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Evidence for Isofunctional Enzymes Used in *m*-Cresol and 2,5-Xylenol Degradation via the Gentisate Pathway in *Pseudomonas alcaligenes*

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Study of the reaction sequence by which *Pseudomonas alcaligenes* (P25X1) and derived mutants degrade *m*-cresol, 2,5-xylenol, and their catabolites has provided indirect evidence for the existence of two or more isofunctional enzymes at three different steps. Maleylpyruvate hydrolase activity appears to reside in two different proteins with different specificity ranges, one of which (MPH1) is expressed constitutively; the other (MPH11) is strictly inducible. Two gentisate 1,2-dioxygenase activities were found, one of which is constitutively expressed and possesses a broader specificity range than the other, which is inducible. From oxidation studies with intact cells, there appear to be two activities responsible for the 6-hydroxylation of 3-hydroxybenzoate, and again a broadly specific activity is present regardless of growth conditions; the other is inducible by 3-hydroxybenzoate. Three other enzyme activities are also detected in uninduced cells, viz., xylenol methylhydroxylase, benzylalcohol dehydrogenase, and benzaldehyde dehydrogenase. All apparently possess broad specificity. Fumarylpyruvate hydrolase was also detected but only in cells grown with *m*-cresol, 3-hydroxybenzoate, or gentisate. Mutants, derived either spontaneously or after treatment with mitomycin C, are described, certain of which have lost the ability to grow with *m*-cresol and 2,5-xylenol and some of which have also lost the ability to form the constitutive xylenol methylhydroxylase, benzylalcohol dehydrogenase, benzaldehyde dehydrogenase, 3-hydroxybenzoate 6-hydroxylase, and gentisate 1,2-dioxygenase. Such mutants, however, retain ability to synthesize inducibly a second 3-hydroxybenzoate 6-hydroxylase and gentisate 1,2-dioxygenase, as well as maleylpyruvate hydrolase (MPH11) and fumarylpyruvate hydrolase; MPH1 was still synthesized. These findings suggest the presence of a plasmid for 2,5-xylenol degradation which codes for synthesis of early degradative enzymes. Other enzymes, such as the second 3-hydroxybenzoate 6-hydroxylase, gentisate 1,2-dioxygenase, maleylpyruvate hydrolase (MPH1 and MPH11), and fumarylpyruvate hydrolase, appear to be chromosomally encoded and, with the exception of MPH1, strictly inducible.

The microbial degradation of *m*-cresol, 2,5-xylenol, and 3,5-xylenol to gentisic acid, 4-methylgentisic acid, and 3-methylgentisic acid, respectively, by two different species of *Pseudomonas* has been described by Hopper and Chapman (11). It was reported that alkyl-substituted gentisates were metabolized to the corresponding alkylmaleylpyruvates that were metabolized further by a maleylpyruvate hydrolase activity (maleylpyruvase [13]) to citraconate and pyruvate (13). Early reactions of the pathway used for the alkyl-substituted compounds appeared analogous to that shown for gentisate degradation by Hopper et al. (12), which differed from that reported by Lack (16), who found that maleylpyruvate was isomerized to fumarylpyruvate before hydrolysis to fumarate and pyruvate. The pathway established by Hopper et al. (11, 13) is shown in Fig. 1.

The work of Hopper et al. (11, 13) suggested that the degradative sequence was catalyzed by a series of enzymes of broad substrate specificity. This report presents evidence to show that, in one of the species studied by Hopper and his coworkers, *Pseudomonas* NCIB 9867, three of the six reactions leading to maleic acid or its corresponding alkyl analog (Fig. 1) are catalyzed by separate sets of isofunctional enzymes.

MATERIALS AND METHODS

Organism and method of cultivation. The organism used was a nonfluorescent species of *Pseudomonas* which has been described by Hopper and Chapman (11). It was characterized as a strain of *Pseudomonas alcaligenes* (M. Rhodes, personal communication) and designated as NCIB 9867; it will be referred to as P25X1. The strain was maintained on a basal salts medium that contained 2.5 mM 2,5-xylenol and grown at 30°C. The basal medium used was the min-

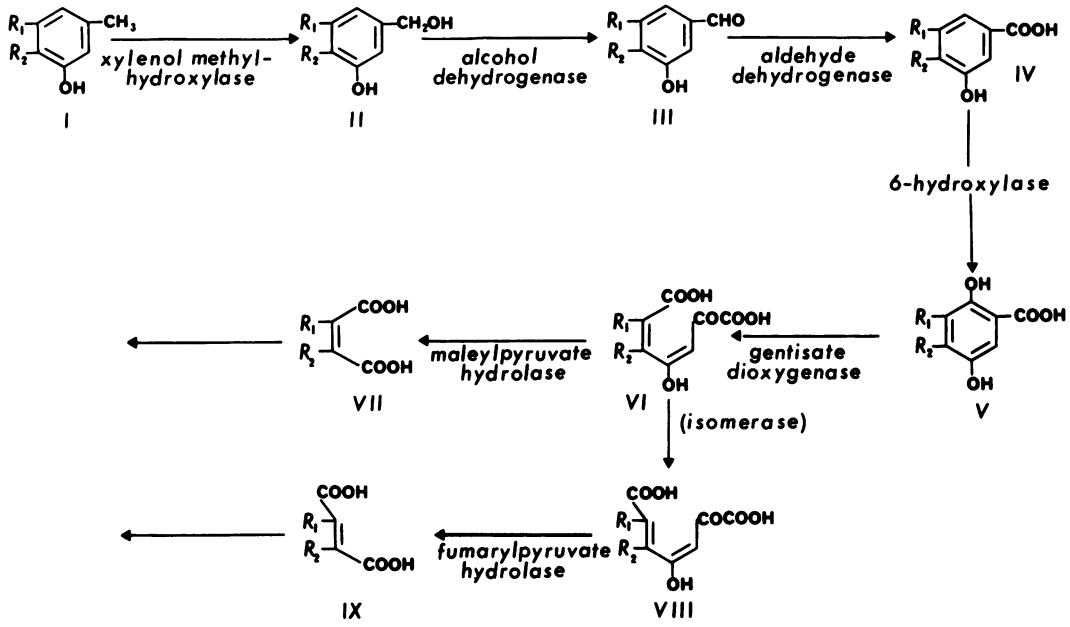


FIG. 1. *The degradation of 2,5-xylenol, 3,5-xylenol, and m-cresol by P. alcaligenes NCIB 9867. R_1 = H, R_2 = H. I, m-Cresol; II, 3-hydroxy-benzylalcohol; III, 3-hydroxy-benzaldehyde; IV, 3-hydroxybenzoic acid; V, gentisic acid; VI, maleylpyruvic acid; VII, maleic acid; VIII, fumarylpyruvic acid; IX, fumaric acid. R_1 = H, R_2 = CH_3. I, 2,5-Xylenol; II, 3-hydroxy-4-methylbenzylalcohol; III, 3-hydroxy-4-methylbenzaldehyde; IV, 3-hydroxy-4-methylbenzoic acid; V, 4-methylgentisic acid; VI, 5-methylmaleylpyruvic acid; VII, citraconic acid. R_1 = CH_3, R_2 = H. I. 3,5-Xylenol; II, 3-Hydroxy-5-methylbenzylalcohol; III, 3-hydroxy-5-methylbenzaldehyde; IV, 3-hydroxy-5-methylbenzoic acid; V, 3-methylgentisic acid; VI, 6-methylmaleylpyruvic acid; VII, citraconic acid.*

eral salts base of Hegeman (10) with appropriate addition of sources of carbon. When an aromatic compound was used either as a sole source of carbon or as an inducer, it was added to a final concentration of 2.5 mM. Fumarate, pyruvate, L-malate, succinate, and asparagine were all used at 10 mM, and D,L-lactate was used at 20 mM. The final pH of the medium was 7.4. Nutrient broths were prepared from nutrient broth (no. 2, Oxoid Ltd., London, England), and solid media were prepared by the addition of 1.2% agar (Difco Laboratories, Detroit, Mich.) to the liquid media.

Cells used for the detection of enzyme activities were grown in 500 ml of medium in 2-liter flasks in an orbital incubator (200 rpm), and growth was followed turbidimetrically at 580 nm in a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.); cells grown on a sole carbon source were harvested in the late exponential phase. For induction experiments, cells were grown to early exponential phase (absorbance, 0.1; 580 nm) in basal medium containing lactate, at which time the inducer was added, and incubation continued for 3 to 4 h when the absorbance was 0.5 to 0.7. Cells were collected and washed twice with cold 0.05 M Tris-hydrochloride buffer (pH 7.4).

Preparation of cell extracts. Washed cells were suspended in 2 volumes of 0.05 M Tris-hydrochloride buffer (pH 7.4) and disrupted in a French pressure cell (American Instrument Co., Inc., Silver Spring, Md.) under a pressure of 10,000 lb/in^2. Cell debris was removed by centrifugation at 26,000 × *g* for 40 min at 2 to 4°C, and the cell extract was kept in an ice bath until assayed on the same day; this will be referred to as crude extract. When enzyme assays were to be carried out in the presence of either NADH (reduced form) or NADPH (reduced form), crude extracts were centrifuged at 100,000 × *g* for 90 min; these will be referred to as ultracentrifuged extracts. Protein concentration in cell extracts was determined by the method of Lowry et al. (17) with bovine albumin as a standard.

Mutagenesis and selection of mutant strains. Strains subsequently found to be defective in xylenol hydroxylase were obtained by mutagenesis with ethylmethane sulfonate (EMS), as described by Carhart and Hegeman (3), and were selected by plating onto a basal medium that contained 0.2% D-(−)-fructose and 2.5 mM 2,5-xylenol and incubated for 72 h. Small colonies were transferred to nutrient agar and then replicated onto basal medium containing 2.5 mM 2,5-xylenol and basal medium containing 20 mM lactate. Colonies that grew on lactate but not 2,5-xylenol were selected for further study. Other classes of mutant strains unable to grow on 2,5-xylenol were selected by patching colonies that had grown on basal medium containing lactate onto basal medium containing 2.5 mM 2,5-xylenol. Colonies that failed to grow on 2,5-xylenol after 4 days of incubation were selected. Additional mutant strains were obtained by treatment with mitomycin C (0.1 μg/ml), as described by Chakrabarty (4), and were selected for their inability to

grow on 2,5-xylenol, while retaining the ability to grow on lactate. Further derivatives of certain mutant strains were obtained by mutagenesis with EMS and selection by a modification of the penicillin-D-cycloserine method (19) described by Wigmore et al. (21). The selective carbon source was lactate, and the counterselective carbon source was either 3-hydroxybenzoate or gentisate. The phenotypic properties of the wild-type strain (P25X1) and the mutant strains derived from it, together with the methods by which they were obtained, are shown in Table 1.

Isolation of revertants of mutant strains. The strains were grown overnight in nutrient broth, washed twice with sterile basal medium, plated onto basal medium containing 2.5 mM 2,5-xylenol, and incubated for 4 days. Spontaneous reversion to wild-type phenotype was obtained from mutant strain P25X2 at a frequency of about 1 per 10^9 cells plated. Despite repeated attempts, spontaneous revertants could not be obtained from either P25X3, -5, -7, or -8.

Chemicals. The following alkyl- and halogen-substituted gentisic acids were prepared as described previously: 3-methyl-, 4-methyl-, 3-ethyl- and 3,4-dimethylgentisate (11); 3-chloro-, 4-chloro-, 4-fluoro- and 3-bromogentisate (7); 3-isopropylgentisate (13).

Maleylpyruvate and its analogs (Fig. 1, VI) were prepared by oxidation of the corresponding gentisate with gentisate 1,2-dioxygenase that was free from maleylpyruvate hydrolase and fumarylpyruvate hydrolase. (One systematic name for maleylpyruvate is *cis*-2,4-diketo-hept-5-ene-1,7-dioic acid, and its derivatives would require similarly long designations. For simplicity we have chosen to show this compound and its analog by reference to a common structural formula with R_1 and R_2 substituents at C_5 and C_6, respectively [see Table 7]. By this means, the ring fission product of 3-methylgentisate is described as 6-methylmaleyl pyruvate and that from 4-methylgentisate as 5-methylmaleyl pyruvate.) For oxidation of gentisate and its 3-alkyl-substituted analogs, the source of gentisate 1,2-dioxygenase was salicylate-grown cells of bacterium OA3 (7). (The designation of this organism as a strain of *Moraxella osloensis* in an earlier publication [7] appears to be incorrect. Attempts to repeat the transformation assay reported earlier [7] have been unsuccessful, and no transformation of Trp auxotrophs of either *M. osloensis* or *Acinetobacter calcoaceticus* has been obtained [Juni, personal communication]. Other attempts to repeat the work were also unsuccessful (Chapman, personal communication). The organism is a gram-negative, oxidase-positive, nonmotile bacillus and will be referred to as bacterium OA3.) Crude extract was brought to 70% saturation with ammonium sulfate, and the resultant precipitate was suspended in 0.1 M Tris-hydrochloride buffer (pH 7.4). For oxidation of other analogs of gentisate, gentisate 1,2-dioxygenase was prepared from 3-hydroxybenzoate-grown cells of P25X1, as described by Hopper et al. (13). To 20 μmol of the gentisate to be oxidized in 20 ml of 0.1 M Tris-hydrochloride buffer (pH 7.4) was added gentisate 1,2-dioxygenase (approximately 20 mg of protein). The reaction mixture was shaken at 30°C for 15 min and brought to pH 2 with perchloric acid (6% vol/vol), the resultant precipitate was removed by centrifugation, and the supernatant fluid was adjusted to pH 7.2 with sodium hydroxide. Solutions stored at 2 to 4°C were stable for 2 to 3 days.

The wavelengths of maximum absorption and molar extinction coefficients of maleylpyruvate and its analogs are shown in Table 2. These values were obtained as follows: to 2.8 ml of 0.1 M Tris-hydrochloride buffer (pH 7.4) in a Clark oxygen electrode was added 0.1 ml of activated gentisate dioxygenase (13) and 0.8 μmol of gentisate or the required gentisate analog. After oxygen uptake had ceased, the wavelength of maximum absorption of the reaction mixture was determined, and molar extinction coefficients were calculated on the basis that 1 mol of product was accumulated per mol of substrate oxidized. The value obtained for maleylpyruvate by this method (E = 12,500) correlates well with the value of E = 13,000 reported by Lack (16).

Fumarylpyruvate was prepared by acid isomerization of maleylpyruvate (16). Immediately before use in assays, the solution of fumarylpyruvic acid was brought to pH 7.4 with sodium hydroxide. Although hydrogen ions catalyzed the *cis-trans* isomerization of maleylpyruvate to fumarylpyruvate, there was no indication that either alkyl- or halogen-substituted analogs of maleylpyruvate underwent a similar isomerization.

All other chemicals were from commercial sources and were purified as deemed necessary.

Enzyme assays. All spectrophotometric assays were carried out at 23°C in silica cuvettes of 1-cm path-length with a Hitachi-124 recording spectrophotometer. Assays involving uptake of oxygen were carried out with a Clark oxygen electrode held at 30°C

TABLE 1. *Derivation and phenotypic properties of P25X1 and mutant strains*

Strain	Immediate parent	Mutagen used	Growth substrate: Lactate	2,5-Xylenol, 3,5-xylenol, *m*-cresol	3-Hydroxy-4-methylbenzoate	3-Hydroxybenzoate	Gentisate	*p*-Cresol
P25X1			+	+	+	+	+	+
P25X2	P25X1	EMS	+	−	+	+	+	+
P25X3	P25X1	Spontaneous	+	−	−	+	+	+
P25X5	P25X1	Mitomycin C	+	−	−	+	+	+
P25X7	P25X3	EMS	+	−	−	−	−	+
P25X8	P25X1	Spontaneous	+	−	−	+	+	+

TABLE 2. *Molar extinction coefficients for the products of ring-cleavage of gentisic acid and some alkyl- and halogen-substituted analogs*

Substrate[a]	Reaction product	
	Absorption maximum (nm) at pH 7.4	Molar extinction coefficient
Gentisate	330	12,500
3-Methylgentisate	327	11,900
4-Methylgentisate	316	11,400
3-Ethylgentisate	327	11,200
3-Isopropylgentisate	325	11,200
3-Bromogentisate	335	13,000
3-Chlorogentisate	335	14,000
3-Fluorogentisate	331	11,700

[a] When 4-chloro-, 4-fluoro- and 3,4-dimethylgentisate were used as substrates, the reaction products usually decomposed rapidly. The respective values obtained in a single determination only were: 4-chlorogentisate, 335 nm, 12,400; 4-fluorogentisate, 337 nm, 11,800; 3,4-dimethylgentisate, 330 nm, 9,700.

(Rank Brothers, Bottisham, Cambridge, England) as described by Bayly and McKenzie (2). Gentisate 1,2-dioxygenase (EC 1.13.11.4) in crude cell extracts was assayed by measurement of the rate of uptake of oxygen in the presence of the assay substrate. Before assay, crude cell extracts were incubated with ferrous ammonium sulfate (10^{-3} M) for 10 min at 23°C. Reaction mixtures contained 0.25 μmol of substrate in 3 ml of 0.1 M Tris-hydrochloride buffer (pH 7.4), and the reaction was initiated by the addition of crude cell extract. Specific activities were calculated on the basis that 1 mol of gentisate (or its analogs) was oxidized per 1 mol of oxygen consumed.

Maleylpyruvate hydrolase activity was determined by following the decrease in absorbance at the λ_{max} of the substrate used (Table 2). Reaction mixtures contained approximately 0.3 μmol of substrate in 3 ml of 0.1 M Tris-hydrochloride buffer (pH 7.4), and the reaction was initiated by the addition of enzyme. Specific activities were calculated with the molar extinction coefficients shown in Table 2. Fumarylpyruvate hydrolase activity was assayed by following the decrease in absorbance at 340 nm of a reaction mixture that contained approximately 0.3 μmol of fumarylpyruvate in 3 ml of Tris-hydrochloride buffer (pH 7.4). Specific activity was calculated with E = 9,400 (20).

Uptake of oxygen when either 2,5-xylenol, 3,5-xylenol, *m*-cresol, 3-hydroxybenzoate, or 3-hydroxy-4-methylbenzoate was substrate was determined in reaction mixtures that contained 2.8 ml of 0.1 M sodium-potassium phosphate buffer (pH 7.4), 100 μl of 0.01 M substrate, and 100 μl of washed cell suspension. The NAD^+-dependent alcohol dehydrogenase was assayed spectrophotometrically by following the reduction of NAD^+ at 340 nm. 3-Hydroxy-benzylalcohol was used routinely as the assay substrate since the true intermediate of 2,5-xylenol metabolism, 3-hydroxy-4-methylbenzylalcohol, was unavailable. The reaction mixture contained in 1 ml, 0.7 ml of 0.05 M Tris-hydrochloride buffer (pH 7.6), 1 μmol of NAD^+, 1 μmol of 3-hydroxy-benzylalcohol, and ultracentrifuged extract. The $NADP^+$-dependent aldehyde dehydrogenase was assayed similarly, except that $NADP^+$ replaced NAD^+, and the substrate was 3-hydroxy-benzaldehyde. 3-Hydroxy-4-methylbenzaldehyde, the true intermediate of 2,5-xylenol metabolism, was unavailable. Specific activities of both the preceding enzymes are expressed as units per milligram of protein where 1 unit corresponds to the reduction of 1 μmol per min of NAD^+ or $NADP^+$, respectively. An extinction coefficient of 6.22 $\times 10^3$ mol^{-1} cm^{-1} for NAD(P)H at 340 nm was used.

RESULTS

Constitutive synthesis of enzymes. When P25X1 was grown on lactate as a sole source of carbon, the following enzyme activities of the gentisate pathway were detected: xylenol methylhydroxylase, alcohol dehydrogenase, aldehyde dehydrogenase, gentisate 1,2-dioxygenase, maleylpyruvate hydrolase. Oxygen uptake occurred immediately when either 3-hydroxybenzoate or 3-hydroxy-4-methylbenzoate was used as a substrate. The levels of these activities are shown in Tables 3–7 where activities of these enzymes from cells grown on various carbon sources are also shown. Similar results were obtained when lactate was replaced by either fumarate, pyruvate, succinate, L-malate, or asparagine, or when nutrient broth was the growth medium.

Synthesis of xylenol methylhydroxylase. When either 2,5-xylenol, 3,5-xylenol, or *m*-cresol was used as the sole carbon source, the activity of xylenol methylhydroxylase, as determined by oxygen uptake with whole cells in the presence of 2,5-xylenol, increased by two- to threefold above the constitutive level (Table 3). Growth on 3-hydroxy-4-methylbenzoate resulted in a similar increase in activity to that observed when 2,5-xylenol was the growth substrate, whereas with cells grown on 3-hydroxybenzoate, the activity was no greater than that seen with lactate. Cells grown on *p*-cresol oxidized the growth substrate at a high rate but attacked the other assay substrates tested at a similar rate to lactate-grown cells.

When mutant strain P25X2 was grown on lactate in the presence of either 2,5-xylenol, 3,5-xylenol, or *m*-cresol, no activity towards any of those compounds was detected, but all other enzymes shown in Fig. 1 were present at about the same level as in P25X1 when lactate was the sole source of carbon. Lactate-grown cells of P25X2 also oxidized *p*-cresol at about the same rate as did wild-type cells grown on lactate. Cells of P25X2 grown on either 3-hydroxybenzoate or 3-hydroxy-4-methylbenzoate failed to oxidize either 2,5-xylenol, 3,5-xylenol, or *m*-cresol, whereas *p*-cresol-grown cells oxidized *p*-cresol at

a high rate, but no oxidation of either 2,5-xylenol, 3,5-xylenol, or *m*-cresol was observed. Revertant strains of P25X2 isolated on either 2,5- or 3,5-xylenol grew on both these compounds and also on *m*-cresol, and such strains were indistinguishable from P25X1.

Synthesis of alcohol- and aldehyde-dehydrogenases. When P25X1 was grown on either 2,5-xylenol, *m*-cresol, 3-hydroxybenzoate, 3-hydroxy-4-methylbenzoate, 3-hydroxy-benzylalcohol, or 3-hydroxy-benzaldehyde, the activities of the two dehydrogenases were approximately the same as that observed in lactate-grown cells (Table 4).

The ultracentrifuged extracts also attacked benzylalcohol and 4-hydroxy-benzylalcohol, and aldehyde dehydrogenase activity towards benzaldehyde was also observed. No activity was detected against 4-hydroxy-benzaldehyde.

As shown in Table 4, the ratio of attack on the two aldehyde substrates did not vary with the carbon source used for growth, and this was also the situation when benzylalcohol and 3-hydroxy-benzylalcohol were the assay substrates. However, when 4-hydroxy-benzylalcohol was the assay substrate, a dehydrogenase activity was found whose activity was dependent on the growth substrate, lactate, 2,5-xylenol- and *m*-cresol-grown cells giving a different ratio of activity to cells grown on either 3-hydroxybenzoate, 3-hydroxy-4-methylbenzoate, 3-hydroxybenzylalcohol, or 3-hydroxy-benzaldehyde.

When P25X5 and P25X8 were examined under the same conditions as for P25X1 (Table 4), no activity of either dehydrogenase was detected, whereas in P25X3 the activities of both enzymes were about the same as in wild type.

Formation of enzymes catalyzing oxygen uptake with 3-hydroxybenzoate and 3-hydroxy-4-methylbenzoate. To determine whether both a 6-hydroxylase and a 4-hydroxylase activity against 3-hydroxybenzoate (9, 18) were synthesized by P25X1, ultracentrifuged extracts were assayed separately in the presence of either NADH or NADPH. These extracts catalyzed the uptake of oxygen with the two substrates when either NADH or NADPH was present, the rates of attack being slightly lower with NADPH. Using *m*-cresol-grown cells of P25X1, Hopper and Chapman (11) identified gentisate and 4-methylgentisate as metabolites of *m*-cresol and 2,5-xylenol, respectively. Formation of these compounds would require that their respective precursors, 3-hydroxybenzoate and 3-hydroxy-4-methylbenzoate, were hydroxylated at the C_6 position. Protocatechuate was not detected as a metabolite of *m*-cresol (Chapman, personal communication). Although the

TABLE 3. *Rates of oxidation of 2,5-xylenol, 3,5-xylenol, and m-cresol by cell suspensions of P25X1 grown on various carbon sources*[a]

Assay substrate	Growth substrate					
	Lactate	2,5-Xylenol	3,5-Xylenol	*m*-Cresol	3-Hydroxy-benzoate	3-Hydroxy-4-methylbenzoate
2,5-Xylenol	149	318	224	375	90	394
3,5-Xylenol	122	300	257	438	125	315
m-Cresol	46	74	113	271	76	140

[a] Values, corrected for endogenous oxidation, are expressed as microliters of oxygen per milligram (dry weight) of cells per hour.

TABLE 4. *Comparative specific activities of alcohol and aldehyde dehydrogenases synthesized by P25X1 grown on various carbon sources*

Assay substrate	Carbon source (sp act[a])						
	Lactate	2,5-Xylenol	*m*-Cresol	3-Hydroxybenzoate	3-Hydroxy-4-methylbenzoate	3-Hydroxy-benzylalcohol	3-Hydroxy-benzaldehyde
Benzylalcohol	1 (0.126)	1 (0.064)	1 (0.063)	1 (0.108)	1 (0.082)	1 (0.115)	1 (0.141)
3-Hydroxy-benzylalcohol	0.9	1.4	1.4	1.1	1.2	1.2	1.5
4-Hydroxy-benzylalcohol	19.9	15.3	15.4	6.7	9.3	5.8	6.5
Benzaldehyde	1 (0.124)	1 (0.059)	1 (0.042)	1 (0.083)	1 (0.042)	1 (0.131)	1 (0.159)
3-Hydroxy-benzaldehyde	0.30	0.34	0.36	0.30	0.29	0.37	0.32

[a] Expressed as the ratio of specific activity against benzylalcohol and benzaldehyde, respectively. Figures in parentheses are the specific activities expressed as units mg protein^{-1}. No activity was detected when 4-hydroxybenzaldehyde was used as substrate.

uptake of oxygen against 3-hydroxybenzoate (Table 5) could be attributed to hydroxylation at either C_4 or C_6, the isolation of gentisate as a metabolite makes it highly unlikely that the activity found was due to hydroxylation at C_6. Table 5 shows the rates of oxygen uptake when P25X1 was grown on different sources of carbon. Uptake of oxygen occurred when either 3-hydroxybenzoate or 3-hydroxy-4-methylbenzoate was an assay substrate, but the ratio of attack on these compounds was dependent upon the carbon source utilized for growth.

When the mutant strains P25X3 and P25X5 were grown on lactate or exposed to either 2,5-xylenol, *m*-cresol, or 3-hydroxy-4-methylbenzoate, no oxygen uptake was detected with either assay substrate, whereas cells grown on 3-hydroxybenzoate gave oxygen uptake with 3-hydroxy-4-methylbenzoate at 12 to 14% of the rate obtained with 3-hydroxybenzoate (Table 5). When P25X7, a mutant strain derived from P25X3, was examined under the same conditions, no activity against either assay substrate was detected.

Gentisate 1,2-dioxygenase. The ratio of specific activities of gentisate 1,2-dioxygenase synthesized when P25X1 was grown on different sources of carbon and assayed with several substrates is shown in Table 6. No gentisate dioxygenase activity was detected in lactate-grown cells of P25X3 and P25X5 or when these strains were exposed to either 2,5-xylenol, *m*-cresol, or 3-hydroxy-4-methylbenzoate. Not shown in Table 6 are the data for cells of P25X7. No activity for the tabulated test substrates could be detected when this strain was grown on lactate in the presence of either 3-hydroxybenzoate or gentisate.

When P25X3 and P25X5 were grown in the presence of either 3-hydroxybenzoate or gentisate, the ratio of activities against the substrates tested differed from that observed with P25X1. No activity was detected against either 4-chloro- or 4-methylgentisate, but the ratio of oxidation of 3-fluorogentisate compared with that of gentisate had increased.

Maleylpyruvate hydrolase. Growth of P25X1 on lactate resulted in activity against maleylpyruvate and several of its analogs (Table 7). Similar results were obtained when lactate was replaced by either fumarate, L-malate, pyruvate, succinate, or asparagine and when nutrient broth was the growth medium. After growth on either 2,5-xylenol or 3-hydroxy-4-methylbenzoate, activity was observed against the same substrates in a ratio similar to that obtained with nonaromatic compounds, such as lactate.

When 3-hydroxybenzoate was the carbon source, maleylpyruvate hydrolase activity against the same substrates was present but in ratios differing from those found with the other growth substrates (Table 7). The largest differences were seen with 6-bromo-, 5-chloro-, 6-methyl-, and 6-chloromaleylpyruvates. Similar results were obtained when either *m*-cresol or gentisate was the carbon source. These results suggested that two maleylpyruvate hydrolases were synthesized, and this has been confirmed by characterization of the two enzymes that will be described elsewhere. (Manuscripts in prepa-

TABLE 5. *Comparative rates of oxidation of 3-hydroxybenzoate and 3-hydroxy-4-methylbenzoate*[a]

Assay substrate and strain used	Carbon source used as growth substrate or inducer				
	Lactate	*m*-Cresol	3-Hydroxy-benzoate	2,5-Xylenol	3-Hydroxy-4-methyl-benzoate
A. P25X1					
3-Hydroxybenzoate	1 (51)	1 (281)	1 (82)	1 (51)	1 (70)
3-Hydroxy-4-methylbenzoate	2.35	0.65	0.14	3.12	3.21
B. P25X3					
3-Hydroxybenzoate	ND	ND[b]	1 (170)	ND[b]	ND[b]
3-Hydroxy-4-methylbenzoate	ND	ND[b]	0.13	ND[b]	ND[b]
C. P25X5					
3-Hydroxybenzoate	ND	ND[b]	1 (121)	ND[b]	ND[b]
3-Hydroxy-4-methylbenzoate	ND	ND[b]	0.12	ND[b]	ND[b]
D. P25X7					
3-Hydroxybenzoate	ND	ND[b]	ND[b]	ND[b]	ND[b]
3-Hydroxy-4-methylbenzoate	ND	ND[b]	ND[b]	ND[b]	ND[b]

[a] By cell suspensions of P25X1 and some mutant strains when grown on, or exposed to, different sources of carbon. Figures in brackets are absolute values expressed as microliters of oxygen per milligram (dry weight) cells per hour and are corrected for endogenous uptake. ND, No activity detected.

[b] Exposed to the carbon source.

ration). The influence of the growth substrate on the specificity of the maleylpyruvate hydrolase activity, expressed as the ratio of activity of 6-bromomaleylpyruvate to maleylpyruvate, was examined by transferring washed cells of lactate-grown P25X1 to separate flasks of basal medium that contained either (i) lactate or (ii) 3-hydroxybenzoate. After 6 h of incubation, crude extract of the lactate culture showed no change in the ratio of activities, whereas extracts prepared from the 3-hydroxybenzoate culture showed a ratio that changed from 3.5:1 at the time of transfer to 1.3:1. When cells grown initially on 3-hydroxybenzoate were examined similarly, no change in the ratio of activity was observed in cells transferred to 3-hydroxybenzoate medium, whereas when transferred to lactate medium, the ratio changed from 1.3:1 to 3.5:1. This alteration in ratios also indicated the presence of two maleylpyruvate hydrolases.

The enzyme formed during growth on either lactate, 2,5-xylenol, or 3-hydroxy-4-methylbenzoate will be termed the constitutive maleylpyruvate hydrolase and designated MPH1, whereas that formed during growth on, or induction by, either *m*-cresol, 3-hydroxybenzoate, or gentisate will be termed the inducible maleylpyruvate hydrolase and designated MPH11.

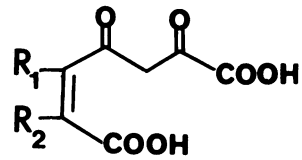
Mutant strains, P25X3, -5, and -8, when grown on either 3-hydroxybenzoate or gentisate had maleylpyruvate hydrolase activity with the ratio of specificities characteristic of MPH11. When grown on lactate in the presence of *m*-cresol, these strains had maleylpyruvate hydrolase activity with the same ratio of specificities as MPH1.

When mutant P25X7 was grown in the presence of either 3-hydroxybenzoate or gentisate, maleylpyruvate activity with the substrate specificities of MPH1, but not MPH11, was detected.

Fumarylpyruvate hydrolase. No activity of this enzyme was detected in crude extracts of P25X1 grown on the nonaromatic carbon sources tested earlier, on 2,5-xylenol, or 3-hydroxy-4-methylbenzoate. Hydrolase activity was detected when either *m*-cresol, 3-hydroxybenzoate, or gentisate was the growth substrate (Table 8). In mutant strains P25X3 and P25X5, fumarylpyruvate hydrolase activity was detected only in those conditions of growth that resulted in the formation of MPH11. No activity of fumarylpyruvate was detected when P25X7 was grown in the presence of either *m*-cresol, 3-hydroxybenzoate, gentisate, or 3,5-xylenol. In the absence of any analog of fumarylpyruvate, the possibility that different carbon sources induced different patterns of specificity was not assessed.

Pleiotropic properties of mutant strains P25X3, P25X5, and P25X8. These strains, selected for their inability to grow on 2,5-xylenol,

TABLE 7. *Comparative rates of activity versus maleylpyruvate and some alkyl- and halogen-substituted analogs of crude extracts of P25X1 grown on different carbon sources*[a]

Substrate		Growth substrate	
		Lactate	3-Hydroxybenzoate
			
R_1	R_2		
H	H	1 (0.011)	1 (0.116)
CH_3	H	0.7	1.1
F	H	0.9	1.0
Cl	H	3.5	1.3
Br	H	4.4	1.3
H	CH_3	4.9	1.2
H	Cl	4.4	0.8

[a] The figures in parentheses are the absolute values expressed as micromoles of substrate degraded per milligram of protein per minute.

TABLE 6. *Comparative rates of oxidation of gentisic acid and some alkyl and halogenated analogs by crude extracts of P25X1, P25X3, and P25X5 grown on different carbon sources*[a]

Assay substrate	P25X1				P25X3			P25X5		
	Lactate	3-Hydroxybenzoate	3-Hydroxy-4-methylbenzoate	Gentisate	Lactate	3-Hydroxybenzoate	Gentisate	Lactate	3-Hydroxybenzoate	Gentisate
Gentisate	1 (24)	1 (27)	1 (30)	1 (30)	ND	1 (21)	1 (6.0)	ND	1 (14)	1 (6.8)
3-Fluorogentisate	0.34	0.50	0.30	0.33	ND	0.86	1.28	ND	0.88	0.90
3-Bromogentisate	0.33	0.40	0.35	0.30	ND	0.39	0.74	ND	0.33	0.51
3-Ethylgentisate	1.46	1.18	2.2	1.04	ND	0.52	0.81	ND	0.65	0.59
4-Chlorogentisate	0.24	0.30	0.36	0.29	ND	ND	ND	ND	ND	ND
4-Methylgentisate	1.11	1.30	1.5	1.0	ND	ND	ND	ND	ND	ND

[a] Figures in parentheses are the rate of oxidation expressed as microliters of oxygen per milligram of protein per minute. ND, No activity detected.

TABLE 8. *Specific activities of maleylpyruvate hydrolase 11 (MPH11)*[a,b] *and fumarylpyruvate hydrolase (FPH) in crude extracts of strains P23X1 and P23X3 grown on, or in the presence of, various carbon sources*[d]

Strain[e]	Enzyme	Carbon source			
		m-Cresol	3-Hydroxybenzoate	Gentisate	3,5-Xylenol
P25X1	MPH11	0.160	0.140	0.070	0.088
	FPH	0.068	0.140	0.056	0.061
P25X3	MPH11	ND	0.129	0.049	ND
	FPH	ND	0.107	0.093	ND

[a] Substrate, maleylpyruvate.

[b] Characterized by the ratio of 6-bromomaleylpyruvate to maleylpyruvate (1.3:1).

[c] Substrate, fumarylpyruvate.

[d] Specific activities are expressed as micromoles of substrate used per milligram of protein per minute.

[e] Strains P25X5 and P25X8 gave the same pattern of activities as P25X3. No activity of MPH11 or FPH was detected in P25X7.

were defective in more than one enzyme activity of the gentisate pathway. Changes in the ability of P25X3 and P25X5 to oxidize constitutively 3-hydroxybenzoate and gentisate are shown in Tables 5 and 6, respectively, and they have also lost the ability to oxidize 2,5-xylenol, *m*-cresol, and 3,5-xylenol (not shown). The same results were obtained with P25X8. P25X5 and P25X8 also have both lost detectable alcohol and aldehyde dehydrogenase activities, whereas in P25X3 these activities were about the same as in P25X1.

DISCUSSION

A common feature of several aromatic degradation pathways is the presence of enzymes that possess broad substrate specificities. The work of Hopper et al. (11, 13) on the degradations of xylenols by P25X1 via a gentisic acid pathway suggested that several enzymes of that pathway possessed broad substrate specificities. The results presented in this report show that the apparent broad specificity of some steps in that pathway is due to the presence of isofunctional enzymes. Our results suggest strongly that the first reaction in the pathway is catalyzed by a nonspecific methylhydroxylase. Mutant strain P25X2 lost the ability to oxidize the methyl group in 2,5-xylenol, 3,5-xylenol, and *m*-cresol (Fig. 1), suggesting that the reaction was mediated by the same enzyme, and this conclusion was supported by the isolation of a revertant of P25X2 that regained the ability to oxidize all three compounds. That this mutant still retained the ability to grow on *p*-cresol showed that there was a separate methylhydroxylase for hydroxylation of *p*-cresol. Contrary to the findings of Hopper et al. (11), we consistently detected constitutive synthesis of xylenol methylhydroxylase in cells grown on either lactate or any of the other nonaromatic carbon sources tested. Constitutive methylhydroxylase activity against *p*-cresol was absent in P25X2, suggesting that the methylhydroxylase induced by *p*-cresol in P25X2 was specific for *p*-cresol. Keat and Hopper (14) reported that the NADH-dependent 3,5-xylenol methylhydroxylase synthesized by *Pseudomonas putida* NCIB 9869 grown on 3,5-xylenol was not active against *p*-cresol. This strain also synthesized two enzymes that oxidized the methyl group of *p*-cresol, one elicited during growth on 3,5-xylenol, the other during growth on *p*-cresol. These latter enzymes are not monooxygenases but hydroxylases. Without purification of the two methylhydroxylases synthesized by P25X1, it is not known whether these enzymes are similar types to those reported in *P. putida* (14).

A constant ratio of activity was found when benzylalcohol and 3-hydroxy-benzylalcohol were used as assay substrates for cells grown on any of the carbon sources shown in Table 4. However, when 4-hydroxy-benzylalcohol was used as a substrate, cells grown on either lactate, 2,5-xylenol, or *m*-cresol showed a different ratio of attack compared to cells grown on either 3-hydroxybenzoate, 3-hydroxy-4-methylbenzoate, 3-hydroxy-benzylalcohol, or 3-hydroxy-benzaldehyde. This may indicate the presence of more than one NAD^+-dependent alcohol dehydrogenase in P25X1. Purification studies by Keat and Hopper on enzymes of *P. putida* NCIB 9869 have shown that three aromatic alcohol dehydrogenases are formed by that strain (15). The ratio of the $NADP^+$-dependent aldehyde dehydrogenase activities against benzaldehyde and 3-hydroxy-benzaldehyde was constant regardless of the carbon source used for growth. Oxygen uptake studies with the wild-type and mutant strains showed that P25X1 synthesized two separate enzymes, both of which were active against 3-hydroxy-benzoate and 3-hydroxy-4-methylbenzoate. One monooxygenase was synthesized constitutively and oxidized 3-hydroxy-4-methylbenzoate three times faster than 3-hydroxybenzoate. The monooxygenase synthesized in 2,5-xylenol and 3-hydroxy-4-methylbenzoate-grown cells had similar substrate specificity to the constitutively synthesized enzyme. *m*-Cresol- and 3-hydroxybenzoate-grown cells synthesized a monooxygenase with greater activity against 3-hydroxybenzoate than 3-hydroxy-4-methylbenzoate (Table 5). Since the difference in substrate specificity observed was based solely

on oxygen uptake by whole cells, the presence of specific permeases that affect the availability of substrates for their respective enzymes cannot be overlooked. However, it is unlikely that a specific permease for the substrates of the monooxygenase that has the higher activity toward 3-hydroxy-4-methylbenzoate could have been eliminated consistently to produce independently isolated mutant strains identical in genotype to P25X3 and P25X5.

The presence of two enzymes in the wild type active against 3-hydroxybenzoate and 3-hydroxy-4-methylbenzoate was strengthened by substrate specificity studies of oxygen uptake by whole cells of P25X3 and P25X5. Neither strain synthesized the constitutive monooxygenase, but an inducible monooxygenase was formed when cells were grown on 3-hydroxybenzoate. 3-Hydroxy-4-methylbenzoate failed to induce any monooxygenase activity in these strains.

The work of Hopper et al. (13) suggested that P25X1 synthesized a gentisate dioxygenase that attacked both alkyl and halogen analogs of gentisate, and no difference in substrate specificity of gentisate dioxygenase was detected between cells of this strain grown on the various carbon sources used for growth (Table 6). However, when mutant strains P25X3 and P25X5 were tested, two changes were observed. First, constitutive activity had been lost, and second, the substrate specificity of gentisate dioxygenase induced by either 3-hydroxybenzoate or gentisate was altered. In these mutant strains, the inducible enzyme was not active against either 4-chloro- or 4-methylgentisate, and activity against 3-fluorogentisate relative to gentisate was increased. It is unlikely that the mutation that affected the substrate specificity would at the same time affect the expression of the enzyme in two independently isolated mutant strains. The occurrence of a constitutive broadly specific and of an inducible narrowly specific gentisate dioxygenase therefore is consistent with the specificity and inducibility of the monooxygenases acting on the acids.

When the maleylpyruvate hydrolase activity of wild-type lactate-grown cells was compared to that of either *m*-cresol- or 3-hydroxybenzoate-grown cells, a difference in the ratios of attack on maleylpyruvate and some alkyl- and halogen-substituted analogs was found. Growth on either 2,5-xylenol or 3-hydroxy-4-methylbenzoate resulted in a maleylpyruvate hydrolase activity of the same specificity as that found in lactate-grown cells. Maleylpyruvate hydrolase activity was inducible when the wild type was grown on either *m*-cresol or 3-hydroxybenzoate. The inability of mutant strains P25X3 and P25X5, which are unable to grow on *m*-cresol, to synthesize MPH11 in the presence of *m*-cresol, showed that the enzyme must be induced by an intermediate of the pathway below *m*-cresol. The reason for the synthesis of fumarylpyruvate hydrolase during growth of P25X1 on either *m*-cresol, 3-hydroxybenzoate, or gentisate is not clear since, unless maleylpyruvate is isomerized to fumarylpyruvate, the *trans* hydrolase has no function in the degradation of the former compounds. It has been reported (6) and confirmed by Chapman (personal communication) that P25X1 may form a low level of a glutathione-dependent activity towards maleylpyruvate that could result in the formation of fumarylpyruvate. However, the extent of the involvement of an isomerase in maleylpyruvate degradation is uncertain for two reasons. First, the MPH11 activity induced during growth on *m*-cresol, 3-hydroxybenzoate, or gentisate is high and this, together with its high affinity for its substrate (1), would result in rapid degradation of maleylpyruvate. Second, it has been shown (13) that approximately stoichiometric amounts of D-malate and pyruvate are formed from gentisate by *m*-cresol-grown cells and isomerization of maleylpyruvate does not occur in that pathway. Hopper et al. (13) reported that the carbon source used for growth by P25X1 affected the relative activities of maleyl- and fumarylpyruvate hydrolase, but no data were presented. However, our results suggest that a revised interpretation of the ratio of the two enzymes by Hopper et al. (13) should now take into consideration the fact that synthesis of two different maleylpyruvate hydrolases can occur. We have consistently found that fumarylpyruvate hydrolase was not detected when MPH1 was synthesized. Approximately equal activities of MPH11 and fumarylpyruvate hydrolase were present in P25X1 grown on either *m*-cresol or 3,5-xylenol, whereas mutant strains that were unable to metabolize either *m*-cresol or 3,5-xylenol produced neither of these enzymes. Therefore, neither *m*-cresol nor 3,5-xylenol acts as an inducer for either of these enzymes.

Pleiotropic mutant strains P25X3, -5, and -8 retained the ability to grow on 3-hydroxybenzoate and gentisate while being unable to utilize either 2,5-xylenol or *m*-cresol. Two explanations are possible for the growth of these strains on 3-hydroxybenzoate. 3-Hydroxybenzoate may either undergo hydroxylation at C_4 to form protocatechuate that is then dissimilated via an ortho pathway or 3-hydroxybenzoate may be hydroxylated at C_6 to form gentisate that is metabolized as shown in Fig. 1. In *Pseudomonas aeruginosa* and *Pseudomonas testosteroni*, it was possible to differentiate between hydroxylation at C_4 or C_6 of 3-hydroxybenzoate by the

difference in the effect on catalytic activity by NADH and NADPH (9, 18). In P25X1, oxygen uptake in the presence of 3-hydroxybenzoate occurred with both NADH and NADPH, and therefore we were unable to differentiate between the two possibilities on this basis. The growth of the three pleiotropic mutants on 3-hydroxybenzoate and gentisate can be explained by the presence of a second set of enzymes, a monooxygenase and a dioxygenase, that are induced by either 3-hydroxybenzoate or gentisate. This was confirmed by the isolation from P25X3 of a putative regulatory mutant strain, P25X7, that is noninducible by either 3-hydroxybenzoate or gentisate. Growth of this strain in the presence of either of these two compounds did not elicit formation of either a monooxygenase, gentisate dioxygenase, MPH11, or fumarylpyruvate hydrolase. Failure of P25X7 to grow on 3-hydroxybenzoate and the absence of gentisate pathway enzymes in that strain make it unlikely that growth of P25X3 on 3-hydroxybenzoate was via the protocatechuate pathway.

The high frequency of occurrence of spontaneous mutants (approximately 4%), the ease of isolation of pleiotropic mutants by the use of mitomycin C, and the non-revertibility of both these classes of mutants (C. L. P., unpublished observations) would be explained if the genes lost in the above mutants were located on a plasmid. If this was the case, then the metabolism of 2,5-xylenol, 3,5-xylenol, and *m*-cresol by this strain of *P. alcaligenes* would involve the interaction of both plasmid and chromosomal gene products. In *P. putida* strain PpG6, an interaction of plasmid and chromosomal gene products is responsible for the degradation of alkanes (8). In this case, there is a duplication of an alcohol dehydrogenase activity: that encoded by the chromosome is NAD^+ dependent, whereas the plasmid-encoded activity is $NAD(P)^+$ independent. If the situation suggested above for *P. alcaligenes* is correct, then our data suggest that the genes encoding the synthesis of five constitutive enzymes (xylenol methylhydroxylase, alcohol and aldehyde dehydrogenases, a monooxygenase, and a gentisate dioxygenase) are plasmid borne, whereas the remainder of the gentisate pathway genes, including MPH1, are located on the chromosome. The location of the genes for a second monooxygenase and dioxygenase on the chromosome would parallel the alcohol dehydrogenase situation in the alkane pathway (8). A difference between these two situations is that the proposed plasmid-encoded enzymes for xylenol degradation are constitutive and the chromosomal-encoded enzymes are inducible, whereas the reverse situation occurs for the alkane pathway.

Our findings would also be consistent with the existence of insertion sequences in the close vicinity of a chromosomal operon causing deletions of some of the genes encoding for the constitutive enzyme functions.

Preliminary information showing that the genes encoding the five constitutive enzymes lost in spontaneous and mitomycin C-derived mutant strains are plasmid borne has been reported (Poh and Bayly, 3rd Int. Symp. Gen. Ind. Micro. abstr. no. 56 P28, 1978) and a detailed report is in preparation (Poh and Bayly, manuscript in preparation).

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