction of hydrogen peroxide, formed from molecular oxygen, with the phenol substrate (29). The diphenolase activity, through the oxidative dehydrogenation of the catechol, was shown to be responsible for the formation of a highly unstable quinone methide intermediate that is subsequently nonenzymatically hydrated (29, 30). The product of hydration depends on whether the side chain of the catechol can be cyclized (29). The diphenolase activity of insect cuticular phenol oxidase and mushroom tyrosinase resulted in the formation of 3,4-dihydroxymandelic acid and 3,4-dihydroxybenzaldehyde from 3,4-

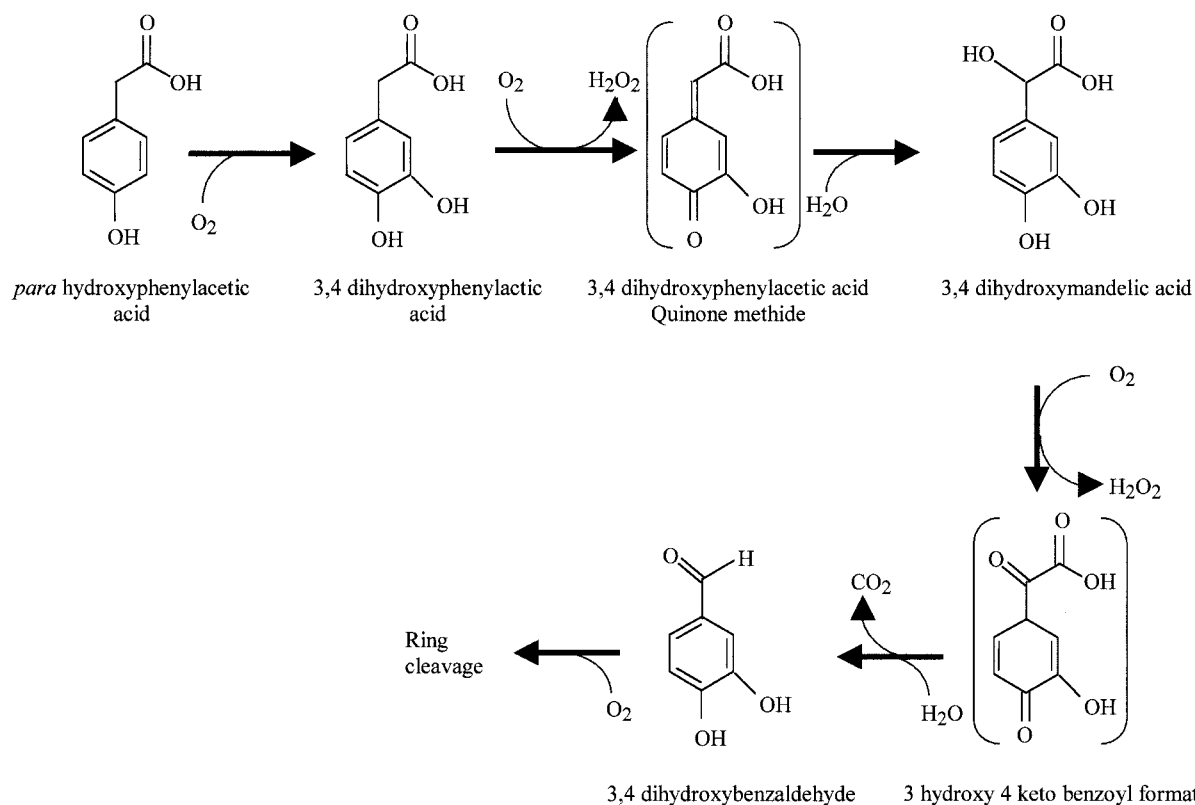


FIG. 4. The proposed pathway for *para* hydroxyphenylacetic acid metabolism in *P. putida* F6. The compounds in parentheses are proposed intermediates and were not detected in this study.

dihydroxyphenylacetic acid (34, 35). Based on the enzyme activities described for tyrosinase, it is possible that a similar enzyme could catalyze a series of reactions for the production of 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxymandelic acid, and 3,4-dihydroxybenzaldehyde from P-OHPA in *P. putida* F6. Stoichiometric analysis of P-OHPA and 3,4-dihydroxyphenylacetic acid consumption by cell extracts of *P. putida* F6 showed a 1.3- and a 1.5-fold increase, respectively, in the ratio of oxygen to substrate consumption in enzyme assays upon the addition of ethanol. An increase in the ratio of oxygen to substrate consumption in response to the addition of ethanol to enzyme assays generally indicates the involvement of an oxidase (4). Further evidence of a common mechanism between tyrosinase and the enzyme activities in *P. putida* F6 was seen in enzyme assays with 3,4-dihydroxyphenylacetic acid as substrate in the presence of ^{18}O water. The results showed the incorporation of ^{18}O in 3,4-dihydroxymandelic acid which support the assertion that 3,4-dihydroxyphenylacetic acid is converted to 3,4-dihydroxymandelic acid through biological oxidation followed by chemical hydration. The subsequent conversion of 3,4-dihydroxymandelic acid to 3,4-dihydroxybenzaldehyde in enzyme assays of *P. putida* F6 is similar to the oxidative decarboxylation of 3,4-dihydroxymandelic acid by mushroom tyrosinase observed by Sugumaran (34). The onset of color formation with *P. putida* F6 enzyme activity is similar to that reported for phenol oxidase activity (30, 35). Color formation in insects and fruit due to phenol oxidase activity is attributed to melanin formed through 1, 6 Michael additions between quinone methides and protein nucleophiles (8, 9, 18,

22, 25, 28, 30, 35). The effects of quinone methide complex formation may explain the 2.3- to 2.6-fold faster disappearance of 3,4-dihydroxymandelic acid in substrate consumption assays with stronger color formation (P-OHPA- and 3,4-dihydroxyphenylacetic acid-consumption assays [125 to 142 nmol min^{-1} per $\text{mg of protein}^{-1}$] compared to 3,4-dihydroxymandelic acid consumption assay [55 nmol min^{-1} $\text{mg of protein}^{-1}$]) (Fig. 1, 2, and 3). Although results from enzyme assays indicate the presence of a phenol oxidase-type enzyme in *P. putida* F6 cell extracts, further evidence is required to support the hypothesis of a multistep process catalyzed by a single enzyme in *P. putida* F6.

The final step in the aromatic ring metabolism is the NAD(P)^+ -independent conversion of 3,4-dihydroxybenzaldehyde. Several observations support the existence of an extradiol ring cleavage dioxygenase enzyme specific for 3,4-dihydroxybenzaldehyde, notably the strict oxygen dependency of catalysis, the production of a soluble yellow color, and the stoichiometric study showing no effect of ethanol on the ratio of oxygen to 3,4-dihydroxybenzaldehyde consumption (indicating that no oxidase activity was present). The observed (low) rate of 3,4-dihydroxybenzoic acid consumption in cell extracts is possibly due to the dioxygenase for 3,4-dihydroxybenzaldehyde acting on 3,4-dihydroxybenzoic acid.

The metabolism of P-OHPA through the series of reactions described above may be energetically more favorable than the previously described pathways involving NAD(P)H -dependent hydroxylation; no NAD(P)H is consumed, and so more NAD(P)H is available for ATP generation. It remains to be seen to what extent the *in vivo* formation of (colored) polymers could

benefit this bacterial strain in analogy to its function in other organisms, such as tanning and hardening of insect pupa, insect immunity, and browning in fruits (2, 8, 30, 35, 36).

One of the products of P-OHPA degradation, 3,4-dihydroxybenzaldehyde, is a potential precursor of the anti-Parkinson drug Levodopa. The biotechnological potential of this organism in producing a cofactor-independent biocatalyst which may be used for the industrial production of Levodopa and other value-added synthons is currently under investigation.

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