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Pathways for the Degradation of *m*-Cresol and *p*-Cresol by *Pseudomonas putida*

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A comparison of the oxidation rates of various compounds by whole cells of *Pseudomonas putida* 3,5 indicated that *m*-cresol is metabolized by oxidation to 3-hydroxybenzoate followed by hydroxylation to gentisate, the ring-fission substrate, when grown with 3,5-xylenol. However, when *m*-cresol was the growth substrate, similar experiments suggested a different pathway involving a methyl-substituted catechol and ring-fission by *meta* cleavage. Assays of ring-fission enzymes in cell-free extracts confirmed that different pathways are induced by the two growth substrates. 3,5-Xylenol-grown cells contained high levels of gentisate oxygenase and only very small amounts of catechol oxygenase, whereas gentisate oxygenase could not be detected in *m*-cresol-grown cells, but levels of catechol oxygenase were greatly increased. Extracts of *m*-cresol-grown cells also contained 2-hydroxymuconic semialdehyde dehydrogenase and hydrolase, whose specificities enable them to metabolize the ring-fission products from catechol, 3-methylcatechol, and 4-methylcatechol. This catechol pathway is also used by *m*-cresol-grown cells for *p*-cresol metabolism. In contrast, the results for cells grown with *p*-cresol point to an alternative pathway involving oxidation to 4-hydroxybenzoate and hydroxylation to protocatechuate as ring-fission substrate. Extracts of these cells contained high levels of protocatechuate oxygenase and only small amounts of catechol oxygenase.

Previous studies have shown that 3,5-xylenol is metabolized in both a fluorescent *Pseudomonas* (*Pseudomonas putida* 3,5; NCIB 9869) and a nonfluorescent species (*Pseudomonas* 2,5) by an initial oxidation of a methyl group to carboxyl. This is followed by hydroxylation of the ring to give 3-methylgentisate (2,5-dihydroxy-3-methylbenzoate), which then serves as substrate for a ring-fission dioxygenase (4, 5). The enzymes involved appear to have broad specificities, since cells grown with 3,5-xylenol were also able to oxidize a number of other methyl-substituted phenols including *m*-cresol. The intermediates for this compound are 3-hydroxybenzoate and gentisate (2,5-dihydroxybenzoate). Similarly, when *Pseudomonas* 2,5 was grown with *m*-cresol it readily oxidized these other phenols including 3,5-xylenol. In contrast, *m*-cresol-grown cells of *P. putida* 3,5 oxidized only the growth substrate rapidly and to completion (4). Metabolism of *m*-cresol by hydroxylation to 3-methylcatechol and subsequent *meta* cleavage of the ring in another strain of *P. putida* (*Pseudomonas* U) have also been described (1, 2). *p*-Cresol, with 4-methylcatechol as intermediate, was also degraded by this *meta* cleavage pathway, which was induced by growth with phenol or any one of the three cresols.

In the present work we show that when *m*-cresol rather than 3,5-xylenol is the growth substrate for our strain of *P. putida* it is degraded not by the gentisate pathway but by the catechol *meta* cleavage pathway. A comparable situation is demonstrated for the metabolism of *p*-cresol, which can also be oxidized by the catechol pathway but which is degraded by a different route when used as growth substrate.

MATERIALS AND METHODS

Isolation and growth of microorganism. The organism was isolated from River Hull mud by selective culture in liquid medium containing 3,5-xylenol (0.3 g/liter) as the sole carbon source. It was previously referred to as *Pseudomonas* 3,5 (4, 5); M. E. Rhodes further examined the organism and identified it as a strain of *P. putida*. It was deposited with the British National Collection of Industrial Bacteria under the collection number NCIB 9869. Stock cultures were maintained on nutrient agar slants at 4 C and subcultured monthly. Cells were grown in 1-liter batches in 2-liter Erlenmeyer flasks on a rotary shaker at 30 C in a medium that contained (per liter):

Na_2HPO_4, 4.33 g; KH_2PO_4, 2.65 g; NH_4Cl, 2.0 g; nitrilotriacetic acid, 0.1 g; salts solution (12), 4.0 ml; carbon source, 0.3 g. Cells were harvested by centrifugation at 4,400 × *g* for 20 min, washed with 0.05 M KH_2PO_4-Na_2HPO_4 buffer (phosphate buffer; pH 7.5), and resuspended in a small volume of the same buffer.

Preparation of cell-free extracts. Washed cells from 1 liter of medium were resuspended in 5.0 ml of phosphate buffer (pH 7.5), and extracts were prepared by sonic treatment at 0 C for 4 min using a 20-kHz Dawe Soniprobe (type 7530A; Dawe Instruments Ltd., London) at setting of 4 (ca. 60 W) and fitted with a microtip. The sonically treated extract was centrifuged for 60 min at 50,000 × *g* and the supernatant, referred to as crude extract, used for enzyme assays of oxygenases.

For assay of 2-hydroxymuconic semialdehyde hydrolase, crude extract was further treated to remove nicotinamide adenine dinucleotide (NAD^+) by passage through a Sephadex G-25 column at 4 C. Crude extract (2.0 ml) was applied to a Sephadex G-25 column (10 by 1.7 cm) and eluted with phosphate buffer, pH 7.5. Fractions of 2.0 ml were collected, and those containing the bulk of the protein, as indicated by absorption at 280 nm, were pooled and are referred to as Sephadex-treated extracts.

Heat-treated extract was prepared by heating crude extract of phenol-grown cells to 55 C for 10 min followed by centrifugation at 2 C and 28,000 × *g* for 10 min.

Enzyme assays and chemical determinations. Catechol 2,3-oxygenase (EC 1.13.11.2; catechol:oxygen 2,3-oxidoreductase), protocatechuate oxygenase (EC 1.13.11.3; protocatechuate:oxygen 3,4-oxidoreductase), and gentisate oxygenase (EC 1.13.11.4; gentisate:oxygen oxidoreductase) were assayed by following the rate of O_2 utilization with a Clark-type O_2 electrode in a stirred vessel at 30 C (oxygen monitor model 53; Yellow Springs Instrument Co., Yellow Springs, Ohio). The 3.0-ml reaction volume contained, in 0.05 M phosphate buffer (pH 7.5), 2.5 μmol of substrate and crude extract. For gentisate oxygenase the crude extract was preincubated with 2 mM ferrous ammonium sulfate for 5 min at 0 C. The oxygenases were assayed immediately after preparation of the extract.

Catechol 2,3-oxygenase was also assayed spectrophotometrically by measuring the rate of formation of 2-hydroxymuconic semialdehyde at 375 nm (10). A 1-cm path length cuvette contained 3.0 μmol of catechol and cell-free extract in 3.0 ml of phosphate buffer, pH 7.5. The same reaction mixture was used to assay catechol 1,2-oxygenase (EC 1.13.11.1; catechol:oxygen 1,2-oxidoreductase) by following the formation of *cis,cis*-muconic acid at 260 nm (8).

2-Hydroxymuconic semialdehyde hydrolase and 2-hydroxymuconic semialdehyde dehydrogenase were both assayed spectrophotometrically by following the decrease in absorption in a 1-cm path length cuvette at the λ_{max} of the appropriate ring-fission product: 375 nm for 2-hydroxymuconic semialdehyde (from catechol), 388 nm for 2-hydroxy-6-oxohepta-2,4-dienoic acid (from 3-methylcatechol), and 382 nm for 2-hydroxy-5-methylmuconic semialdehyde (from 4-methylcatechol) (1). The hydrolase reaction mixture contained, in 3.0 ml of phosphate buffer (pH 7.5), 0.1 μmol of substrate and Sephadex-treated extract. The dehydrogenase reaction mixture contained, in addition, 0.5 μmol of NAD^+, and crude extract was used. The specific activity of 2-hydroxymuconic semialdehyde dehydrogenase was determined by subtracting the hydrolase activity from that of extracts assayed in the presence of NAD^+.

All spectrophotometric assays were carried out at 30 C in a Gilford 2400-S recording spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio). Enzyme units are defined as the activity required to convert 1 μmol of substrate per min.

Oxygen uptake measurements with whole cells were made in conventional Warburg respirometers.

β-Oxoadipic acid was detected by the Rothera reaction (13).

Protein was determined by the method of Lowry et al. (7).

Chemicals. The ring-fission products of catechol, 3-methylcatechol and 4-methylcatechol, were prepared in solution immediately prior to use by incubation of the catechols with heat-treated extract. Such an extract retains the catechol oxygenase activity but has lost the ability to degrade the ring-fission products (1). A 10-ml volume of 0.05 M phosphate buffer (pH 7.5) containing 2.0 μmol of the appropriate catechol was gently shaken at room temperature with 0.3 ml of heat-treated extract until the absorption of the ring-fission product reached a maximum. This solution was then used for assays of the ring-fission product-degrading enzymes.

NAD^+ was obtained from the Boehringer Corporation (London) Ltd.

RESULTS

Oxidation of compounds by intact cells. Washed suspensions of cells grown with 3,5-xylenol as the sole carbon source rapidly oxidized this compound and also *m*-cresol, at a slower but significant rate (Fig. 1a). It has been shown previously that this organism degrades 3,5-xylenol by a pathway involving 3-hydroxy-5-methylbenzoate and 3-methylgentisate as intermediates (4). It was suggested that *m*-cresol is metabolized by a parallel series of reactions employing the same enzyme system, and this is supported by the rapid oxidation of the appropriate intermediates, 3-hydroxybenzoate and gentisate, by these cell suspensions. It is significant that there was no oxidation of catechol.

m-Cresol was also rapidly oxidized when it was used as growth substrate (Fig. 1b). In this case, however, there was only very slow oxidation of 3,5-xylenol, and the low rates of oxygen uptake with 3-hydroxybenzoate and gentisate suggest that the gentisate pathway was not operating. In contrast, there was a rapid oxidation of catechol and, although not shown in Fig.

1, 3-methylcatechol and 4-methylcatechol were also rapidly metabolized. Transient yellow colors, characteristic of 2-hydroxymuconic semialdehyde and its homologues, formed as a result of *meta* cleavage, were observed in the Warburg flasks for all three catechols. A transient yellow color, of lower intensity, was also seen in the reaction mixture for *p*-cresol, which was oxidized rapidly and to about the same extent as *m*-cresol, as also were *o*-cresol and, with a slightly lower final uptake, phenol. Rates and extents of oxygen uptake for phenol, the cresols, and the catechols, similar to those for *m*-cresol-grown cells, were obtained for cells grown with phenol or *o*-cresol. However, growth with *p*-cresol gave rather different results (Fig. 1c). The rate of catechol metabolism was much lower, and now there was rapid O_2 uptake with both 4-hydroxybenzoate and protocatechuate.

m-Cresol, *p*-cresol, and 3,5-xylenol were not oxidized under the experimental conditions given for Fig. 1 when succinate-grown cells were used.

Levels of ring-fission oxygenases. As a further indication of the aromatic pathway involved after growth with a number of these phenolics, the levels of three ring-fission oxygenases were measured (Table 1). The results for extracts from phenol- and *m*-cresol-grown cells were very similar, with high levels of catechol oxygenase but no detectable gentisate oxygenase. Although the assay used for the catechol-cleaving enzyme does not distinguish between catechol 1,2-oxygenase of the *ortho* pathway and catechol 2,3-oxygenase of the *meta* pathway, a yellow color appeared in the reaction mixture, indicative of *meta* fission. This was confirmed in spectrophotometric assays, which showed an increase in absorption at 375 nm, the λ_{max} of 2-hydroxymuconic semialdehyde, but not at 260 nm for *cis,cis*-muconic acid, the *ortho* cleavage product. Moreover, after treatment of the extract with H_2O_2 as described by Nakazawa and Yokota (9), less than 10% of the catechol oxygenase activity remained. This treatment inactivates the *meta* cleavage enzyme but does not affect catechol 1,2-oxygenase.

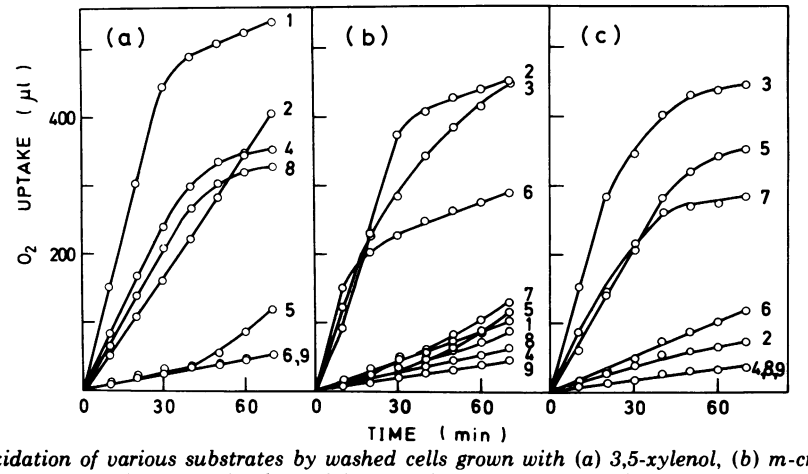

FIG. 1. *Oxidation of various substrates by washed cells grown with (a) 3,5-xylenol, (b) m-cresol, and (c) p-cresol. Each flask contained 2.5 ml of bacterial suspension (ca. 5 mg dry weight) in 0.05 M phosphate buffer (pH 7.0), 0.3 ml of 0.01 M substrate tipped from the side-arm, and 0.2 ml of 20% (wt/vol) KOH in the center well. Substrates were: (1) 3,5-xylenol, (2) m-cresol, (3) p-cresol, (4) 3-hydroxybenzoate, (5) 4-hydroxybenzoate, (6) catechol, (7) protocatechuate, (8) gentisate, and (9) water. The temperature was 30 C. The gas phase was air.*

TABLE 1. *Specific activities of ring-fission oxygenases in cell-free extracts of P. putida 3,5*

Enzymes	Sp act[a] on growth substrate:					
	Phenol	*m*-Cresol	*p*-Cresol	3,5-Xylenol	3-Hydroxy-benzoate	Succinate
Catechol oxygenase	316	206	20	4.5	7	0
Protocatechuate oxygenase .	19.2	16.7	2,040	135	190	94
Gentisate oxygenase	0	0	0	3,500	2,750	0

[a] Milliunits per milligram of protein.

The results for *p*-cresol-grown cells were quite different with relatively low levels of catechol oxygenase and a large increase of protocatechuate oxygenase over the basal amount of succinate-grown cells. When 0.5 ml of crude extract (15 mg of protein per ml) was gently shaken with 3.0 μmol of protocatechuate in 2.0 ml of 0.05 M phosphate buffer (pH 7.0) for 10 min at 30 C, the product gave a persistent purple color in the Rothera reaction, an indication of β-oxoadipic acid, the product of the *ortho* cleavage pathway (11).

Low levels of catechol oxygenase were also found for 3,5-xylenol- and 3-hydroxybenzoate-grown cells, but in these extracts it was gentisate oxygenase that showed high activity.

Activities and specificities of ring-fission product-degrading enzymes. The strain of *P. putida* used by Bayly et al. (1) in studies on cresol metabolism has since been shown to contain two different enzymes for the further metabolism of the catechol ring-fission products (15). These are a hydrolase, which is most active with the 3-methylcatechol product, an intermediate of *o*- and *m*-cresol metabolism, and an NAD^+-dependent dehydrogenase acting on the products from catechol and 4-methylcatechol, intermediates of phenol and *p*-cresol metabolism, respectively. In an *Azotobacter* species lack of the hydrolase leads to an inability to utilize 3-methylcatechol or compounds which would yield this intermediate (14). Altered specificities or lack of one of these enzymes could result in the pathway operating for *m*-cresol but not *p*-cresol.

The levels of both enzymes in extracts of our organism grown on various substrates are shown in Table 2.

DISCUSSION

The proposed pathways for the metabolism of 3,5-xylenol, *m*-cresol, and *p*-cresol under different growth conditions are shown in Fig. 2. The 3,5-xylenol route is that previously reported for both this organism and *Pseudomonas* 2,5 by Hopper and Chapman (4), who also presented evidence for the degradation of *m*-cresol by this pathway in both organisms. This does indeed seem to be the case for 3,5-xylenol-grown cultures. Whole cells rapidly oxidized *m*-cresol and the proposed intermediates 3-hydroxybenzoate and gentisate (Fig. 1), and cell-free extracts contained high levels of gentisate oxygenase but no catechol oxygenase (Table 1). The gentisate oxygenase of this organism has been shown to be almost as active with gentisate as with 3-methylgentisate (5). *Pseudomonas* 2,5 also uses this pathway when grown with *m*-cresol, and there was evidence to suggest that *P. putida* 3,5 did likewise. For example, extracts were capable of the breakdown of gentisate to pyruvate and malate (6). However, we show here that it is enzymes of the catechol *meta* cleavage pathway, rather than the gentisate pathway, that are induced by growth with *m*-cresol, and this is reflected by the altered range of substrates oxidized by whole cells.

The earlier results probably arose from the use of a culture growing with 3,5-xylenol as inoculum for *m*-cresol medium. Even after several generations there could be measurable levels of the gentisate enzymes remaining, and these would be maintained at high levels by any accumulation of 3-hydroxybenzoate or gentisate formed from *m*-cresol during the induction period for the catechol enzymes. We show

TABLE 2. *Specific activities of ring-fission product-metabolizing enzymes in cell-free extracts of P. putida 3,5*

Growth substrate[a]	Assay substrate	Sp act[b] of enzyme:	
		2-Hydroxymuconic semialdehyde hydrolase	2-Hydroxymuconic semialdehyde dehydrogenase
Phenol	2-Hydroxymuconic semialdehyde	9.8	80.5
	2-Hydroxy-6-oxohepta-2,4-dienoic acid	298	0
	2-Hydroxy-5-methylmuconic semialdehyde	5.3	86.2
m-Cresol	2-Hydroxymuconic semialdehyde	15.6	66.8
	2-Hydroxy-6-oxohepta-2,4-dienoic acid	350	0
	2-Hydroxy-5-methylmuconic semialdehyde	5.0	108.3
p-Cresol	2-Hydroxymuconic semialdehyde	0.5	3.7
	2-Hydroxy-6-oxohepta-2,4-dienoic acid	12	0
	2-Hydroxy-5-methylmuconic semialdehyde	2.5	3.5

[a] No activity was detected in extracts of cell grown with 3,5-xylenol or 3-hydroxybenzoate.
[b] Milliunits per milligram of protein.

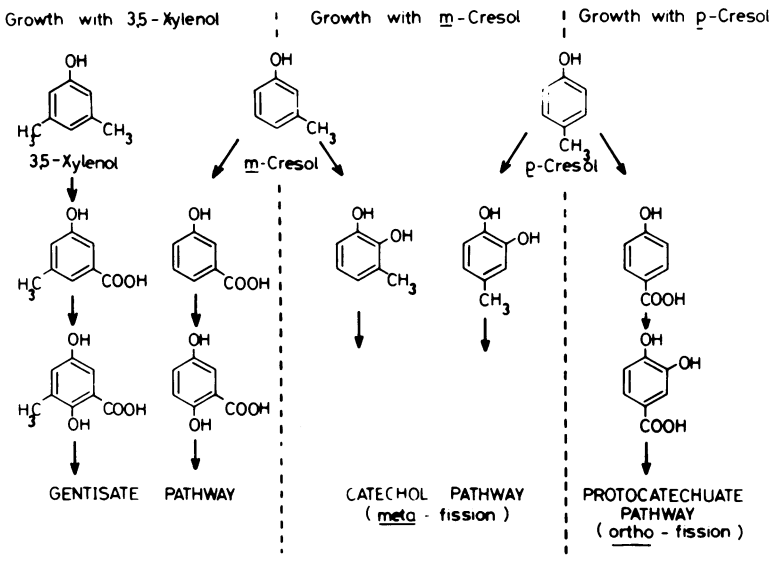

FIG. 2. *Proposed pathways for the metabolism of 3,5-xylenol, m-cresol and p-cresol by P. putida 3,5 after growth with each of these compounds.*

(Table 1) that gentisate oxygenase was induced by growth with 3-hydroxybenzoate. This does suggest that the pathway for unsubstituted gentisate has some physiological importance in this organism. In the present experiments, inocula for growth with each aromatic substrate were taken from either a nutrient agar slant or a liquid culture grown with succinate. Under these circumstances, there was no whole-cell oxidation of 3,5-xylenol, 3-hydroxybenzoate, or gentisate by *m*-cresol-grown cells (Fig. 1), and extracts did not contain gentisate oxygenase. Oxidation of the catechols and high levels of catechol 2,3-oxygenase, however, point to the pathway of Dagley et al. (2) for *m*-cresol degradation with 3-methylcatechol as intermediate and metabolism of this by the *meta* cleavage pathway.

m-Cresol-grown cells oxidize not only the growth substrate but also phenol and the other cresols. Furthermore, this pattern of oxygen uptake was also seen when cells were grown with phenol or *o*-cresol and extracts of phenol-grown cells contained catechol 2,3-oxygenase and the 2-hydroxymuconic semialdehyde-degrading enzymes. In these respects, *P. putida* 3,5 resembles *Pseudomonas* U (1). However, whereas for this latter organism *meta* cleavage pathway was induced by phenol or any of the three cresols (3), *P. putida* 3,5 appears to use a quite different pathway for growth with *p*-cresol. This involves oxidation to 4-hydroxybenzoate and hydroxylation of this to protocatechuate, which is metabolized by *ortho* cleavage. This is indicated by the rapid oxidation of these compounds by *p*-cresol-grown cells and the high levels of protocatechuate 3,4-oxygenase in cell-free extracts. *m*-Cresol and catechol were only slowly oxidized by these cells, and cell extracts contained low levels of catechol oxygenase and the 2-hydroxymuconic semialdehyde-degrading enzymes.

It is unlikely that this pathway operates for the metabolism of *p*-cresol by *m*-cresol-grown cells, which have only low levels of protocatechuate oxygenase and oxidized 4-hydroxybenzoate very slowly. Yet, such cells rapidly oxidized *p*-cresol, and the yellow color observed in the Warburg flask is indicative of hydroxylation to 4-methylcatechol and fission by *meta* cleavage as is found in *Pseudomonas* U (1). Certainly the catechol oxygenase was able to cleave 4-methylcatechol, and the product was a substrate for the 2-hydroxymuconic semialdehyde dehydrogenase and hydrolase whose specificities were similar to those reported for *Pseudomonas* U (15). Although later enzymes of the *meta* cleavage pathway may have narrower capabilities, it appears from the extent of oxidation that *p*-cresol can be completely degraded and that it is at induction where a stricter specificity is shown. Similarly, although the enzymes of 3,5-xylenol metabolism allow degradation of the structurally similar compound *m*-cresol, they are not induced by growth with this substrate.

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