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Control of Catechol *meta*-Cleavage Pathway in *Alcaligenes eutrophus*

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Alcaligenes eutrophus 335 (ATCC 17697) metabolizes phenol and *p*-cresol via a catechol *meta*-cleavage pathway. Studies with mutant strains, each defective in an enzyme of the pathway, showed that the six enzymes assayed are induced by the primary substrate. Studies with a putative polarity mutant defective in the expression of aldehyde dehydrogenase suggested that the structural genes encoding this and subsequent enzymes of the pathway exist in the same operon. From studies with mutant strains that constitutively synthesize catechol 2,3-oxygenase and subsequent enzymes and from the coordination of repression of these enzymes by *p*-toluate, benzoate, and acetate, it is proposed the catechol 2,3-oxygenase structural gene is situated in this operon (2,3-oxygenase operon). Studies with regulatory mutant strains suggest that the 2,3-oxygenase operon is under negative control.

The absence of suitable genetic techniques has hampered detailed elucidation of the regulation of gene expression in apparently chromosomally encoded *meta* pathways. Models for the regulation of *meta*-cleavage pathways, such as in *Pseudomonas putida* U (24) and *Pseudomonas aeruginosa* T1 (17) for the degradation of phenol and methyl analogs of phenol and in *P. putida* P23X1 for the degradation of 4-hydroxyphenylacetate (2), have come largely from studies of mutant strains with presumed structural or regulatory gene mutations. Detailed studies of the plasmid-encoded *meta*-cleavage pathway for the degradation of toluene, *m*-xylene, and *p*-xylene in *P. putida* mt-2 have resulted from extensive deletion and transposon insertion mutagenesis and from cloning experiments (6, 9, 11, 14).

Alcaligenes eutrophus 335 (ATCC 17697) degrades phenol and *p*-cresol via a catechol *meta*-cleavage pathway (Fig. 1), as suggested by Johnson and Stanier (12) and now confirmed by the present study. This paper reports a study of some regulatory and structural mutant strains defective in enzymes A to F (Fig. 1) of the catechol *meta*-cleavage pathway in *A. eutrophus* 335.

MATERIALS AND METHODS

Microorganisms. The bacterial strains used are listed in Table 1. *A. eutrophus* 335 was supplied by P. J. Chapman, University of Minnesota, St. Paul, Minn.

Media and culture conditions. Media and methods for cultivation have been described previously (4), except that DL-lactate (20 mM) was used as a noninducing carbon source and the period of induction was 8 to 10 h. Unless otherwise stated, all other carbon sources were used at a concentration of 2.5 mM. Sodium thioglycolate, at a concentration of 0.5% (wt/vol), was incorporated routinely into media containing 4-methylcatechol, as this retarded its autooxidation for up to 60 h. Neither the rate nor the extent of growth of *A. eutrophus* strains was affected by this addition.

Mutagenesis. *A. eutrophus* 335 was treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) as previously described (12), with the following modifications: (i) before mutagenesis, cells were grown in nutrient broth; (ii) all washings were done with minimal medium (MM) without a carbon source; (iii) cells were exposed to 100 μg of NTG per ml for 60 min; (iv) after mutagenesis, inocula of 0.1 ml were made into 10 ml of MM containing 2 mM lactate and incubated overnight, except that when auxotrophic mutants were sought, inocula of 0.1 ml were made into 10 ml of nutrient broth.

Selection for mutant strains. (i) Strains deficient in phenol hydroxylase. Mutant strains were selected by the toxic metabolite enrichment method (25, 26) with an enrichment medium containing 2 mM 4-chlorophenol and 1.5 mM phenol.

(ii) Strains deficient in catechol 2,3-oxygenase, aldehyde dehydrogenase, isomerase, decarboxylase, or benzoate oxidase. Mutants were selected by the modification of the D-cycloserine–penicillin selection method (15) described by Wigmore et al. (23) with the following modifications: (i) the selective carbon source was 2 mM lactate and the contraselective carbon source was phenol, and (ii) incubation in the presence of the contraselective carbon source before the addition of 10^5 U of penicillin G per ml and 0.4 mg of D-cycloserine per ml was done for 8 h. The contraselective carbon source for selection of benzoate oxidase mutants was benzoate.

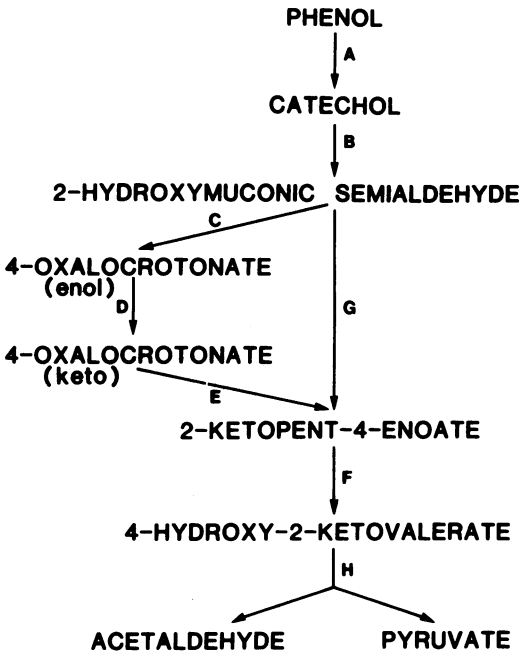

FIG. 1. *meta*-Cleavage pathway for the degradation of phenol. A, Hydroxylase; B, 2,3-oxygenase; C, aldehyde dehydrogenase; D, isomerase; E, decarboxylase; F, hydratase; G, hydrolase; H, aldolase. Enzymes A to F and H mediate the degradation of phenol and *p*-cresol in *A. eutrophus* 335; enzyme G is not detectable.

(iii) Isolation of mutant strains constitutive for catechol 2,3-oxygenase. Two methods were used for isolation of constitutive catechol 2,3-oxygenase mutants: alternate subculturing and growth on 4-methylcatechol. Alternate subculturing was based upon that described by Parke and Ornston (16). After strain 335 was subcultured alternately for 15 cycles in MM containing phenol or lactate, the cultures were screened for potential constitutive mutants by spraying single colonies on MM containing lactate with 10 mM 4-methylcatechol. Colonies which turned yellow within 30 s were retained as putative constitutive catechol 2,3-oxygenase mutants.

4-Methylcatechol cannot act as a growth substrate for strain 335, although it is capable of entering the cell as evidenced by O_2 uptake with whole-cell suspensions of strain 335 grown in the presence of either phenol or *p*-cresol. Since mutant strains constitutive for enzymes B to F, isolated by alternate subculturing, can grow on 4-methylcatechol, the following method was used to screen for such mutants. After NTG mutagenesis, the expressed culture was centrifuged and suspended in MM without a carbon source, and approximately 10^9 cells were spread onto each of 10 plates of MM containing 1 mM 4-methylcatechol and 0.5% sodium thioglycolate. Colonies arising on the medium were purified on the same medium and then screened as described above by spraying with 4-methylcatechol.

Enzyme assays. The enzyme assays were performed by previously described methods with, as substrates, unless specified otherwise, the compounds shown in parentheses: catechol 2,3-oxygenase (catechol), aldehyde dehydrogenase (2-hydroxymuconic semialdehyde), and hydrolase (2-hydroxy-6-keto-2,4-hepta-

TABLE 1. *A. eutrophus* strains

Strain	Relevant phenotype[a]	Parent strain	Derivation
335 (ATCC 17697)	Phl^+ $Pcre^+$ Ben^+		
RA1258	Phl^- $Pcre^-$[b]	335	NTG/PC[c]
RA1259	Phl^+ $Pcre^-$	RA1258	Growth on phenol
RA1260	Phl^+ $Pcre^-$	RA1258	Growth on phenol
RA1261	Phl^+ $Pcre^+$	RA1259	Growth on *p*-cresol
RA1265	Phl^+ $Pcre^+$ Ben^-	335	NTG/PC[d]
RA1270	Phl^+ $Pcre^-$	335	Toxic metabolite enrichment[e]
RA1276	Phl^- $Pcre^-$	335	As RA1258
RA1280	Phl^- $Pcre^-$	335	As RA1258
RA1281	Phl^- $Pcre^-$	335	As RA1258
RA1284	Phl^- $Pcre^-$	335	As RA1258
RA1286	Phl^- $Pcre^-$	335	As RA1258
RA1300	Phl^- $Pcre^-$	RA1303	See text
RA1303	Phl^- $Pcre^-$	335	As RA1258
RA1308	Phl^+ $Pcre^+$	RA1276	Growth on phenol
RA1344	Phl^+ $Pcre^+$	335	Growth on 4-methylcatechol
RA1345	Phl^+ $Pcre^+$	335	Alternate subculture[f]

[a] Phl, Phenol; Pcre, *p*-cresol; Ben, benzoate.
[b] All derivatives of strain 335, except RA1265, were Ben^+.
[c] PC, Penicillin/D-cycloserine enrichment; with phenol as the contraselective carbon source.
[d] With benzoate as the contraselective carbon source.
[e] Carbon sources were 4-chlorophenol and phenol.
[f] Alternate carbon sources were phenol and lactate.

dienoate) (4); hydratase (2-ketopent-4-enoate) (7); phenol hydroxylase (phenol) (3); 4-oxalocrotonate (keto) decarboxylase (4-oxalocrotonate-keto) (18). 4-Oxalocrotonate-keto-enol-isomerase was assayed as described by Sala-Trepat and Evans (18), with 4-oxalocrotonate (enol) prepared by the method of Wigmore and Bayly (21). This enzyme previously has been termed a tautomerase, but, for the reasons given by Sparnins et al. (20) and Barbour and Bayly (1), we have chosen to term it an isomerase. Assays for aldolase were not carried out. Protein concentrations were determined by the method of Lowry et al. (13).

Determination of catechol and 4-methylcatechol. The concentrations of catechol and 4-methylcatechol in the supernatant fluid of cultures were determined as described previously, with a heat-treated extract of *P. putida* grown in the presence of phenol (5).

RESULTS

Induction pattern in *A. eutrophus* 335. When strain 335 was grown on lactate in the presence of phenol or *p*-cresol, enzymes A to F (Fig. 1) were detected, but such enzymes were not at detectable levels when lactate was the sole carbon source. When *o*- and *m*-cresol and *o*-, *m*-, and *p*-fluoro- or chlorophenol were tested as potential inducers of enzymes A to F, each enzyme was induced, but the levels in all cases were only about 10% of those found when phenol or *p*-cresol was used (data not shown).

As reported earlier (R. L. Bayly, P. L. Venegas, and P. J. Chapman, Abstr. Annu. Meet. Am. Soc. Microbiol. 1974, P133, p. 166), 2-hydroxymuconic semialdehyde hydrolase (Fig. 1, G) has not been detected in extracts of phenol-grown strain 335, nor could we detect it when any of the analogs of phenol described above were used. The following compounds did not result in the induction of enzymes A to G: 4-hydroxyphenylacetate, benzoate, *p*-hydroxybenzoate, protocatechuate, *o*-, *m*-, and *p*-toluate, catechol, 3- and 4-methylcatechol. With the exception of catechol and its methyl analogs, the above-mentioned compounds failed to act as substrates for enzyme A or B, as evidenced by the absence of O_2 uptake in their presence when strain 335 was grown in the presence of phenol or *p*-cresol. We confirmed the observation of Johnson and Stanier (12) that growth of strain 335 in the presence of high (>5 mM) concentrations of benzoate results in the accumulation of 2-hydroxymuconic semialdehyde. This indicates the presence of catechol 2,3-oxygenase, although we could not detect activities of this or any other enzymes shown in Fig. 1. When a catechol 2,3-oxygenase-defective mutant strain, RA1303, was grown under the same conditions, no 2-hydroxymuconic semialdehyde was detected. It is possible that failure to detect catechol 2,3-oxygenase in strain 335 grown on benzoate was due to the fact that the level of catechol 2,3-oxygenase was too low to be detected by the assay system.

Mutant strains defective in enzymes B to E. When mutant strains RA1303, RA1280, RA1286, and RA1284 (Table 1) were grown in the presence of phenol or *p*-cresol, enzymes A to F were at wild-type levels, except for catechol 2,3-oxygenase in RA1303, aldehyde dehydrogenase in RA1280, isomerase in RA1286, and decarboxylase in RA1284 (Table 2). Each mutant strain reverted to growth on phenol at a frequency of about 10^{-7} per cells plated, and the revertants were indistinguishable phenotypically and by enzyme analysis from the wild-type strain.

Constitutive mutant strains. Enzymes B to F were detected at phenol-induced wild-type levels in crude extracts of mutant strains RA1344 and RA1345 grown on lactate (Table 3), and activities were not elevated when the strains were grown in the presence of phenol or *p*-cresol. Phenol hydroxylase was detected only when growth occurred in the presence of phenol or *p*-cresol. Both RA1344 and RA1345 utilized catechol and 4-methylcatechol as sole carbon sources.

Mutant strains with defective induction of enzymes A to F. When RA1276 was grown in the presence of phenol or *p*-cresol, hydroxylase activity was detected at wild-type-induced levels, but no activity of enzymes B to F was detected. Catechol and 4-methylcatechol accumulated in the supernatant fluid when RA1276 was grown in the presence of phenol and *p*-cresol, respectively. RA1276 reverted to growth on phenol or *p*-cresol, at a frequency of appearance of about 10^{-5} per cells plated, and such revertants were indistinguishable phenotypically and by enzyme analysis. One such revertant, RA1308, retained inducible phenol hydroxylase activity and constitutively expressed activities of enzymes B to F at wild-type phenol-induced levels (Table 4).

The mutational defect in mutant RA1258 resulted in the complete loss of activity of enzymes A to F. Revertants of this strain selected for growth on *p*-cresol appeared at a frequency of about 10^{-9} per cells plated, regained the ability to grow on phenol, had inducible enzyme synthesis at wild-type levels of enzymes A to F and were indistinguishable from the wild type. Revertants selected for growth on phenol appeared at a frequency of 10^{-6} per cells plated and were of three classes, as typified by the following revertant strains: (i) RA1261, indistinguishable phenotypically and by enzyme analysis from the wild type; (ii) RA1260, which remained unable to grow on *p*-cresol and synthesized constitutively the activities of enzymes B to F at wild-type levels—enzyme A had strict inductive and substrate specificity for phe-

TABLE 2. Specific activities of enzymes of the catechol *meta*-cleavage pathway in crude extracts of *A. eutrophus* 335 and mutants RA1303, RA1280, RA1286, and RA1284[a]

Strain	Sp act of:					
	Hydroxylase[b]	2,3-Oxygenase[c]	Dehydrogenase[c]	Isomerase[d]	Decarboxylase[e]	Hydratase[c]
335[f]	152	0.51	0.08	0.29	0.36	0.41
RA1303	127	ND[g]	0.07	0.28	0.39	0.30
RA1280	176	0.47	ND	0.36	0.42	0.49
RA1286	132	0.49	0.05	ND	0.32	0.38
RA1284	140	0.52	0.09	0.40	ND	0.26

[a] All grown on lactate in the presence of phenol.
[b] Microliters of oxygen consumed per hour per milligram (dry weight) of cells.
[c] Micromoles of product formed or substrate used per minute per milligram of protein.
[d] Decrease in absorbance at 295 nm per minute per milligram of protein.
[e] Decrease in absorbance at 350 nm per minute per milligram of protein.
[f] No activities were detected when strain 335 was grown on lactate.
[g] ND, Not detected.

nol only; (iii) RA1259, which also remained unable to grow on *p*-cresol, but, when grown in the presence of phenol, enzymes B to F were detected at 20 to 30% of wild-type-induced levels. Enzyme A had strict inductive and substrate specificity for phenol only. The enzyme activities of these strains are shown in Table 5.

Mutant strain defective in hydroxylation of *p*-cresol. When RA1270 was grown in the presence of either phenol or *p*-cresol, activities of enzymes B to F were detected at wild-type-induced levels; however, hydroxylase activity has strict inductive and substrate specificity for phenol only. Revertants selected for growth on *p*-cresol appeared at a frequency of about 10^{-8} per cell plated and were indistinguishable phenotypically and by enzyme analysis from the wild type.

Putative polarity mutant. When strain RA1281 was grown in the presence of either phenol or *p*-cresol, enzyme C was not detected, and enzymes D to F were detected at less than 5% of wild-type phenol-induced levels. Enzymes A and B were detected at wild-type-induced levels. Revertants selected for growth on phenol appeared at a frequency of approximately 10^{-6} per cells plated and were indistinguishable phenotypically and by enzyme analysis from the wild type.

Effect of *p*-toluate and benzoate on expression of enzymes A to F. When strain 335 was grown in the presence of both 2.5 mM phenol and 4 mM *p*-toluate, no activity of enzyme A was detected, and activities of enzymes B to F were 40 to 50% of wild-type phenol-induced levels (Table 6). Similar reductions in the levels of activities were obtained when RA1265, a strain defective in benzoate oxidase, was grown in the presence of both 2.5 mM phenol and 6 mM benzoate. Neither *p*-toluate (4 mM) nor benzoate (6 mM)

TABLE 3. Specific activities of enzymes of the catechol *meta*-cleavage pathway in *A. eutrophus* 335 and mutants RA1344 and RA1345[a]

Strain	Carbon source	Sp act of:					
		Hydroxylase[b]	2,3-Oxygenase[c]	Dehydrogenase[c]	Isomerase[d]	Decarboxylase[e]	Hydratase[c]
335[f]	Phenol	165	0.46	0.06	0.21	0.28	0.38
RA1344	Phenol	143	0.55	0.07	0.25	0.31	0.25
	Lactate	ND[g]	0.47	0.07	0.23	0.33	0.23
RA1345	Phenol	120	0.39	0.04	0.19	0.27	0.30
	Lactate	ND	0.42	0.05	0.21	0.28	0.29

[a] All grown on lactate or in the presence of phenol.
[b] Microliters of oxygen consumed per hour per milligram (dry weight) of cells.
[c] Micromoles of product formed or substrate used per minute per milligram of protein.
[d] Decrease in absorbance at 295 nm per minute per milligram of protein.
[e] Decrease in absorbance at 350 nm per minute per milligram of protein.
[f] No activities were detected when strain 335 was grown on lactate.
[g] ND, Not detected.

TABLE 4. Specific activities of enzymes of the catechol *meta*-cleavage pathway in *A. eutrophus* 335, mutant strain RA1276, and revertant strain RA1308[a]

Strain	Carbon source	Sp act of:						
		Hydroxylase[b]		2,3-Oxygenase[c]	Dehydrogenase[c]	Isomerase[d]	Decarboxylase[e]	Hydratase[c]
		Phenol	*p*-Cresol					
335[f]	Phenol	173	109	0.39	0.05	0.25	0.36	0.24
	p-Cresol	83	63	0.33	0.04	0.18	0.39	0.16
RA1276[f]	Phenol	158	94	ND[g]	ND	ND	ND	ND
	p-Cresol	72	80	ND	ND	ND	ND	ND
RA1308	Phenol	162	89	0.39	0.05	0.36	0.30	0.27
	p-Cresol	94	69	0.32	0.05	0.32	0.20	0.21
	Lactate	ND	ND	0.34	0.06	0.29	0.26	0.38

[a] All grown on lactate or in the presence of phenol or *p*-cresol.
[b] Microliters of oxygen consumed per hour per milligram (dry weight) of cells. Phenol and *p*-cresol were substrates.
[c] Micromoles of product formed or substrate used per minute per milligram of protein.
[d] Decrease in absorbance at 295 nm per minute per milligram of protein.
[e] Decrease in absorbance at 350 nm per minute per milligram of protein.
[f] No activities were detected when strains 335 and RA1276 were grown on lactate.
[g] ND, Not detected.

inhibited the activity of enzymes A to F in cultures of strains 335 and RA1265 grown in the presence of phenol. When strain 335 was grown in the presence of phenol or *p*-cresol together with high concentrations of acetate (>20 mM), enzyme A was not detected, but enzymes B to F were at 50 to 60% of wild-type phenol-induced levels (data not shown). When DL-lactate was substituted for acetate, the levels of enzymes A to F (Fig. 1) were the same as levels obtained in phenol-grown cells.

Growth of RA1303, defective in catechol 2,3-oxygenase activity, on MM containing lactate and phenol resulted in the colonies and medium

TABLE 5. Specific activities of enzymes of the catechol *meta*-cleavage pathway in *A. eutrophus* 335, mutant strain RA2158, and revertant strains RA1259, RA1260, and RA1261[a]

Strain[b]	Carbon source	Sp act of:						
		Hydroxylase[c]		2,3-Oxygenase[c]	Dehydrogenase[d]	Isomerase[e]	Decarboxylase[f]	Hydratase[d]
		Phenol	*p*-Cresol					
335	Phenol	173	109	0.29	0.05	0.25	0.26	0.24
	p-Cresol	86	63	0.32	0.04	0.18	0.29	0.16
RA1258	Phenol	ND[g]	ND	ND	ND	ND	ND	ND
RA1259	Phenol	125	ND	0.12	0.01	0.05	0.09	0.08
RA1260	Phenol	158	ND	0.31	0.04	0.26	0.15	0.23
	p-Cresol	ND	ND	0.46	0.04	0.20	0.18	0.20
	Lactate	ND	ND	0.31	0.05	0.17	0.21	0.19
RA1261	Phenol	129	72	0.49	0.06	0.37	0.45	0.48
	p-Cresol	63	52	0.35	0.05	0.23	0.38	0.32

[a] All grown on lactate or in the presence of phenol or *p*-cresol.
[b] Activities were not detected when strains 335, RA1258, RA1259, and RA1261 were grown on lactate and when strains RA1258 and RA1259 were grown in the presence of *p*-cresol.
[c] Microliters of oxygen consumed per hour per milligram (dry weight) of cells. Phenol and *p*-cresol were substrates.
[d] Micromoles of product formed or substrate used per minute per milligram of protein.
[e] Decrease in absorbance at 295 nm per minute per milligram of protein.
[f] Decrease in absorbance at 350 nm per minute per milligram of protein.
[g] ND, Not detected.

TABLE 6. Percentage activities of enzymes of the catechol *meta*-cleavage pathway in *A. eutrophus* 335 and RA1265[a]

Strain[b]	Carbon source	% Activity (sp act) of:					
		Hydroxylase[c]	2,3-Oxygenase[d]	Dehydrogenase[d]	Isomerase[e]	Decarboxylase[f]	Hydratase[d]
335	Phenol	100 (162)	100 (0.49)	100 (0.08)	100 (0.30)	100 (0.34)	100 (0.39)
	Phenol + 4 mM *p*-toluate	0	53	51	40	47	45
RA1265	Phenol	100 (174)	100 (0.33)	100 (0.05)	100 (0.57)	100 (0.40)	100 (0.41)
	Phenol + 6 mM benzoate	7	39	42	33	41	35

[a] All grown in the presence of phenol with or without the addition of either *p*-toluate or benzoate.
[b] No activity of catechol 1,2-oxygenase or catechol 2,3-oxygenase was detected when strains 335 and RA1265 were grown in the presence of *p*-toluate and benzoate, respectively.
[c] Microliters of oxygen consumed per hour per milligram (dry weight) of cells.
[d] Micromoles of product formed or substrate used per minute per milligram of protein.
[e] Decrease in absorbance at 295 nm per minute per milligram of protein.
[f] Decrease in absorbance at 350 nm per minute per milligram of protein.

turning black, and this was shown to be due to the accumulation of catechol. Increasing concentrations of *p*-toluate (0 to 4 mM) in the above-mentioned medium resulted in a decrease in the extent of blackening, and this was consistent with a reduction in the level of phenol hydroxylase activity. Since mutant strains resistant to repression by *p*-toluate could be expected to express phenol hydroxylase constitutively, such mutant strains were screened for as follows. An expressed, mutagenized culture of strain RA1303 was plated onto MM containing lactate, phenol, and 4 mM *p*-toluate to give 100 to 150 colonies per plate. Under these growth conditions, catechol would not accumulate due to repression of phenol hydroxylase; therefore, any colonies that were black may express phenol hydroxylase activity constitutively. Putative *p*-toluate repression-resistant mutants appeared at a frequency of 10^{-5}, and each was indistinguishable phenotypically and by enzyme analysis; RA1300 is a representative of this group. Activities of enzymes A to F in RA1300 were inducible to wild-type-induced levels when the strain was grown in the presence of phenol with or without *p*-toluate but were not at detectable levels when RA1300 was grown solely on lactate or in the presence of either *p*-cresol or *p*-toluate. Phenol hydroxylase had activity against phenol only.

DISCUSSION

Results show that enzymes A to F (Fig. 1) were induced coincidentally when *A. eutrophus* 335 was grown in the presence of either phenol or *p*-cresol. This mode of induction has been shown in catechol *meta*-cleavage pathways in *P. putida* U (24) and *P. aeruginosa* T1 (17) for the degradation of phenol and in *P. putida* mt-2 (29) and MT20 (27, 30) for the degradation of toluene. Coincident synthesis of enzymes A to E induced in the presence of either phenol or *p*-cresol was confirmed by studies with mutants separately blocked in each of these enzymes and with mutant strains RA1344 and RA1345, which synthesized constitutively catechol 2,3-oxygenase and enzymes C to F. The properties of RA1281 are similar to those of *P. putida* putative polarity mutants described in studies on the *meta*-degradative pathways of phenol (22) and of 4-hydroxyphenylacetic acid (2). The loss of aldehyde dehydrogenase activity and reduction in activities of enzymes D to F to less than 5% of wild-type phenol-induced levels in strain RA1281 is consistent with a polar mutation at or near the aldehyde dehydrogenase structural gene. This would place the structural genes encoding enzymes C to F within one operon. The inclusion of the catechol 2,3-oxygenase structural gene within this operon seems reasonable on the basis of studies with the constitutive mutants and the coordinate repression of enzymes B to F by *p*-toluate, benzoate, or acetate. The present data do not allow an order to be ascribed to the genes encoding enzymes B to F.

The mutational defect in RA1270 resulted in a loss of phenol hydroxylase activity against the methyl-, fluoro-, and chloro- analogs of phenol in cells grown in the presence of phenol or *p*-cresol. Activity of phenol hydroxylase against phenol and activities of enzymes B to F were at wild-type-induced levels. Such a phenotype could have resulted from one of two events: diminished affinity of phenol hydroxylase to substituted phenols or a mutation resulting in loss of a phenol hydroxylase and leaving a hydroxylase with strict substrate specificity for phenol only. This differs from that found by Wigmore et al. (24) in *P. putida* U and by Ribbons (17) in *P. aeruginosa* T1. Their phenol

hydroxylase-deficient strains contraselected against growth on phenol resulted in loss of hydroxylase activity against phenol and the isomers of cresol.

The mutational defect in RA1258 resulted in a complete loss in activities of enzymes A to F when the strain was grown in the presence of either phenol or *p*-cresol. The constitutive nature of some revertants of RA1258 is inconsistent with repair of a lesion within a permease structural gene, but rather the original lesion is within a single regulatory gene controlling all six enzymes. A single regulatory gene controlling all catechol *meta*-cleavage enzymes has been proposed by Wigmore and Bayly (21) on the basis of mutant L2 of *P. putida* U in which a mutation results in the reduced expression of all catechol *meta*-cleavage enzymes.

The mutational defect in strain RA1276, in which activities of enzymes B to F were lost, may have resulted from a lesion at or near a regulatory region of the 2,3-oxygenase operon. Revertants constitutively expressed enzymes B to F to wild-type phenol-induced levels, and we consider this expression consistent, as suggested for the lactose system in *Escherichia coli* by Willson et al. (28), with the 2,3-oxygenase operon being under negative control. This control is different for *meta*-cleavage pathways in *P. putida* mt-2 for the degradation of toluene (10, 11, 29) and in *P. putida* P23X1 (2) for the degradation of 4-hydroxyphenylacetate, for which the 2,3-oxygenase operons are reported to be under positive control. Revertants of noninducible mutants of the arabinose (19) and amidase (8) systems were consistent with these systems being under positive control. Revertants of RA1258 all regained fully inducible phenol hydroxylase activity and hence may be under positive control. The model for the regulation of the catechol *meta*-cleavage pathway in *P. putida* U proposed by Wigmore et al. (24) has similarities to strain 335: (i) separation of structural genes encoding phenol hydroxylase activity and enzymes B to F into different regulatory regions, (ii) evidence suggesting that at least one regulatory gene exerts a controlling effect over the expression of all *meta*-pathway enzymes, and (iii) evidence suggesting that the 2,3-oxygenase operon is under negative control and hydroxylase gene(s) are under positive control.

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