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Phenol and Benzoate Metabolism by *Pseudomonas putida*: Regulation of Tangential Pathways

CAROL F. FEIST[1] AND G. D. HEGEMAN

Department of Bacteriology and Immunology, University of California, Berkeley, California 94720

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Catechol occurs as an intermediate in the metabolism of both benzoate and phenol by strains of *Pseudomonas putida*. During growth at the expense of benzoate, catechol is cleaved *ortho* (1,2-oxygenase) and metabolized via the β-ketoadipate pathway; during growth at the expense of phenol or cresols, the catechol or substituted catechols formed are metabolized by a separate pathway following *meta* (2,3-oxygenase) cleavage of the aromatic ring of catechol. It is possible to explain the mutually exclusive occurrence of the *meta* and *ortho* pathway enzymes in phenol- and benzoate-grown cells of *P. putida* on the basis of differences in the mode of regulation of these two pathways. By use of both nonmetabolizable inducers and blocked mutants, gratuitous synthesis of some of the *meta* pathway enzymes was obtained. All four enzymes of the *meta* pathway are induced by the primary substrate, cresol or phenol, or its analogue. Three enzymes of the *ortho* pathway that catalyze the conversion of catechol to β-ketoadipate enol-lactone are induced by *cis,cis*-muconate, produced from catechol by 1,2-oxygenase-mediated cleavage. Observations on the differences in specificity of induction and function of the two pathways suggest that they are not really either tangential or redundant. The *meta* pathway serves as a general mechanism for catabolism of various alkyl derivatives of catechol derived from substituted phenolic compounds. The *ortho* pathway is more specific and serves primarily in the catabolism of precursors of catechol and catechol itself.

Of the aromatic compounds that support the growth of fluorescent pseudomonads (*Pseudomonas aeruginosa*, *P. putida*, and *P. fluorescens*), a considerable number are catabolized via the common diphenolic intermediate, catechol (Fig. 1). As a general rule, the growth of a fluorescent pseudomonad at the expense of a catechol precursor elicits the induction of catechol 1,2-oxygenase and associated enzymes of the β-ketoadipate pathway (Fig. 2). It has occasionally been reported, however, that *Pseudomonas* strains of the fluorescent group can decompose catechol by an alternate inducible pathway (Fig. 3) involving ring cleavage by a catechol 2,3-oxygenase (4). We shall term the former pathway the *ortho* cleavage pathway, and the latter, the *meta* cleavage pathway. Many of the strains capable of decomposing catechol by the *meta* cleavage pathway also possess the genetic capability to decompose this compound through the *ortho* cleavage pathway. The phenotypic expression of the alternate pathways appears to be determined by the chemical nature of the aromatic catechol precursor with which the organisms are grown. For example, in naphthalene-decomposing fluorescent pseudomonads, the enzymes of the *meta* cleavage pathway are induced after growth with naphthalene or salicylate, but the enzymes of the *ortho* cleavage pathway are induced after growth with benzoate (6). Similarly, in fluorescent pseudomonads which decompose arylsulfonates, the enzymes of the *meta* cleavage pathway are induced by benzenesulfonate, but the enzymes of the *ortho* cleavage pathway are induced by benzoate (3).

A taxonomic analysis of the fluorescent pseudomonads (21) showed that strains known to be capable of degrading catechol by the *meta* cleavage pathway are predominantly strains of *P. putida;* unpublished observations by N. J. Palleroni on catechol cleavage mechanisms, subsequently extended by us, revealed that such strains are relatively rare within this species. Forty-one strains belonging to biotypes A and B of *P. putida* were examined. Only eight of these strains were capable of performing a *meta* cleavage of catechol. They could be subdivided into three physiological groups in terms of the growth conditions responsible for the induction of catechol 2,3-oxygenase activity (Table 1).

[1] Present address: Department of Genetics, University of California, Berkeley, Calif. 94720.

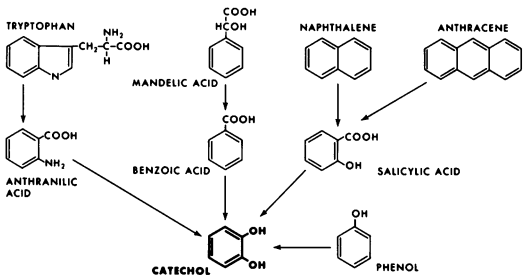

FIG. 1. *Central role of catechol in the oxidation of aromatic compounds by fluorescent pseudomonads.*

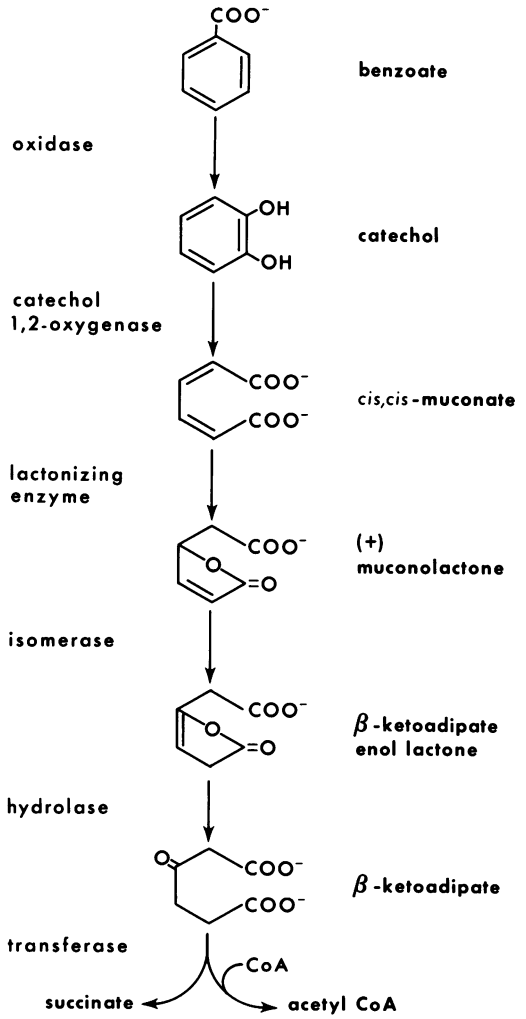

FIG. 2. *The ortho cleavage pathway (β-ketoadipate pathway) for oxidation of benzoate.*

In the strains of groups I and II, growth with benzoate always elicits synthesis of catechol 1,2-oxygenase, but growth with salicylate (group I) or phenol (group II) elicits the synthesis of catechol 2,3-oxygenase. The strains of group III, one of which was previously designated *P. arvilla* mt-2 and used as material for the purification of catechol 2,3-oxygenase (17), can perform a *meta* cleavage of catechol even when grown in the absence of aromatic substrates. They are the only strains which appear to decompose benzoate through a *meta* cleavage of catechol and apparently lack completely the enzymes of the *ortho* cleavage pathway.

The observations summarized above show that the pathway of catechol metabolism in strains of *P. putida* that possess the genetic capability to decompose catechol through both the *ortho* and *meta* cleavage pathways is determined by the nature of the primary aromatic substrate. However, the regulatory mechanisms that operate to determine the selection of alternate pathways have not been established. The regulation of the synthesis of catechol 1,2-oxygenase has been studied in strains of *P. putida* (18) and *P. aeruginosa* (11) which metabolize catechol exclusively through the *ortho* pathway. In both cases, it has been shown that catechol 1,2-oxygenase is product-induced by *cis,cis*-muconate and that catechol itself is not an inducer for *P. aeruginosa*. Accordingly, when such strains are grown at the expense of catechol precursors (e.g., benzoate), the induction of catechol 1,2-oxygenase results from the intracellular accumulation of *cis,cis*-muconate, formed metabolically from catechol through the low basal activity of the oxygenase.

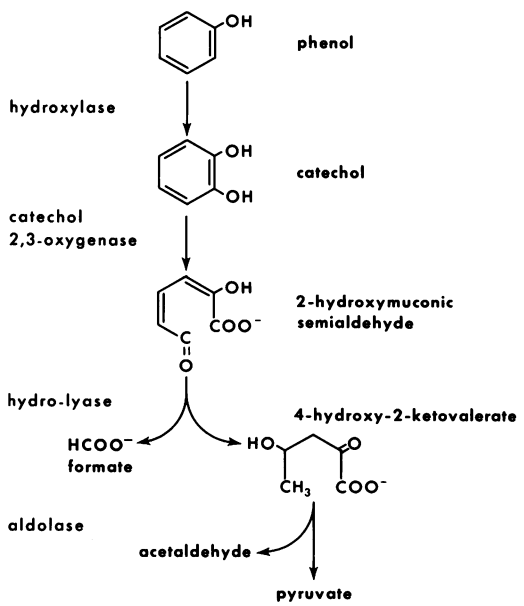

FIG. 3. *The meta cleavage pathway for oxidation of phenol.*

TABLE 1. *Strains of P. putida able to perform a meta (2,3) cleavage of catechol*

Group	Strain no.[a]	Constitutive catechol oxygenase	Nature of catechol oxygenase induced by growth at the expense of:		
			Benzoate	Phenol	Salicylate
I	110	None	1,2-	2,3-	2,3-
	111	None	1,2-	No growth	2,3-
	187	2,3-	No growth	No growth	2,3-
	109	2,3-	No growth	No growth	2,3-
II	144	None	1,2-	2,3-	No growth
	505	None	1,2-	2,3-	No growth
III	503 (*P. arvilla* mt-2)	Partial 2,3-	2,3-	No growth	No growth
	145	Partial 2,3-	2,3-	No growth	No growth

[a] Strain numbers given are those of Stanier, Palleroni, and Doudoroff (21).

Since *cis,cis*-muconate is also the inducer of the two other enzymes of the catechol branch of the β-ketoadipate pathway, the formation of *cis,cis*-muconate within the cell triggers the synthesis of no less than three enzymes specific to the *ortho* cleavage pathway. The synthesis of the enzymes of the alternate (*meta*) cleavage pathway is, therefore, probably induced by the specific primary substrates (e.g., salicylate, phenol, cresols, and benzenesulfonate) which are decomposed through this pathway.

To examine this question, we selected for detailed study a strain of group II. The strain was originally isolated by cresol enrichment and has been shown by Dagley et al. (4) to decompose phenol and substituted phenols (*o*-, *m*- and *p*-cresols) through *meta* cleavage pathways. Its pathway of phenol metabolism is shown in Fig. 3. Bayly and Dagley (2), in addition, recently found evidence that 2-ketopent-4-enoic acid is produced as an intermediate during the conversion of 2-hydroxymuconic semialdehyde to 4-hydroxy-2-ketovalerate. The substituted phenols are decomposed through analogous substituted intermediates. Benzoate, however, is decomposed strictly by the *ortho* cleavage pathway (Fig. 2).

MATERIALS AND METHODS

Organisms and methods of cultivation. *P. putida* (biotype A) strain U of Dagley (5), NCIB 10105, ATCC 17514, strain 144 of Stanier, Palleroni, and Doudoroff (21), was the primary organism used in this study. In addition, a number of *Pseudomonas* strains were obtained from the collection of Stanier, Doudoroff, and Palleroni for comparative studies. The origins and characteristics of these strains have been described (21). The mineral base described by Hegeman (9) was used to prepare all media. The final *p*H of the medium used was 6.8. Substrates were separately sterilized and added to the autoclaved mineral base. Phenol and catechol were used at a concentration of 2.5 mM, sodium benzoate at 5.0 mM, sodium succinate at 10 mM, and sodium acetate at 20 mM. Cultures were incubated at 30 C on a rotary shaker, and the turbidity of cultures was measured with a Klett-Summerson colorimeter fitted with a no. 66 filter. A culture of 100 Klett units contained approximately 3.6×10^8 cells/ml or 0.7 mg (dry weight)/ml.

Extraction of cells and enzymological methods. Cultures were harvested by centrifugation in the cold, and the cells were washed once with 0.003 M tris(hydroxymethyl)aminomethane(Tris)-hydrochloride, *p*H 7.6. In most cases, the prepared cell paste was stored at −20 C before extraction. Extracts were made in 0.033 M Tris-hydrochloride, *p*H 7.6. Cells were resuspended in this buffer (about 0.25 g, wet weight, of cells in 1.0 ml of buffer) and disintegrated with a 20-kc, 60-w, probe-type sonic oscillator (Measuring and Scientific Equipment Co., Ltd., London, England). Three minutes of treatment at maximal power broke 80 to 90% of the cells. The cell suspension was kept in an ice bath during sonic treatment. A 20-min centrifugation at 37,000 × *g* at 0 C removed whole cells and large debris from the extract. Reduced nicotinamide adenine dinucleotide (NADH)-oxidase activity, which interfered with the assay of 4-hydroxy-2-ketovalerate aldolase, was removed by sedimentation at 96,000 × *g* for 90 min.

Protein concentration in extracts was determined by the method of Lowry et al. (14) with bovine serum albumin (fraction V; Armour Pharmaceuticals, Kankakee, Ill.) as a standard.

Measurements of enzyme activity were performed with a recording spectrophotometer (model 2000; Gilford Instrument Laboratories Inc., Oberlin, Ohio) equipped with a cell compartment maintained at 25.0 C. All measurements were made in cuvettes of 1-cm path length in a final reaction volume of 3.0 ml.

Catechol 1,2-oxygenase (EC 1.99.2.2) was assayed by measuring the rate of formation of *cis,cis*-muconate as described by Hegeman (9). The oxidation of 0.1 μmole of catechol to *cis,cis*-muconate causes an increase in absorbancy at 260 nm of 0.56 absorbance units.

Muconate lactonizing enzyme (EC 5.5.1.1) was assayed by measuring the decrease in absorbancy at 260 nm after adding a sample of extract to a cuvette containing *cis,cis*-muconate. Assay conditions were those specified by Ornston (18). A decrease in ab-

sorbancy at 260 nm of 0.575 units was equivalent to the complete disappearance of 0.1 μmoles of *cis,cis*-muconate.

Catechol 2,3-oxygenase (EC 1.99.2.a) was measured by determining the rate of accumulation of 2-hydroxymuconic semialdehyde. The activity of 2-hydroxymuconic semialdehyde hydrolyase in crude extracts is so low under the conditions of this assay that it does not interfere with the accumulation of 2-hydroxymuconic semialdehyde. Reaction mixtures contained 100 μmoles of Tris-hydrochloride buffer (*p*H 7.6) and 0.2 μmoles of catechol. Oxidation of 0.1 μmoles of catechol to 2-hydroxymuconic semialdehyde results in an absorbancy increase of 0.98 optical density units at 375 nm.

2-Hydroxymuconic semialdehyde hydrolyase was determined by measuring the rate of decrease in absorbancy at 375 nm as the strongly absorbing substrate was converted to nonabsorbing products. Substrate for this reaction was produced by adding heated (53 C for 10 min) crude extract of phenol-grown cells of *P. putida* U to 3 to 10 mM catechol in 0.003 M Tris-hydrochloride (*p*H 8.0 or 8.8). Dagley, Evans, and Ribbons (5) discovered that maintaining extracts of phenol-grown cells at 53 C for 10 min destroyed 2-hydroxymuconic semialdehyde hydrolyase but did not affect the activity of catechol 2,3-oxygenase. Incubation of the catechol with heated extract was continued until 2-hydroxymuconic semialdehyde production stopped. The incubation medium was then extracted with diethyl ether to remove residual catechol, acidified (*p*H 2.0), centrifuged to remove the precipitated protein, and reextracted with ether to remove the 2-hydroxymuconic semialdehyde from the aqueous mixture. The ether layer containing the substrate was extracted with a suitable volume of Tris-hydrochloride buffer (*p*H 7.6). Substrate was recovered in the buffer at concentrations of 3 to 4 mM.

The addition of substrate amounts of NAD^+ (0.18 μmole in a reaction) was observed to increase the rate of conversion of 2-hydroxy muconic semialdehyde to nonabsorbing products in a reaction catalyzed by extracts of phenol-grown *P. putida*. Nishizuka et al (16) observed a similar phenomenon in the oxidation of *ortho* cresol via the *meta* cleavage pathway by a pseudomonad, and concluded that a dicarboxylic acid occurred as an intermediate. The NAD^+-stimulated activity catalyzing the disappearance of 2-hydroxymuconic semialdehyde was found only in phenol-grown but not in succinate or acetate-grown cells, and was therefore specifically inducible. Nicotinamide adenine dinucleotide (NAD) could not be replaced by nicotinamide adenine dinucleotide phosphate, acetyl-NAD, or these compounds in the reduced form. Sephadex chromatography indicated that the NAD-dependent and NAD-independent activities could be resolved, but they were never completely separated. All rates reported here for hydrolyase activity were measured in the absence of added NAD.

4-Hydroxy-2-ketovalerate aldolase (no EC number) was assayed indirectly, by measuring pyruvate production by the rate of oxidation of NADH in the presence of an excess of lactic dehydrogenase. The lactonized precursor of the substrate, 4-methyl-2-ketobutyrolactone, was synthesized according to Rossi and Schinz (19) and Stacy and Wagner (20). The lactone was hydrolyzed to yield 4-hydroxy-2-ketovalerate by the method of Dagley and Gibson (5). The assay mixture contained 100 μmoles of Tris-hydrochloride (*p*H 8.8), 2 μmoles of $MgCl_2$, 0.3 μmoles of NADH, and one unit of lactic dehydrogenase. Only 25% of the synthetic substrate could be converted to pyruvate. Enough of the delactonized material was added to provide 10^{-3} M utilizable substrate.

Mutagenesis and selection of mutants. Ethylmethane sulfonate (Distillation Products Industries, Rochester, N.Y.) was used at a concentration of 2% (v/v) as described by Loveless and Howarth (13). *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co., Milwaukee, Wis.) was used at a final concentration of 50 μg/ml in 0.1 M sodium citrate buffer (*p*H 5.5). Mutants were isolated from populations of cells treated for a period sufficient to reduce the viable count by 50 to 99.7%. In some trials, cells were transferred to nutrient medium after mutagen treatment and permitted several doublings for expression and segregation before plating.

Mutagen-treated cells were plated on yeast extract plates and, when growth occurred, replicas were prepared (12) on each of three plates of mineral medium containing benzoate, phenol, and succinate, respectively, as sole sources of carbon and energy. Mutants able to grow on benzoate and unable to grow on phenol and those that were unable to grow on benzoate but that retained the ability to grow on phenol were selected for further study.

Manometry. A model GRP 20 differential respirometer (Gilson Medical Electronics, Middleton, Wis.) was used in all manometric experiments. Flasks contained 1.75 ml of cells (6×10^8/ml) that had been washed and suspended in 0.05 phosphate buffer (*p*H 7.0). Freshly prepared 20% KOH (0.2 ml) was placed in the center well, and 0.25 ml of a 10 or 20 mM solution of substrate was added from the side arm. Oxygen uptake was measure directly in microliters at 30 C and converted to microliters at standard conditions.

RESULTS

Specificity of induction among phenol-utilizing strains of P. putida. Of the 11 phenol-utilizing strains of *P. putida* (21) which were tested, only three strains (110, 144, and 505) exhibited a *meta* cleavage mechanism after growth on phenol. Of these strains, only two were also able to grow at the expense of *o*-, *m*-, and *p*-cresol. When strain 144 (*Pseudomonas* U) was grown on phenol, cell extracts contained activities of the *meta* pathway enzymes but no detectable levels of *ortho* pathway enzymes (Table 2). Extracts of the same strain grown on benzoate contained only enzymes of the *ortho* pathway. When catechol was used as a carbon source, the *ortho* pathway enzymes were induced, and no *meta* pathway enzymes could be detected. Enzymes of both pathways could be

TABLE 2. *Specific activities[a] of enzymes in extracts of wild-type P. putida U grown on various substrates*

Enzymes	Substrates			
	Sodium succinate (10 mM); phenol (2 mM)	Sodium benzoate (5.0 mM)	Catechol (2.5 mM)	Sodium succinate (10 mM); pheno (2.5 mM) catechol (2.5 mM)
meta Pathway				
Catechol 2,3-oxygenase	0.66	≤0.001	≤0.001	0.49
2-Hydroxymuconic-semialdehyde hydrolyase	0.015	<0.001	<0.001	+[c]
2 Keto-4-hydroxyvalerate aldolase	0.017	<0.006	NT[b]	NT
ortho Pathway				
Catechol 1,2-oxygenase	<0.005	0.47	0.46	0.15
cis,cis-Muconate-lactonizing enzyme	<0.005	0.55	+	0.47

[a] The minimum detectable levels of activity are indicated by the less-than-or-equal-to signs (≤). Specific activities are expressed as micromoles of product formed or substrate consumed per minute per milligram of protein in a reaction.

[b] NT indicates not tested.

[c] +, Indicates a positive qualitative determination of the induced level of the enzyme.

found in extracts of cells which had grown in medium containing both catechol and phenol (Table 2).

Compounds which act as inducers of the meta cleavage pathway enzymes. Twenty-three compounds chosen as analogues of phenol were screened for the ability to induce enzymes of the *ortho* and *meta* pathways in strain 144. 2-Ethyl- and 3-ethylphenol, 2-allylphenol, and 2-methoxy-4-methylphenol all induce an enzyme capable of mediating a *meta* cleavage of catechol. These substituted phenols were metabolized to varying extents in reactions mediated by the induced enzymes. The ethyl phenols seemed to support some growth of *P. putida* U, but this growth may be attributable to small amounts of methylphenols or other substances capable of supporting growth that contaminate the commercial materials. Allylphenol and 2-methoxy-4-methylphenol did not support growth but were hydroxylated and cleaved by the enzymes they induced. A wide range of halogenated phenols and hydroxymethylpyridines and pyrimidines did not support growth or induce an enzyme capable of cleaving catechol.

Two compounds, benzyl alcohol and 2,6-dimethylphenol, are nonmetabolizable inducers of the *meta* pathway enzymes. These phenol analogues are not oxidized by phenol-induced cells, but catechol and phenol are immediately oxidized by cells grown in the presence of either of them. The addition of chloramphenicol (up to 1 mg/ml) to suspensions of cells grown in the presence of either of these compounds does not prevent the subsequent oxidation of catechol or phenol at rates equivalent to those observed in the absence of chloramphenicol (Table 3), although, under these conditions, the drug prevents induced enzyme synthesis.

Mutants of P. putida U blocked in enzymes of the meta pathway. A mutant designated 5P is not able to use phenol as a sole source of carbon and energy. After growth on acetate in the presence of phenol (2.5 mM), whole cells of 5P are unable to oxidize phenol, even though they retain the ability to oxidize catechol (Fig. 4). Enzyme activities in extracts of phenol-induced cells are shown in Table 4. The mutant lacks phenol oxidase activity. A spontaneous revertant of 5P is able to oxidize phenol normally. A second mutant, unable to grow at the expense of phenol, was designated 2P. When grown in the presence of phenol, it can oxidize both phenol and catechol without a lag. Extracts of phenol-induced mutant 2P have normal induced levels of catechol 2,3-oxygenase and 2-hydroxymuconic semialdehyde hydrolyase but contain no 4-hydroxy-2-ketovalerate aldolase activity (Table 5). The consumption of only 1 μmole of oxygen per μmole of added catechol indicated that the lesion prevents the conversion of catechol to pyruvate and acetaldehyde.

Strains of mutant 2P which had regained the ability to grow on phenol were selected by plating large numbers of cells on solid medium containing phenol as sole source of carbon and energy. Extracts of several of these phenol-grown apparent revertants had not regained 4-hydroxy-2-ketovalerate aldolase activity; instead, they were found to lack catechol 2,3-oxygenase activity. Catechol 1,2-oxygenase and muconate-lactoniz-

TABLE 3. *Rates of oxidation of phenol and catechol in the presence of chloramphenicol (1 mg/ml) by phenol-, benzyl alcohol-, and 2,6-dimethylphenol-induced cells of P. putida U*

Cells grown on:	Oxygen uptake[a]	
	Oxidation of phenol	Oxidation of catechol
Acetate (20 mM) + phenol (2.5 mM)	0.43	0.74
Acetate (20 mM) + benzyl alcohol (2.5 mM)	0.4	0.4
Acetate (20 mM) + 2,6-dimethylphenol (2.5 mM)	0.05	0.14

[a] Expressed as micromoles per minute per milligram of protein.

ing enzyme were present in extracts of phenol-grown revertants, although these enzymes are never observed in extracts of phenol-grown wild-type cells (Table 5). It was concluded that these apparent revertants in fact represented a curious case of extragenic suppression. In these mutant strains, loss of the second enzyme, catechol 2,3-

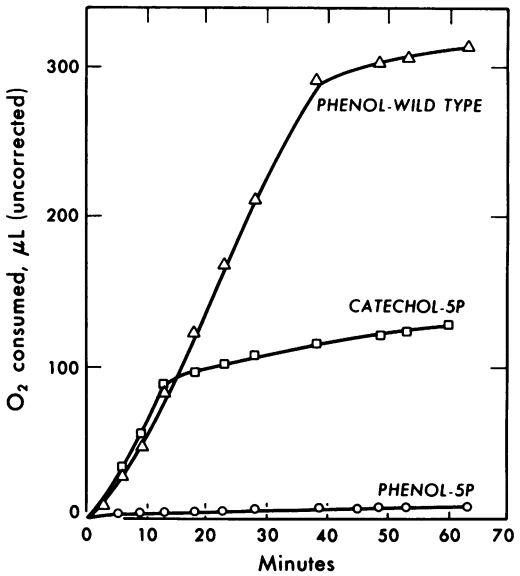

FIG. 4. *Kinetics of oxygen uptake by suspensions of cells of mutant 5P and the wild-type strain from which it was derived. The substrates indicated were added at zero time.*

TABLE 4. *Specific activities[a] of enzymes in extracts of wild-type and mutant P. putida U grown on 20 mM sodium acetate in the presence of 2.5 mM phenol*

Enzymes	Wild type	Mutant 5P	Revertant 5PR
meta Pathway			
Phenol oxidation (whole cells)	+	−	+
Catechol 2,3-oxygenase	0.5	0.8	0.53
2-Hydroxymuconic semialdehyde hydrolyase	0.03	0.07	0.05
4-Hydroxy-2-ketovalerate aldolase	0.02	0.014	0.01
ortho Pathway			
Catechol 1,2-oxygenase	≤.005[b]	≤.005[b]	≤.005[b]
Muconate-lactonizing enzyme	≤.005[b]	≤.005[b]	≤.005[b]

[a] Expressed as micromoles per minute per milligram of protein.
[b] Limit of the sensitivity of the assay.

TABLE 5. *Specific activities[a] of enzymes in extracts of wild-type and mutant P. putida U grown on 10 mM sodium succinate in the presence of 2.5 mM phenol*

Enzymes	Wild type	Mutant 2P	Revertant 2PR
meta Pathway			
Catechol 2,3-oxygenase	0.66	0.3	0.001[b]
2-Hydroxymuconic semialdehyde hydrolyase	0.015	0.02	0.02
4-Hydroxy-2-ketovalerate aldolase	0.017	≤0.006[b]	≤0.006[b]
ortho Pathway			
Catechol 1,2-oxygenase	≤0.005[b]	≤0.005[b]	0.5
Muconate-lactonizing enzyme	≤0.005[b]	≤0.005[b]	0.48

[a] Expressed as micromoles per minute per milligram of protein.
[b] Limit of sensitivity of the assay.

oxygenase, evidently permits growth on phenol via the *ortho* pathway.

Rate of growth and cell yield. The apparent revertant 2PR that uses the *ortho* pathway grows in phenol-mineral medium with a generation time (105 min) similar to that of wild-type cells growing on phenol via the *meta* pathway (100 min). Wild-type cells using the *ortho* pathway to metabolize catechol derived from benzoate have a generation time of 70 min. Therefore, the rate of phenol catabolism seems to be limited by the phenol hydroxylase reaction.

Two independently isolated revertants of the mutant 2P that utilize phenol by the *ortho* cleavage pathway produced the same amount of cell material per unit substrate as the wild-type strain metabolizing phenol by the *meta* pathway. There is, therefore, no apparent functional difference if the *ortho* rather than the *meta* pathway is used to metabolize the catechol derived from phenol.

DISCUSSION

Nature of the control of phenol metabolism. The enzymes and functional inducers of the *ortha* and *meta* pathways in *P. putida* are shown in Fig. 5. Phenol induces phenol hydroxylase-less mutant 5P to synthesize the three remaining *meta* pathway enzymes. Since this strain cannot metabolize phenol, and other pathway intermediates cannot serve as functional inducers, it may be concluded that the synthesis of the first four enzymes of the *meta* pathway is controlled by the initial substrate. It seems probable that the cresols and phenol are functionally equivalent as inducers of *meta* pathway enzymes.

Studies with analogues support the view that phenol and cresols can act as inducers of the enzyme group comprising phenol hydroxylase and the three *meta* pathway enzymes measured. Both benzyl alcohol, a phenol analogue, and 2,6-dimethylphenol, an *ortho* cresol analogue, can induce *meta* pathway enzymes. Neither of these two compounds are metabolized by *P. putida* U.

Catechol and methyl catechols do not induce enzymes of the *meta* pathway. The presence of only *ortho* pathway enzymes in catechol-grown cells of *P. putida* U indicates that catechol, or

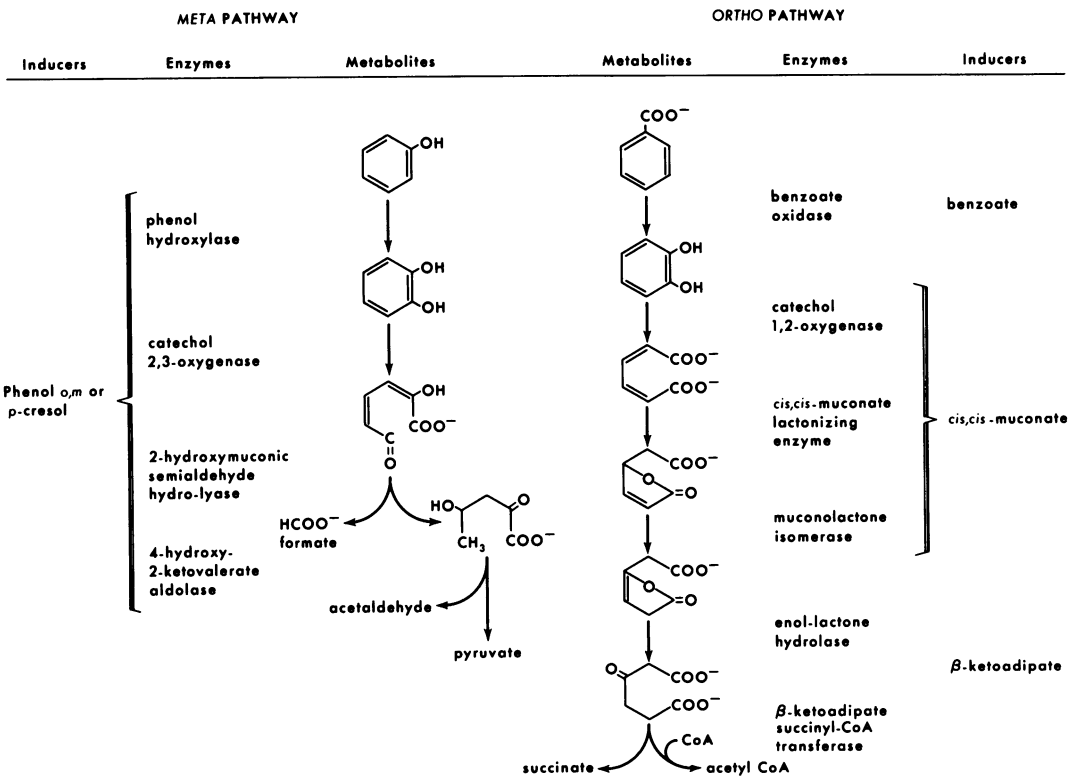

FIG. 5. *Metabolites, enzymes, and inducers of the ortho and meta pathways in P. putida. The regulation of the ortho pathway was described by Ornston (18). Enzymes, the names of which are enclosed by brackets, share a common inducer.*

cis,cis-muconate formed from it, acts as an inducer for enzymes of the *ortho* pathway but not the *meta* pathway. This conclusion is supported by phenotypic analysis of apparent revertants of mutant 2P that regained the ability to grow on phenol by the mutational loss of a second *meta* pathway enzyme. Mutant 2P, which lacks 4-hydroxy-2-ketovalerate aldolase, cannot use the *ortho* pathway for growth on phenol because neither phenol nor 4-hydroxy-2-ketovalerate is able to induce catechol 1,2-oxygenase. Subsequent mutational loss of catechol 2,3-oxygenase activity by the apparent revertants, however, results in the accumulation of catechol from phenol and concomitant synthesis of *ortho* pathway enzymes which then serve to further catabolize the catechol produced from phenol. If the pattern of induction of *ortho* pathway enzymes in *P. putida* U is the same as that of *P. aeruginosa* (11) and *P. putida* A.3.12 (18), *cis,cis*-muconate formed from the accumulated catechol by the low uninduced level of catechol 1,2-oxygenase is the actual inducer. Thus, catechol 2,3-oxygenase is induced by the primary substrate of the pathway, and catechol 1,2-oxygenase is induced by the product of its action. As originally suggested by Stanier (Discussion to reference 3, p. 143), the difference in the mechanism of regulation of the two pathways explains why enzymes of only one pathway are synthesized during growth on a particular substrate even though a common intermediate is formed. In the case of *P. putida* U, growth on benzoate does not induce enzymes of the *meta* pathway because these are induced only by phenols or cresols. Growth at the expense of phenol does not induce *ortho* pathway enzymes because the high level of phenol-induced catechol 2,3-oxygenase prevents catechol and, therefore, *cis,cis*-muconate accumulation.

2-Hydroxymuconic semialdehyde that accumulates during growth on phenol does not induce measurable levels of catechol 1,2-oxygenase. This contrasts with observations made by Farr and Cain (8) on induction of catechol 1,2-oxygenase in a strain of *P. aeruginosa*. They demonstrated that purified samples of 2-hydroxymuconic semialdehyde induced catechol 1,2-oxygenase. Some *Pseudomonas* strains synthesize enzymes of both the *ortho* and *meta* pathways when they are grown on salicylate or naphthalene (1). Although salicylate as the primary substrate induces enzymes of the *meta* pathway, enough *cis,cis*-muconate may accumulate through the activity of a low endogenous level of catechol 1,2-oxygenase to cause induction of *ortho* pathway enzymes.

Differences in the specificity of function and mode of regulation of the two pathways which serve to catabolize catechol lead us to suggest that they are not in fact redundant, but have evolved separately to fulfill different metabolic functions. The two catechol cleavage enzymes differ in specificity. Studies with crystalline preparations (17) demonstrate that catechol 2,3-oxygenase from *P. putida* (*arvilla*) mt-2 is capable of catalyzing the cleavage of methylcatechols as rapidly as that of catechol itself. Catechol 1,2-oxygenase, on the other hand, seems to be much more specific (17, 23).

Induction of an entire pathway by the primary substrate as observed in induction of the *meta* pathway by cresol or phenol would seem to be a much less specific control than the induction of the *ortho* pathway by a product of one of the pathway enzymes (18). Phenol, *o*-, *m*-, and *p*-cresol, 2-ethylphenol, 3-ethylphenol, allylphenol, and 3-methoxy-2-methylphenol, as well as benzyl alcohol and 2,6-dimethylphenol, induce a catechol 2,3-oxygenase. Exposure of cells to catechol results in inducation of catechol 1,2-oxygenase, but exposure to 3-methyl or 4-methylcatechol does not lead to synthesis of this enzyme.

The low specificity of the induction and function of *meta* pathway enzymes implies that during its evolution this pathway came to function in the catabolism of a wide variety of derivatives of aromatic compounds which presumably arise during the degradation of natural products. If this is true, catechol is only one of a number of possible intermediates metabolizable via the *meta* pathway. In contrast, the *ortho* pathway seems to be quite specific for catechol. The revertant of mutant 2P which regained the ability to grow on phenol (a precursor of catechol) by using the *ortho* pathway did not simultaneously regain the ability to grow on cresols, precursors of methylcatechols.

It is of interest to observe the occurrence of a complete shift from use of one pathway to the other caused by only two mutational events in strain 2PR. A similar observation was made by Tanaka et al. (22) while studying arabitol degradation by *Aerobacter aerogenes*. Mutants unable to grow on mannitol yielded revertants able to grow on mannitol by virtue of a lesion leading to production of a constitutive arabitol dehydrogenase. This enzyme was able to convert mannitol to fructose, thereby affording the cell a new functional pathway for mannitol degradation. In the present case, the derivation of double mutant 2PR may serve as a model for the divergence of metabolic capabilities among members of a taxonomic group.

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