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Role and Regulation of the *ortho* and *meta* Pathways of Catechol Metabolism in Pseudomonads Metabolizing Naphthalene and Salicylate

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The enzymes of naphthalene metabolism are induced in *Pseudomonas putida* ATCC 17484, PpG7, NCIB 9816, and P_G and in *Pseudomonas* sp. ATCC 17483 during growth on naphthalene or salicylate; 2-aminobenzoate is a gratuitous inducer of these enzymes. The *meta*-pathway enzymes of catechol metabolism are induced in ATCC 17483 and PpG7 during growth on naphthalene or salicylate or during growth in the presence of 2-aminobenzoate, but in ATCC 17484 and NCIB 9816 the *ortho*-pathway enzymes of catechol metabolism are induced during growth on naphthalene or salicylate. 2-Aminobenzoate does not induce any enzymes of catechol metabolism in the latter two organisms. In *Pseudomonas* P_G the *meta*-pathway enzymes are present at high levels under all conditions of growth, but this organism and PpG7 can induce *ortho*-pathway enzymes during naphthalene or salicylate metabolism. Salicylate appears to be the inducer of the enzymes of naphthalene metabolism in all of the organisms studied and, where they are inducible, of the *meta*-pathway enzymes, but the properties of *Pseudomonas* P_G suggest that separate, regulatory systems may exist.

The catabolism of catechol produced during the metabolism of naphthalene by pseudomonads has previously been shown to involve the *meta* (or α-ketoacid) pathway (2, 3) in which the first reaction is catalyzed by catechol 2,3-dioxygenase (EC 1.13.11.2) (Fig. 1E). The induction of catechol 1,2-dioxygenase (EC 1.13.11.1) (Fig. 1H) has also been observed, however, in pseudomonads metabolizing naphthalene (1), and so the present work was undertaken to determine whether this has metabolic significance for catechol degradation. Pseudomonads have sometimes been observed to produce at least some of the enzymes of both the *ortho* and *meta* pathways during the metabolism of compounds that are degraded through catechol (5, 9, 10), but this does not invariably occur (6, 12, 18). It is shown here that in some pseudomonads the *ortho* pathway plays the dominant role during naphthalene and salicylate metabolism, and in other pseudomonads that use the *meta* pathway this may be induced by salicylate.

MATERIALS AND METHODS

Bacterial strains. *Pseudomonas putida* ATCC 17484 and NCIB 9816 and *Pseudomonas* sp. ATCC 17483 were obtained, fresh, from the American Type Culture Collection, Rockville, Md., and the National Collection of Industrial Bacteria, Aberdeen, Scotland, respectively. *P. putida* PpG7 (4) was a gift from I. C. Gunsalus; *Pseudomonas* P_G was from P. A. Williams (17).

Culture conditions. The organisms were grown on the mineral medium described previously (15), containing an appropriate carbon source. Cultures growing on salicylate (3.5 mM) or naphthalene (0.1%, wt/vol, suspension) were most readily started by the addition of a heavy inoculum (from a fresh culture on an agar plate containing the respective carbon source) to the liquid medium to give an initial absorbance of about 0.2 at 600 nm (Beckman DB spectrophotometer). This procedure avoided the unpredictably long lag periods that were invariably associated with growth from a single colony, but the results were identical with those obtained by the latter procedure. Induction of enzymes in cells growing on succinate (8 mM) was effected by the addition of salicylate (3.5 mM), catechol (3 mM), or 2-aminobenzoate (0.35 mM).

Preparation of cell extracts and the measurement of enzymatic activity. Cultures were harvested during the exponential phase of growth, washed, and extracted as previously described (1), except that centrifugation of disrupted bacteria was carried out at 27,000 $\times$ *g* for 30 min at 4 C.

Catechol 2,3-dioxygenase was measured by the method of Feist and Hegeman (6), except that phosphate buffer (pH 7.6) was used and measurements

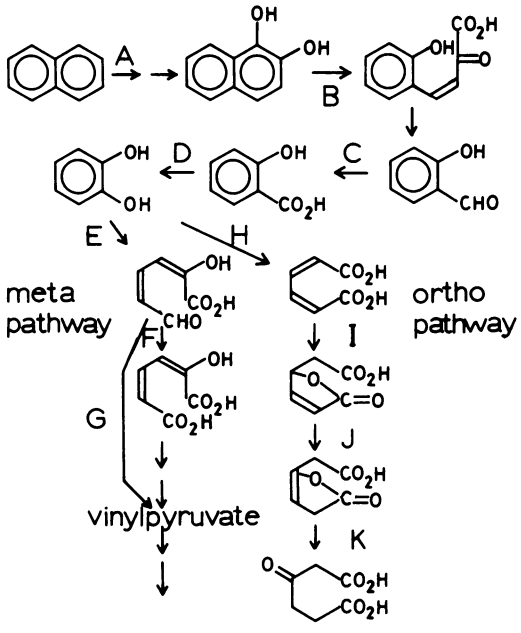

FIG. 1. *Metabolism of naphthalene. The pathway is outlined in terms of the substrates of enzymes measured in this work, and the enzymes are identified by letters: (A) naphthalene oxygenase; (B) 1,2-dihydroxynaphthalene oxygenase; (C) salicylaldehyde dehydrogenase; (D) salicylate hydroxylase; (E) catechol 2,3-dioxygenase; (F) 2-hydroxymuconic acid semialdehyde dehydrogenase; (G) 2-hydroxymuconic acid semialdehyde hydrolase; (H) catechol 1,2-dioxygenase; (I) muconate cycloisomerase; (J) muconolactone isomerase; and (K) β-ketoadipate enol-lactonase. A double arrow indicates two or more enzymatic steps between the given compounds.*

were made immediately after an extract had been centrifuged. Catechol 1,2-dioxygenase was measured by the method of Hegeman (8), using a cell extract that had been subjected briefly to oxidation with H_2O_2. The extract (0.1 ml) was added to 1.1 ml of ice-cold 0.05 KH_2PO_4-NaOH buffer (pH 7.0), and 2 μl of 0.3% H_2O_2 was then added to the mixture. One minute after the addition of H_2O_2, a sample was transferred to the cuvette containing the reagents for the determination of catechol 1,2-dioxygenase. Muconate cycloisomerase (EC 5.5.1.1) (Fig. 1I) was measured by the method of Ornston (11).

Measurements were made of the activity of muconolactone isomerase (EC 5.3.3.4) (Fig. 1J) and β-ketoadipate enol-lactonase (EC 3.1.1.24) (Fig. 1K) together by observing the rate of decrease in absorbance at 230 nm that occurred when the cell extract was added to an equilibrium mixture of *cis,cis*-muconate and *cis,cis*-muconolactone in the presence of an excess of the lactonizing enzyme. To 27 ml of 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.0) were added 1.0 ml of *cis,cis*-muconic acid (3 mM), 0.05 ml of $MnCl_2$ (10 mM), and 3 U of partially purified muconate cycloisomerase (1 U causes the reaction of 1 μmol of substrate per min). When the absorbance at 230 nm had reached the steady state, 2.95 ml of the reaction mixture was transferred to a cuvette, and the rate of decrease in absorbance at 230 nm caused by the addition of bacterial extract was measured. The absorbance at 230 nm of an equilibrium mixture of *cis,cis*-muconic acid and muconolactone at a total concentration of 1 mM is 2.05, whereas the extinction coefficient of β-ketoadipate enol-lactone at 230 nm is 1.61 $mM^{-1}cm^{-1}$ (13). Consequently, a decrease in absorbance may signify the presence of only muconolactone isomerase. Since it was observed, however, that the absorbance with extracts of induced cells always fell below 78% of that in the starting equilibrium mixture, the implication was that the reaction was proceeding to the formation of the nonabsorbing species β-ketoadipate. This was confirmed by the extraction of material from acidified reaction media, which was chromatographically identical with β-ketoadipate and gave a positive Rothera reaction. Therefore, in calculating the activities given in Table 1, I used an extinction coefficient of 2.05 $mM^{-1}cm^{-1}$, which leads to a minimal value for the activity of either enzyme. It should be noted that the concentration of Mn^{2+} used in equilibrating *cis,cis*-muconic acid and its lactone is sufficient to activate the enzyme fully but is less than that used in the routine determination of the activity of the cycloisomerase because, at that concentration, some inhibition of the enzymatic activity measured here is observed.

2-Hydroxymuconic acid semialdehyde hydrolase (Fig. 1G) and dehydrogenase (Fig. 1F) were measured by reported methods (14) in the absence and presence, respectively, of 0.33 mM nicotinamide adenine dinucleotide (NAD). Treatment of cell extracts with NADase was omitted since the apparent hydrolase activities were, in every case, very small. Naphthalene oxygenase (Fig. 1A) (15), 1,2-dihydroxynaphthalene oxygenase (Fig. 1B) (16), salicylaldehyde dehydrogenase (Fig. 1C) (16), and salicylate hydroxylase (Fig. 1D) (EC 1.14.13.1) (reference 1, method 2) were determined by published methods.

Partial purification of muconate cycloisomerase. The purification of muconate cycloisomerase was based on the observations of Ornston (11). *P. putida* NCIB 9816 was grown on a basal mineral medium containing phenol (0.04%), harvested during the exponential phase of growth, washed once with phosphate buffer (pH 7.0), and resuspended in the same buffer at a concentration of 0.1 g (wet weight) per ml. Cells were disrupted in a French pressure cell, and the extract was centrifuged as described above. The supernatant was heated at 60 C for 10 min, cooled, clarified by centrifugation, and fractionated with $(NH_4)_2SO_4$. The fraction that precipitated between 30 and 40% saturation contained 77% of the activity in the original extract, the purification was 27-fold, and the specific activity was 20 μmol/min per mg of protein.

RESULTS AND DISCUSSION

The levels of the enzymes of the *ortho* and *meta* pathways in four pseudomonads grown on

various carbon sources are given in Table 1. *P. putida* ATCC 17483 was unique among all of the organisms studied (see Tables 1 and 2) in that enzymes of the *ortho* pathway were not induced in it. It was reported previously that catechol 1,2-dioxygenase was induced in ATCC 17483 (1) but, when it was observed in the present work that muconate cycloisomerase was not present in cell extracts, the catechol 1,2-dioxygenase activity was re-investigated. An increase in absorbance at 260 nm was observed in the absence of ethylenediaminetetraacetic acid, but subsequent treatment with partially purified muconate cycloisomerase and Mn^{2+} did not cause a decrease in absorbance. The previous report of catechol 1,2-dioxygenase activity in ATCC 17483 was therefore in error, and the absorption changes measured were probably due to the formation of a large concentration of 2-hydroxymuconic acid semialdehyde in the presence of the very active catechol 2,3-dioxygenase. The latter enzyme was inactivated, therefore, by oxidation with H_2O_2 before the determination of catechol 1,2-dioxygenase. Oxidation with H_2O_2 has been used previously (9, 10) to prevent catechol 2,3-dioxygenase from masking catechol 1,2-dioxygenase (i.e., the opposite situated from that suspected here), but the methods used also destroyed a large proportion of the catechol 1,2-dioxygenase in extracts of organisms in which this was the predominant enzyme (ATCC 17484 and NCIB 9816). Therefore, the smallest concentration of H_2O_2 that would just destroy catechol 2,3-doxygenase was used. This was much less (about 0.15 mM) than that (30 mM) used previously (9). Although this procedure would not rule out the presence of a catechol 1,2-dioxygenase that was particularly sensitive to H_2O_2, the conclusion drawn, that catechol 1,2-dioxygenase was absent from ATCC 17483, is in accord with the lack of sensitivity of the reaction product to muconate cycloisomerase.

The induction of the *meta* pathway is mimicked in ATCC 17483 by growth in the presence of 2-aminobenzoate, which is not metabolized (15). Since growth on, or in the presence of, catechol causes only a weak induction of catechol 2,3-dioxygenase (1; Table 1), it appears that salicylate is the inducer of catechol

TABLE 1. *Activity of ortho- and meta-pathway enzymes*

Strain	Carbon source or inducer	Activity (μmol/min per mg of protein) of:				
		ortho-Pathway enzymes			*meta*-Pathway enzymes[a]	
		Catechol 1,2-dioxygenase	Muconate cycloisomerase	Muconolactone isomerase and β-ketoadipate enol-lactonase	Catechol 2,3-dioxygenase	2-Hydroxymuconic acid semialdehyde dehydrogenase
17483	Succinate	<0.003	<0.003	0.019	0.007	0.004
	Naphthalene	<0.003	<0.003	0.020	1.88	0.140
	Salicylate	<0.003	<0.003	0.075	1.04	0.140
	Succinate + 2-aminobenzoate	<0.003	<0.003	0.036	0.505	0.068
	Succinate + catechol	<0.003	<0.003	0.015	0.050	0.004
PpG7	Succinate	<0.003	<0.003	0.014	0.014	0.004
	Naphthalene	0.099	0.132	0.155	1.21	0.166
	Salicylate	0.610	0.705	0.199	0.705	0.132
	Succinate + 2-aminobenzoate	0.003	<0.003	0.029	0.398	0.098
9816	Succinate	<0.003	<0.003	0.016	0.017	0.004
	Naphthalene	0.573	2.07	0.40	0.042	0.007
	Salicylate	0.241	0.915	0.187	0.021	0.010
	Succinate + 2-aminobenzoate	0.003	<0.003	0.027	0.053	0.011
17484	Succinate	0.003	<0.003	0.030	0.014	0.023
	Naphthalene	0.438	1.07	0.386	0.012	0.008
	Salicylate	0.301	1.66	0.515	0.048	0.016
	Succinate + 2-aminobenzoate	0.008	<0.003	0.024	0.083	0.017

[a] The activities of 2-hydroxymuconic acid semialdehyde hydrolase have not been tabulated. The enzyme was frequently not detected, and the greatest activity that was detected was 0.005 μmol/min per mg of protein.

2,3-dioxygenase and, in view of the effect of 2-aminobenzoate, probably of the later *meta*-pathway enzymes as well. Gratuitous induction by 2-aminobenzoate has been observed as far along the pathway as vinylpyruvate hydratase (K. M. Shumsuzzaman and E. A. Barnsley, Proc. Can. Fed. Biol. Soc. **18**:132, 1975). This pattern of induction by the immediate precursor of catechol (in this case, salicylate) is essentially similar to that observed by Feist and Hegeman (6, 7) in certain pseudomonads that oxidize benzoate or phenol through the *meta* pathway.

Similar conclusions about the induction of the *meta* pathway in *Pseudomonas* PpG7 may also be drawn, but in this organism significant levels of the *ortho*-pathway enzymes are produced during growth on naphthalene or salicylate. The concomitant induction of *ortho*- and *meta*-pathway enzymes has been noted previously in pseudomonads growing on other carbon sources (5, 9), the *ortho* pathway being induced when sufficient catechol accumulates to ensure its conversion to the *ortho*-pathway inducer *cis,cis*-muconate (9, 12).

P. putida NCIB 9816 and ATCC 17484 contrast markedly with ATCC 17483 and PpG7 in that the *ortho*-pathway enzymes predominate during growth on naphthalene or salicylate. Although the former strains, when grown on succinate, contain a significant basal quantity of catechol 2,3-dioxygenase, this quantity does not increase to the same level as the *ortho*-pathway enzymes during growth on naphthalene or salicylate. The failure to observe highly elevated levels of catechol 2,3-dioxygenase was probably not due to the instability generally inherent in this enzyme, since the incorporation of 10% (vol/vol) acetone into the medium used to disrupt cells (14) did not alter the activity measured subsequently. Higher enzymatic activities were not found after the use of other buffers in the preparation of extracts or in the assays themselves. It seems likely, therefore, that during growth on naphthalene or salicylate sufficient catechol is converted to *cis,cis*-muconate by basal levels of catechol 1,2-dioxygenase to induce the latter enzyme and the later *ortho*-pathway enzymes (12). Some support for this view is obtained from observations on the metabolism of 2-methylnaphthalene by *P. putida* NCIB 9816. Growth is slower (doubling time, 3.5 h) than growth on naphthalene (doubling time, 1.6 h), catechol 1,2-dioxygenase is not induced, catechol 2,3-dioxygenase remains at the levels given in Table 1, and a large concentration of a yellow compound accumulates in the medium. The pathway of degradation of 2-methylnaphthalene is uncertain, but it is likely to lead to 4-methylcatechol. The failure of the latter to lead to an inducer of the *ortho* pathway, and its own oxidation by catechol 2,3-dioxygenase to a methyl hydroxymuconic acid semialdehyde, would account for the observations made. It is worthy of note that some hydroxymuconic acid semialdehyde does appear in the medium during the metabolism of naphthalene or salicylate by NCIB 9816 and ATCC 17484. Evidently the low level of catechol 2,3-dioxygenase is able to divert some catechol into the beginning of the *meta* pathway and, consequently, it should be borne in mind that the appearance of a yellow color during the metabolism of aromatic compounds may not necessarily indicate the predominance of the *meta* pathway or even a complete pathway.

Pseudomonas P_G was a culture of NCIB 9816 maintained in the laboratory of P. A. Williams (17). The levels of various enzymes found in this organism under different growth conditions are given in Table 2. It responds to growth on naphthalene, salicylate, succinate plus salicylate, and succinate plus 2-aminobenzoate in ways very similar to that of other pseudomonads examined (1). Naphthalene oxygenase, 1,2-dihydroxynaphthalene oxygenase, salicylaldehyde dehydrogenase, and salicylate hydroxylase are all induced when compared with the levels of the enzymes present during growth on succinate. On the other hand, the enzymes of the *meta* pathway are always present at high levels, even during growth on succinate. These results are very different from those reported previously (17), when the induction of 1,2-dihydroxynaphthalene oxygenase by salicylate and the presence of high constitutive levels of *meta*-pathway enzymes were not noted.

The results given in Table 2 are the third set of properties attributed to an organism which, at one time or another, has been labeled NCIB 9816 (compare the data from reference 1 and from Table 1 with that of reference 17 and of Table 2). Apart from the bearing on the problem of maintaining a bacterial strain for long periods in different laboratories, these properties of *Pseudomonas* P_G are of interest in terms of possible mechanisms of the regulation of naphthalene degradation. All of the naphthalene-oxidizing pseudomonads that we examined induce the enzymes of naphthalene oxidation (Fig. 1A through D) in response to salicylate (or gratuitously in response to 2-aminobenzoate). Those strains (ATCC 17483 and *Pseudomonas* PpG7) that can induce high levels of *meta*-pathway enzymes (Fig. 1E onwards) respond in this way to the same two compounds

TABLE 2. *Activity of enzymes in Pseudomonas* P_G

Carbon source/ inducer	Activity (μmol/min per mg of protein) of:							
	Naphthalene oxygenase	1,2-Dihydroxynaphthalene oxygenase	Salicylaldehyde dehydrogenase	Salicylate hydroxylase	Catechol 2,3-dioxygenase	Hydroxymuconic acid semialdehyde dehydrogenase	Catechol 1,2-dioxygenase	Muconate cycloisomerase
Succinate	0.005	0.10	0.080	0.005	1.26	0.134	<0.003	<0.003
Naphthalene	0.090	2.95	0.755	0.323	1.25	0.130	0.014	0.028
Salicylate	0.091	2.66	0.750	0.441	1.10	0.127	0.133	0.406
Succinate + salicylate	0.076	2.06	0.615	0.550	1.00	0.120	<0.003	<0.003
Succinate + 2-aminobenzoate	0.060	3.30	0.990	0.302	1.45	0.140	<0.003	<0.003

(salicylate and 2-aminobenzoate). Consequently, the possibility arises that the enzymes for a very long metabolic sequence not only share a single inductive mechanism, derepression by salicylate or 2-aminobenzoate, but may also be regulated as a unit. Independent genetic evidence has been adduced for the close linkage on a plasmid of the genes for naphthalene metabolism and catechol degradation through the *meta* pathway in *Pseudomonas* PpG7 (4). The clear demonstration in *Pseudomonas* P_G that naphthalene metabolism and catechol metabolism are not regulated as a unit, however, shows that extrapolation from *Pseudomonas* PpG7 to other strains must be made with caution.

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