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# Metabolism of Phenol and Cresols by Mutants of *Pseudomonas putida*

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Mutant strains of *Pseudomonas putida* strain U have been obtained which are deficient in enzymes of the degradative pathways of phenol and cresols. Mutant strains deficient in catechol 2,3-oxygenase accumulated the appropriate catechol derivative from cresols. A mutant strain which would not grow on either phenol or a cresol was shown to be deficient in both 2-hydroxymuconic semialdehyde hydrolase and a nicotinamide adenine dinucleotide, oxidized form, ($NAD^+$)-dependent aldehyde dehydrogenase. When this strain was grown in the presence of phenol or a cresol, the appropriate product of *meta* fission of these compounds accumulated in the growth medium. A partial revertant of this mutant strain, which was able to grow on *ortho*- and *meta*-cresol but not *para*-cresol, was shown to have regained only the hydrolase activity. This strain was used to show that the products of *meta* ring fission of the cresols and phenol are metabolized as follows: (i) *ortho*- and *meta*-cresol exclusively by a hydrolase; (ii) *para*-cresol exclusively by a $NAD^+$-dependent aldehyde dehydrogenase; (iii) phenol by both a $NAD^+$-dependent dehydrogenase and a hydrolase in the approximate ratio of 5 to 1. This conclusion is supported by the substrate specificity and enzymatic activity of the hydrolase and $NAD^+$-dependent aldehyde dehydrogenase enzymes of the wild-type strain. The results are discussed in terms of the physiological significance of the pathway. Properties of some of the mutant strains isolated are discussed.

*Pseudomonas putida* strain U of Dagley and Gibson (7) is able to metabolize phenol and the isomers of cresol by converting them into catechol and methylcatechols, respectively, which then undergo *meta* fission of the benzene nucleus. The product of ring fission of catechol is 2-hydroxymuconic semialdehyde (6), whereas 3-methylcatechol and 4-methylcatechol give 2-hydroxy-6-keto-2,4-heptadienoate (4) and 2-hydroxy-5-methylmuconic semialdehyde (3), respectively.

Until recently, the generally accepted physiologically important metabolic pathway for phenol and the isomers of cresols in *P. putida* U was that in which the products of ring fission give rise to keto-enoic acids with concomitant formation of formate or acetate (1, 2, 7). Earlier (16) it was shown that cell extracts of a *Pseudomonas* species metabolized 2-hydroxymuconic semialdehyde by a nicotinamide adenine dinucleotide, oxidized form, ($NAD^+$)-dependent aldehyde dehydrogenase to 4-oxalocrotonate which was decarboxylated to 4-hydroxy-2-ketovalerate. Feist and Hegeman (10) reported that addition of $NAD^+$ to cell extracts of phenol-induced *P. putida* U resulted in an increase in the rate of disappearance of 2-hydroxymuconic semialdehyde. It has been reported recently (5) that $NAD^+$-dependent and non-$NAD^+$-dependent pathways coexist in a naphthalene-grown pseudomonad, and it was concluded that the former pathway is the one of physiological importance. A similar conclusion as to the physiological significance of the two pathways was reported for *Azotobacter* species (21).

The two pathways by which 2-hydroxymuconic semialdehyde is reported to be metabolized (1, 21) are shown in Fig. 1.

The work now reported describes the metabolism of phenol and isomers of cresol by mutant strains of *P. putida* U, with particular reference to the metabolism of the products of ring fission.

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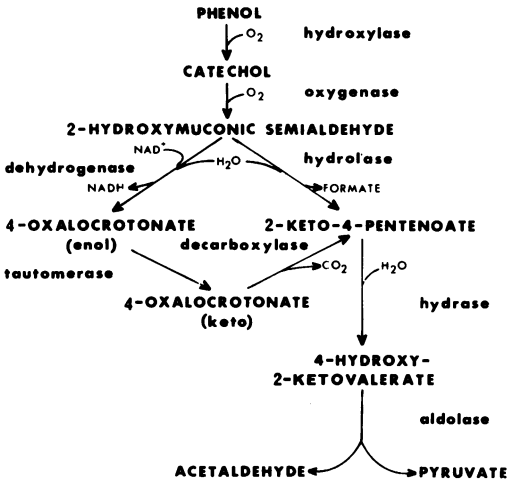


FIG. 1. *The meta fission pathway for the oxidation of phenol showing the alternative modes of metabolism of 2-hydroxymuconic semialdehyde.*

## MATERIALS AND METHODS

**Organism and methods of cultivation.** The organism used was *P. putida* strain U of Dagley and Gibson (7), also referred to as NCIB 10015 and by Stanier, Palleroni, and Doudoroff (24) as strain 144. The strain was stored as lyophilized suspensions and maintained for use as described by Kemp and Hegeman (13). The basal medium was the mineral salts base of Hegeman (12), to which was added different carbon sources as required. When phenol or an isomer of cresol was added the concentration used was 2.5 mM; sodium fumarate or sodium benzoate was added to give a concentration of 10 mM. The final *p*H of the medium was 7.4. Cultures were grown in 500 ml of medium in 2-liter flasks on a rotary incubator (200 rpm) at 30 C. Growth was measured turbidimetrically at 580 nm in a Spectronic 20 spectrophotometer (Bausch and Lomb). For induction experiments, the cells were grown in basal medium with fumarate to the early exponential phase (optical density 0.1–0.2), the inducer was added, and incubation was continued for 2 h. At the end of the period of induction the optical density was 0.6–0.7.

**Mutagenesis and selection of mutant strains.** Cells from an exponential phase nutrient broth culture of *P. putida* strain U were washed twice with 0.02 M phosphate buffer, *p*H 7.4, resuspended in the same buffer and ethylmethanesulfonate (EMS) (Koch-Light, Colnbrook, United Kingdom) added to a final concentration of 2% (vol/vol). After exposure to the mutagen for sufficient time to produce a 95 to 99.5% reduction in viable cells, the cells were washed as before, inoculated into nutrient broth, and incubated overnight at 30 C before plating onto the appropriate medium.

To select mutant strains which were unable to use phenol as a sole source of carbon and energy, the EMS-treated cells were plated onto the basal medium plus 0.5 mM fumarate and 2.5 mM phenol and incubated for 72 h. Small colonies were transferred to nutrient agar and then replicated onto basal medium plus 2.5 mM phenol and basal medium plus 10 mM fumarate.

Mutant strains which lacked a catechol 2,3-oxygenase (EC 1.99.2.a) were selected by plating onto basal medium plus 2.5 mM phenol, incubating at 30 C for 72 h, and spraying with an aqueous solution of 10 mM 4-methylcatechol. Colonies which did not turn bright yellow due to production of 2-hydroxy-5-methylmuconic semialdehyde were selected.

Apparent revertants were isolated from mutant strains unable to utilize phenol by plating onto basal medium which contained phenol or a cresol as a sole source of carbon. Treatment with mutagen was not used.

The strains selected for further study are shown in Table 1.

**Preparation of cell extracts.** After growth or induction for the required periods, cell extracts were prepared by sonic disintegration as described by Dagley and Gibson (7). Cell extracts were treated with 0.1 vol of 2% (w/v) salmine sulfate, and the precipitate was removed by centrifuging at 26,000 × *g* for 15 min at 2 C. Protein concentration in cell extracts was determined by the method of Lowry et al. (14) using bovine albumin (Fraction V powder; Pentex, Kankakee, Ill.) as a standard.

TABLE 1. *Mutant strains derived from wild-type PsU-O*

| Mutant strain | Mutagen | Selection and characteristics | Immediate parent |
|---|---|---|---|
| PsU-5 | EMS | Unable to use phenol as a sole carbon source. | PsU-O |
| PsU-D1 | EMS | Absence of catechol 2,3-oxygenase when grown on phenol; unable to use any cresol as a sole carbon source. | PsU-O |
| PsU-5-R5 | None | Able to grow on phenol; on replica plating able to use each cresol as a sole carbon source. | PsU-5 |
| PsU-5-R11 | None | Able to grow on phenol; on replica plating unable to use any cresol as a sole carbon source. | PsU-5 |
| PsU-5-R21 | None | Able to grow on *o*-cresol; on replica plating able to grow on *m*-cresol but not phenol or *p*-cresol. | PsU-5 |

**Enzyme assays.** All enzyme assays were carried out in silica cuvettes of 1-cm path length by using a Hitachi-124 recording spectrophotometer. The final reaction volume was 3.0 ml, and the temperature was 23 C.

Catechol 2,3-oxygenase was determined by measuring the rate of accumulation of 2-hydroxymuconic semialdehyde at 375 nm; cuvettes contained 120 μmol of phosphate buffer, pH 7.6, 0.1 μmol of catechol and extract. The activity of this enzyme against 3-methylcatechol and 4-methylcatechol was determined similarly by following the increase in absorbance at 388 nm and 382 nm, respectively, due to accumulation of the corresponding products of ring fission (2). In the case of 3-methylcatechol, 1.0 μmol of substrate was added to counter the effect of the relatively rapid breakdown of the product of ring fission; the values thus obtained are lower than those which would be found if heat-treated extracts (7) were used. Extinction coefficients reported by Bayly et al. (2) were used to calculate the specific activities. Catechol 2,3-oxygenase assays were always conducted immediately after preparation of the cell extracts.

Catechol 1,2-oxygenase (EC 1.99.2.2) was determined as described by Hegeman (12).

Muconate lactonizing enzyme (EC 5.5.1.1) was assayed using the conditions described by Ornston (17). Specific activities of catechol 1,2-oxygenase and muconate lactonizing enzyme were calculated as described by Feist and Hegeman (10).

The activity of 2-hydroxymuconic semialdehyde hydrolase (no EC number) was measured by determining the rate of decrease in absorbance at 375 nm. The activities of this enzyme against 2-hydroxy-6-keto-2,4-heptadienoate and 2-hydroxy-5-methylmuconic semialdehyde were determined similarly by following the rate of decrease of absorbance at 388 nm and 382 nm, respectively. Each cuvette contained 0.1 to 0.2 μmol of substrate, 100 μmol of phosphate buffer, *p*H 7.6, and extract.

The combined activity of $NAD^+$-dependent aldehyde dehydrogenase and hydrolase present in the extracts was determined by the addition of 0.3 μmol of $NAD^+$ to the above assay system.

Substrates for the assay of the hydrolase and the dehydrogenase were prepared as described by Bayly and Dagley (1) but, to remove unchanged catechols, an ether extraction was carried out before the reaction mixture was acidified.

Hydroxylation of phenol and the isomers of cresols were measured manometrically. Warburg flasks contained, in 3.0 ml, 120 μmol of phosphate buffer, *p*H 7.4, washed cells (5 mg dry weight), 0.2 ml of 20% KOH in the center well, and 5 μmol of substrate was added from the side arm of the flask. The gas phase was air and the temperature, 30 C.

The presence of 4-oxalocrotonate in reaction mixtures was detected by an increase in absorbance at 350 nm following the adjustment to *p*H 12 with NaOH (21). Formate was detected by the method of Grant (11).

**Chemicals.** *cis,cis*-Muconate was synthesized by the method of Elvidge et al. (8). Catechol, 3-methylcatechol, and 4-methylcatechol were purified by vacuum sublimation. Other chemicals were obtained from commercial sources and used without further treatment.

## RESULTS

A mutant strain PsU-5, which was unable to utilize either phenol or any of the isomers of cresol as a sole source of carbon and energy, was isolated from the wild-type strain PsU-O. When this mutant strain was grown in fumarate basal medium which contained either phenol or a cresol, a yellow product accumulated in the culture fluid. These products were shown to have the same spectral properties as the *meta* ring fission products of phenol and the cresols described by Bayly et al. (2). The rate of accumulation of the product formed from each phenolic compound is shown in Fig. 2. Further evidence that the compounds produced were the *meta* ring fission products of phenol and the cresols was that they were metabolized by extracts of phenol-grown, wild-type cells but not by extracts of cells grown on fumarate or benzoate.

In an attempt to isolate revertants to wild-type phenotype, PsU-5 was plated on basal medium with either phenol or a cresol. The number of organisms which grew on each phenolic compound compared with those which grew on the same medium plus fumarate are shown in Table 2. The ratio of cells which grew on phenol as a sole source of carbon to those which grew on phenol with fumarate was $70/10^6$, but when a cresol was used for growth, the ratio was $2/10^6$. Each apparent revertant was tested for its ability to grow on the alternate phenolic carbon sources by replication with velveteen. Strains PsU-5-R5, PsU-5-R11, and PsU-5-R21 were derived in this manner (Table 1).

From the growth characteristics of these strains (Table 1) it is apparent that PsU-5-R11 and PsU-5-R21 have not reverted to wild-type phenotype and hence must be regarded as partial revertants.

About 3% of the apparent revertants isolated on phenol could grow on each of the cresols as a sole carbon source. The remainder were shown to be partial revertants of the type which Feist and Hegeman (10) showed to be due to an extragenic suppressor mutation so that phenol was metabolized by the tangential *ortho* fission pathway. The absence of catechol 2,3-oxygenase in these revertants was confirmed as described in Materials and Methods.

Cell extracts of eight of these partial revertants (randomly selected), when grown on phe-

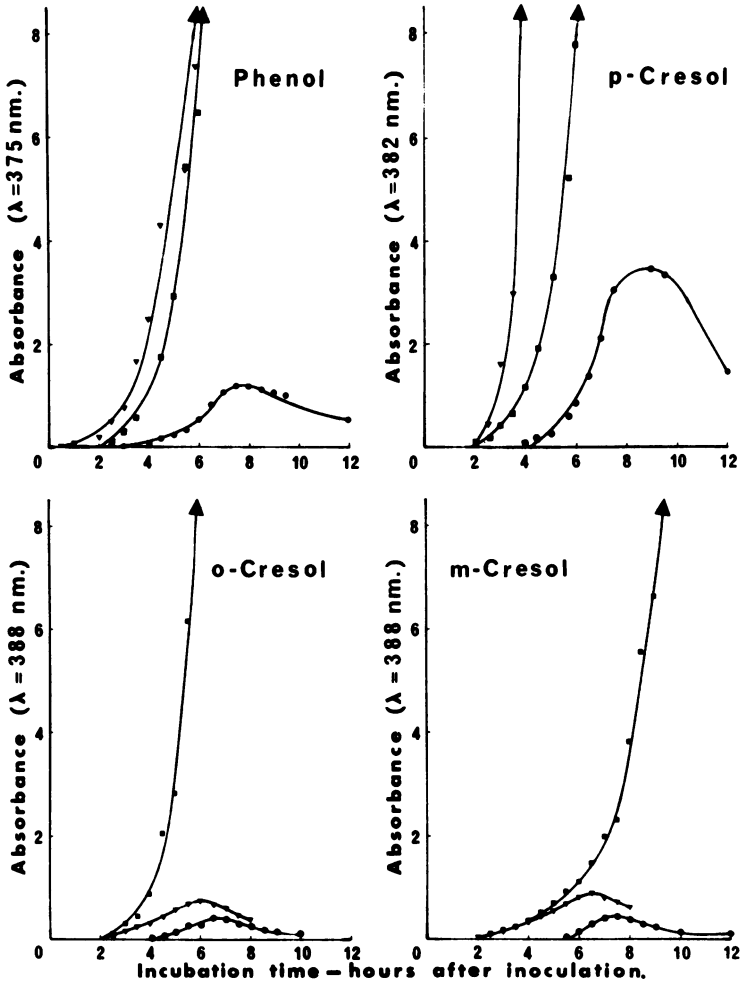


FIG. 2. *The accumulation of products of meta fission during growth of mutants of P. putida in fumarate medium containing either phenol or a cresol. Symbols: PsU-O,* ●——●; *PsU-5,* ■——■; *PsU-5-R21,* ▼——▼.

nol, were shown to have high levels of catechol 1,2-oxygenase and *cis,cis*-muconate lactonizing enzyme but no detectable levels of catechol 2,3-oxygenase, hydrolase, or $NAD^+$-dependent aldehyde dehydrogenase. PsU-5-R11 is a mutant strain of this type.

PsU-D1, like PsU-5-R11, can utilize only phenol and not a cresol as a sole source of carbon. The enzymatic activities of these two strains and of PsU-O are shown in Table 3. After overnight incubation in basal medium containing a cresol, both PsU-D1 and PsU-5-R11 accumulated a catechol which, after an ether extraction of the culture supernatant fluid, was detected as described by Evans (9). The methylcatechol from each cresol was identified by adding heat-treated extract of phenol-grown, wild-type cells (7) to the supernatant fluid of the culture and following the increase in products of ring fission which absorb in the range 360 nm to 420 nm (Table 4). The spectra showed that *ortho-* and *meta*-cresol were metabolized to 3-methylcatechol and that *para*-cresol gave 4-methylcatechol.

Only 4/134 of the apparent revertants selected on cresols would not grow on each of the cresols and phenol. The others, of which PsU-5-R5 is typical, appear to be revertants to wild-type phenotype. The remaining four partial revertants grew well on *ortho-* and *meta*-cresol, poorly on phenol, and not at all on *para*-cresol. Preliminary tests failed to exclude these strains as siblings and one of them, PsU-5-R21, was selected for further study.

When the levels of hydroxylase and catechol 2,3-oxygenase formed by PsU-O and

PsU-5-R21 in response to varying substrates and inducers were compared, it was found that they did not differ significantly (Table 5). This indicated that the growth spectra of PsU-5-R21 could not be due to a change in inducer ability or substrate specificity. In vivo evidence for the presence of hydroxylase and catechol 2,3-oxygenase in this mutant strain was obtained by growth in fumarate basal medium plus each of the phenolic compounds; the accumulation of the products of *meta* ring fission (Fig. 2) show that these two enzymes must be present. The hydrase and aldolase enzymes do not have to be considered to explain the properties of PsU-5-R21 since both phenol, *ortho*- and *meta*-cresol give rise to 2-keto-4-pentenoate and 4-hydroxy-2-ketovalerate, which are the respective substrates for these enzymes (1, 2) (Fig. 1).

TABLE 2. *Spontaneous partial revertants of PsU-5*

| Carbon source used for selection of spontaneous partial revertants | No. of revertants/$10^8$ cells plated | Growth spectra on replica plating |
|---|---|---|
| Phenol | 71 | 9/254 grew on all phenolics; remainder grew only on phenol. |
| *o*-Cresol | 3[a] | [b]43/44 grew on all phenolics; 1 did not grow on phenol or *p*-cresol. |
| *m*-Cresol | 1[a] | [b]52/55 grew on all phenolics; 3 did not grow on phenol or *p*-cresol. |
| *p*-Cresol | 1[a] | [b]35/35 grew on all phenolics. |

[a] As only a total of 16 partial revertants were isolated initially on the cresols, differences and similarities within this group are not significant.
[b] Isolated subsequently.

The finding that PsU-5-R21 grew well in fumarate basal medium plus *para*-cresol (see Table 7) shows that its failure to utilize *para*-cresol as a sole source of carbon is not likely to be due to an enhanced susceptibility to the bacteriostatic or bactericidal effects of the agent. However, this mutant strain grows at a slower rate in fumarate plus phenol medium than does the wild type.

A comparison of the levels of the first four enzymes of the *meta* fission pathway in PsU-O, PsU-5, PsU-5-R5, and PsU-5-R21 after induction with phenol is given in Table 6. The reason for the reduction of oxygen uptake by PsU-5 and PsU-5-R5 is not understood, but at least with PsU-5-R5 it has no physiological signifi-

TABLE 3. *Specific activities[a] of enzymes of the ortho and meta fission pathways when selected strains were grown to stationary phase on phenol as the sole source of carbon*

| Strain | Carbon source | Catechol 1,2-oxygenase | Muconate lactonizing enzyme | Catechol 2,3-oxygenase | Hydrolase | Hydrolase and dehydrogenase |
|---|---|---|---|---|---|---|
| PsU-O | Phenol | <0.001 | <0.001 | 0.177 | 0.006 | 0.036 |
| | Benzoate[b] | 0.065 | 0.023 | <0.001 | <0.001 | <0.001 |
| PsU-D1 | Phenol | 0.047 | 0.017 | <0.001 | 0.004 | 0.019 |
| PsU-5-R11 | Phenol | 0.047 | 0.015 | <0.001 | <0.001 | <0.001 |
| PsU-5-R21 | Phenol | <0.001 | <0.001 | 0.141 | 0.006 | 0.006 |

[a] Expressed as micromoles of substrate used (or product formed) per minute per milligram of protein.
[b] Included to provide a comparison for strains using the *ortho* fission pathway.

TABLE 4. *Increase in absorbance at λmax of products of ring fission following addition of heat-treated phenol-grown cell extract to supernatants of strains incubated overnight in media containing a phenolic compound as the sole source of carbon*

| Strain | Phenolic compound | | | |
|---|---|---|---|---|
| | Phenol | *o*-Cresol | *m*-Cresol | *p*-Cresol |
| PsU-O | 0.1 | 0.1 | 0.1 | 0.1 |
| PsU-5-R11 | 0.1 | 0.88 (λmax 388 nm) | 0.40 (λmax 388 nm) | 0.48 (λmax 382 nm) |
| PsU-D1 | 0.1 | 1.72 (λmax 388 nm) | 1.16 (λmax 388 nm) | 1.59 (λmax 382 nm) |
| PsU-5-R21 | 0.1 | 0.1 | 0.1 | NT[a] |

[a] Could not be tested due to accumulation of 2-hydroxy-5-methylmuconic semialdehyde during incubation.

TABLE 5. *Specific activities of enzymes of the meta fission pathway of PsU-O and PsU-5-R21 after induction with each of the cresols*

| Enzyme | Assay substrate | PsU-O | | | PsU-5-R21 | | |
|---|---|---|---|---|---|---|---|
| | | *o*-Cresol[a] | *m*-Cresol[a] | *p*-Cresol[a] | *o*-Cresol[a] | *m*-Cresol[a] | *p*-Cresol[a] |
| Hydroxylase[b, c] | Phenol | 56 | 70 | 46 | 47 | 50 | 33 |
| | *o*-Cresol | 59 | 95 | 56 | 60 | 93 | 58 |
| | *m*-Cresol | 24 | 46 | 29 | 35 | 50 | 31 |
| | *p*-Cresol | 31 | 52 | 50 | 29 | 44 | 38 |
| Catechol 2,3-oxygenase[c] | Catechol | 0.09 | 0.12 | 0.06 | 0.11 | 0.08 | 0.02 |
| | 3-Methylcatechol | 0.11 | 0.11 | 0.06 | 0.11 | 0.07 | 0.03 |
| | 4-Methylcatechol | 0.41 | 0.39 | 0.16 | 0.41 | 0.29 | 0.08 |
| (i) Hydrolase[d] | (i) 2-Hydroxymuconic semialdehyde | 0.004 | 0.004 | 0.002 | 0.010 | 0.009 | 0.002 |
| (ii) Hydrolase and dehydrogenase[c] | (ii) 2-Hydroxymuconic semialdehyde + $NAD^+$ | 0.014 | 0.014 | 0.006 | 0.010 | 0.009 | 0.002 |
| | (i) 2-Hydroxy-5-methylmuconic semialdehyde | 0.002 | 0.002 | <0.001 | <0.001 | <0.001 | <0.001 |
| | (ii) 2-Hydroxy-5-methylmuconic semialdehyde + $NAD^+$ | 0.017 | 0.012 | 0.007 | <0.001 | <0.001 | <0.001 |
| | (i) 2-Hydroxy-6-keto-2,4-Heptadienoate | 0.053 | 0.047 | 0.022 | 0.047 | 0.041 | 0.021 |
| | (ii) 2-Hydroxy-6-keto-2,4-Heptadienoate + $NAD^+$ | 0.052 | 0.048 | 0.021 | 0.046 | 0.041 | 0.022 |

[a] Inducer.
[b] Expressed as microliters of oxygen taken up per hour per milligram of protein.
[c] Values are corrected for endogenous respiration.
[d] Expressed as micromoles of substrate used (product formed) per minute per milligram of protein.
Note: in the absence of an inducer all hydroxylase levels <5 and all other activities <0.001.

cance as this strain grows well on any of the phenolic compounds.

The absence of detectable levels of hydrolase and $NAD^+$-dependent aldehyde dehydrogenase in PsU-5 accounts for the accumulation of the products of ring fission when this strain is grown in the presence of phenol or a cresol (Fig. 2) and explains its failure to utilize these compounds as growth substrates. The ability to regain both these activities spontaneously at a frequency of $2/10^6$ (Table 2), as exemplified by PsU-5-R5, suggests that the original mutant strain, PsU-5, had a single genetic lesion.

Unlike PsU-5-R5, strain PsU-5-R21 has regained only the hydrolase activity. The growth substrate spectra and ability to accumulate *meta* ring fission products of this mutant strain (Fig. 2) can be explained if the physiologically significant metabolism of 2-hydroxy-6-keto-2,4-heptadienoate is by the hydrolase, whereas that of 2-hydroxymuconic semialdehyde and 2-hydroxy-5-methylmuconic semialdehyde is by the $NAD^+$-dependent aldehyde dehydrogenase. That these enzymes are the significant ones for the respective mutant strains is shown by their specific activities in Table 6.

Although PsU-5-R21 was shown by replica plating to be unable to utilize phenol as a sole source of carbon, it will grow on phenol in liquid medium but with a greatly increased doubling time compared with wild type. In all other growth conditions which were tested (Table 7) there was little difference in the doubling times of wild type and this mutant strain. The ability to grow on phenol was shown to be due not to growth via the *ortho* fission pathway, as does PsU-5-R11, but by the *meta* fission pathway. After growth on phenol (Table 3), the cells contained no detectable levels of catechol 1,2-oxygenase and muconate-lactonizing enzyme but wild-type levels of catechol 2,3-oxygenase and hydrolase.

That the strain had not regained a $NAD^+$-dependent aldehyde dehydrogenase was shown in two ways: first, by the absence of an increased rate of breakdown of 2-hydroxymuconic semialdehyde in the presence of $NAD^+$ (Table 3) and, secondly, that no 4-oxalocrotonate could be detected in the reaction mix-

TABLE. 6. *Specific activities of enzymes of the meta fission pathway after induction of various strains with phenol*

| Enzyme | Assay Substrate | PsU-O | PsU-5 | PsU-5-R5 | PsU-5-R21 |
|---|---|---|---|---|---|
| Hydroxylase[a,b] | Phenol | 154 | 24[c] | 28[c] | 126 |
| | *o*-Cresol | 186 | 33[c] | 38[c] | 168 |
| | *m*-Cresol | 144 | 24[c] | 25[c] | 119 |
| | *p*-Cresol | 105 | 16[c] | 15[c] | 114 |
| Catechol 2,3-oxygenase[d] | Catechol | 0.14 | 0.07 | 0.13 | 0.10 |
| | 3-Methylcatechol | 0.13 | 0.09 | 0.13 | 0.11 |
| | 4-Methylcatechol | 0.52 | 0.25 | 0.42 | 0.39 |
| (i) Hydrolase[d] | (i) 2-Hydroxymuconic semialdehyde | 0.005 | <0.001 | 0.005 | 0.013 |
| (ii) Hydrolase and dehydrogenase[c] | (ii) 2-Hydroxymuconic semialdehyde + $NAD^+$ | 0.016 | <0.001 | 0.020 | 0.012 |
| | (i) 2-Hydroxy-5-methyl-muconic semialdehyde | 0.003 | <0.001 | 0.003 | 0.002 |
| | (ii) 2-Hydroxy-5-methyl-muconic semialdehyde + $NAD^+$ | 0.019 | <0.001 | 0.017 | 0.002 |
| | (i) 2-Hydroxy-6-keto-2,4-heptadienoate | 0.053 | <0.001 | 0.034 | 0.053 |
| | (ii) 2-Hydroxy-6-keto-2,4-heptadinoate + $NAD^+$ | 0.052 | <0.001 | 0.033 | 0.049 |

[a] Expressed as microliters of oxygen taken up per hour per milligram of protein.
[b] Values are corrected for endogenous respiration.
[c] Low levels of hydroxylase are reproducible.
[d] Expressed as micromoles of substrate used (product formed) per minute per milligram of protein.

TABLE 7. *Doubling times of wild-type and mutant PsU-5-R21*[a]

| Carbon source | PsU-O | PsU-5-R21 |
|---|---|---|
| Fumarate | 44 | 45 |
| Fumarate and phenol | 48 | 70 |
| Fumarate and *o*-cresol | 50 | 47 |
| Fumarate and *m*-cresol | 48 | 46 |
| Fumarate and *p*-cresol | 45 | 46 |
| Phenol | 73 | 225 |
| *o*-Cresol | 63 | 82 |
| *m*-Cresol | 62 | 80 |
| *p*-Cresol | 68 | No growth |

[a] Measured in minutes.

ture after all the 2-hydroxymuconic semialdehyde had been metabolized in the presence of $NAD^+$. When 20 μmol of 2-hydroxymuconic semialdehyde was metabolized in the presence of $NAD^+$ by phenol-grown cell extracts of this strain and the reaction mixture was deproteinized and steam distilled as described by Dagley and Gibson (7), formate could be detected. When similar cell extracts of PsU-O were used in the same way, a much lower quantity of formate was detected in the reaction mixture, whereas 4-oxalocrotonate was a major product. These results show that, although PsU-5-R21 can utilize phenol for growth, it does so in a manner different from that of the wild type.

## DISCUSSION

With the exception of PsU-5-R11, the mutant strains described are different from those previously reported for the *meta* fission pathway (10, 20). PsU-5-R11 is a partial revertant of the type described by Feist and Hegeman (10) as being due to an extragenic suppression, but the initial block in this mutant strain is at a different point. PsU-D1, selected directly for its lack of a catechol 2,3-oxygenase, has the same properties as PsU-5-R11 in regard to its ability to metabolize phenol by *ortho* fission. The accumulation of methylcatechols from cresols by PsU-D1 and PsU-5-R11 supports the indirect evidence of cresol metabolism of Bayly et al. (2).

The accumulation of *meta* fission products of phenol and cresols by PsU-5 showed it be a pleiotropic mutant which is deficient in both hydrolase and $NAD^+$-dependent aldehyde dehydrogenase. PsU-5-R5 regained both these activities whereas PsU-5-R21 regained the hydrolase activity only.

The characteristics of the mutant strains tested show that phenol can be metabolized by *P. putida* U in three ways, namely, (i) by conversion to catechol which undergoes *meta* fission to 2-hydroxymuconic semialdehyde which is metabolized to 4-oxalocrotonate; (ii) as in (i) to 2-hydroxymuconic semialdehyde which is metabolized to 2-keto-4-pentenoate;

and (iii) by conversion to catechol which is metabolized by *ortho* fission. The first two pathways probably function simultaneously in wild type while the third method provides an alternative if the other two systems are blocked.

The metabolism of the cresols diverges at the level of the products of ring fission. The product from *ortho*- and *meta*-cresol, 2-hydroxy-6-keto-2,4-heptadienoate, is metabolized by a hydrolase to 2-keto-4-pentenoate. 2-Hydroxy-5-methylmuconic semialdehyde, from *para*-cresol, is metabolized by a $NAD^+$-dependent dehydrogenase to an unidentified product which is probably a methyl derivative of 4-oxalocrotonate. Similar metabolism of the products of *meta* ring fission have been reported in *Azotobacter* species (21), in a naphthalene-grown pseudomonad (5), and in *P. arvilla* grown on benzoate, *meta*-, and *para*-toluate (15). *P. putida* U appears to differ from the *Azotobacter* species in that it has a significant induced level of hydrolase, whereas the activity in *Azotobacter* is very low both before and after induction. In the naphthalene-grown pseudomonad, the hydrolase level after induction is about 5% of that of the $NAD^+$-dependent system against 2-hydroxymuconic semialdehyde.

These recent reports (5, 15, 21) and the results now reported are in contrast to those of Bayly et al. (2) who showed stoichiometric conversion of the ring fission products of phenol and cresols to formate/acetate and hydroxy-keto acids by cell extracts of phenol-grown *P. putida* U. These extracts had undergone $(NH_4)_2SO_4$ precipitation and Sephadex fractionation and were not supplemented with $NAD^+$ during assay. Preliminary investigations (G. J. Wigmore, *unpublished observations*) indicate that such extracts do not contain the $NAD^+$-dependent dehydrogenase so that metabolism of the substrates would have proceeded by the hydrolase. If the $NAD^+$-dependent pathway is the physiological operative one for 2-hydroxymuconic semialdehyde and 2-hydroxy-5-methylmuconic semialdehyde, this also explains why Ribbons (19) and Bayly et al. (2) reported that the rate of breakdown of these compounds was considerably slower than that for 2-hydroxy-6-keto-2,4-heptadienoate, since the assays in both cases were carried out in the absence of $NAD^+$.

It therefore seems probable that, for *P. putida* U, the $NAD^+$-dependent system is the physiologically significant pathway for the metabolism of phenol and *para*-cresol, whereas for *ortho*- and *meta*-cresol the only operative pathway is that in which the hydrolase is concerned.

In a recent study which used the same strain of *P. putida* as in this report, Sala-Trepat et al. (22) arrived at a similar conclusion on the basis of the substrate specificity and enzymatic activity of the hydrolase and $NAD^+$-dependent aldehyde dehydrogenase of the wild-type strain.

Ornston (18) has stated that the *meta* fission pathway of alkyl-substituted diphenols is an example where there is broad specificity of inductive control of groups of enzymes which have a broad substrate specificity. The present results support this view as the ratio of the rate of hydroxylation of phenol and cresols is approximately the same regardless of the inducer used (Table 6), and, in addition, the ratios of the activities of the oxygenase against catechol and methylcatechols are similar regardless of the inducer used.

It is of interest to examine the possible reasons for the pleiotropic-negative mutation of PsU-5. It is unlikely that PsU-5 is a control mutant since two strains derived from it, PsU-5-R5 and PsU-5-R21, regained different enzymatic activities. When the frequency of spontaneous reversion to pseudo wild type is considered, the characteristics of this mutant are also unlikely to be accounted for by either separate mutations or a deletion mutation. The loss of both activities could be due to a single structural gene mutation if both activities reside on a single protein, and this may be possible since both activities utilize the same substrate. Regaining of one or both activities would then depend on the nature of the intragenic suppressor mutation. If, however, the activities were due to two separate proteins, the foregoing explanation would not apply. Feist and Hegeman (10) report a partial separation of the two activities, and unpublished observations (G. J. Wigmore) also indicate that the two activities are separable by protein fractionation procedures. Recently Sala-Trepat et al. (22) provided strong evidence that the two activities in *P. putida* U reside on separate proteins by showing that the activities have different thermal inactivation properties. It is probable therefore that PsU-5 is not a mutant of this type.

An explanation which is consistent with the behavior of PsU-5 is that the mutation resulted in premature termination of either transcription or translation; that is, it is a polarity mutation. If this were the case then the regaining of one or both enzymatic activities, as has happened with PsU-5-R21 and PsU-5-R5, re-

spectively, could occur by the spontaneous creation of either promoter or starter regions within the operon. The creation of two internal promoter-like regions has been described in the tryptophan operon of *Salmonella typhimurium* (25), and Sarabhai and Brenner (23) have reported the creation of an internal starter in bacteriophage $T_4$, but whether the characteristics of PsU-5 are due to such reasons can be resolved only by a genetic analysis.

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