APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1995, p. 2159–2165 Vol. 61, No. 6
0099-2240/95/$04.00+0

Formation of Dimethylmuconolactones from Dimethylphenols by *Alcaligenes eutrophus* JMP 134

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Received 19 December 1994/Accepted 24 March 1995

2,3-, 2,4-, 2,5-, 3,4-, and 3,5-dimethylphenols were cometabolized by 2,4-dichlorophenoxyacetate-grown *Alcaligenes eutrophus* JMP 134 or the constitutive derivative JMP 134-1 via the *ortho* pathway into dimethylmuconolactones as dead-end products. Formation of two distinct lactones from 3,4-dimethylphenol is indicative of 2- as well as 6-hydroxylation. Induction of the *meta*-cleavage pathway by 2,3- and 3,4-dimethylphenols resulted in growth and no accumulation of products. In contrast, 3,5-dimethylphenol is not metabolized by the *meta*-cleavage pathway.

Alcaligenes eutrophus JMP 134 and its derivative JMP 134-1 can mineralize a variety of chloro- and methyl-substituted phenoxyacetates, phenols, and benzoates via the corresponding catechols as central intermediates (12, 35, 37). Three distinct catechol *ortho*-cleavage pathways can be induced by this strain, i.e., a 3-oxoadipate pathway (24, 45), a modified *ortho*-cleavage pathway for the degradation of chlorocatechols (chlorocatechol 1,2-dioxygenase, dichloromuconate cycloisomerase, dienelactone hydrolase, and maleylacetate reductase) (24, 25, 37, 49), and an *ortho*-cleavage pathway for the metabolism of 4-methylcatechol (34). The last of these pathways includes a 4-methylmuconolactone methylisomerase (39) as a key enzyme, together with isoenzymes of the 3-oxoadipate pathway (40). In addition to *ortho*-cleavage activities, JMP 134 exhibits catechol *meta*-cleavage activities (20, 21).

The capability to mineralize phenols results from the occurrence of distinct phenol hydroxylases in this strain (16, 27, 35). 2,4-Dichlorophenol and 4-chloro-2-methylphenol are mineralized by JMP 134-1 via a constitutive chlorophenol hydroxylase and *ortho* cleavage of intermediate chlorocatechols, whereas phenol is transformed by a phenol hydroxylase. High levels of catechol 2,3-dioxygenase in phenol-grown (35) cells indicate a preference for *meta*- over *ortho*-cleavage routes. Whereas 2-methylphenol was also reported to be a growth substrate, 2,4-dimethylphenol was converted into 2,4-dimethylmuconolactone, a dead-end product (36).

Dimethylphenols are by-products of coal conversion processes (10) and constitute important pollutants of waste streams in this industry. Only 2,3- and 3,4-dimethylphenols have been reported to be mineralized via a catechol *meta*-cleavage pathway, with 3,4-dimethylcatechol as an intermediate (42, 48), while 2,4- and 2,5-dimethylphenols were degraded only after oxidation of a methyl substituent (18, 19).

Because different *ortho*-cleavage and *meta*-cleavage routes are present in JMP 134 and because the strain is a very versatile organism, able to mineralize even methylaromatics via *ortho*-cleavage routes (34), it is of potential interest for the development of biotreatment processes. We have therefore investigated its metabolic potential toward dimethylphenols.

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MATERIALS AND METHODS

Organisms. The 2,4-dichlorophenoxyacetate (2,4-D)-degrading organism *A. eutrophus* JMP 134 was isolated by Don and Pemberton (11). *A. eutrophus* JMP 134-1 is a spontaneous mutant of this strain which expresses 2,4-D/α-ketoglutarate dioxygenase, chlorophenol hydroxylase, chlorocatechol 1,2-dioxygenase, dichloromuconate cycloisomerase, dienelactone hydrolase, and maleylacetate reductase constitutively (35).

Culture conditions. Growth in liquid culture was performed with mineral salts medium (13) containing 5 or 2 mM (in the case of substituted phenols) growth or induction substrate. Cells were grown in fluted Erlenmeyer flasks incubated at 30°C on a rotary shaker at 150 rpm. Growth was monitored photometrically.

In the induction experiments, cultures were grown in 500-ml fluted Erlenmeyer flasks containing 50 ml of mineral salts medium and fructose as the carbon source. During late exponential growth, the culture was transferred to a 3-liter flask, containing 500 ml of medium supplemented with fructose and the respective inducer. Cells were harvested after a further incubation period of 15 h.

Preparation of cell extracts. Harvested cells were suspended in 100 mM Tris/HCl buffer (pH 7.5) and disrupted in a French press (Aminco, Silver Spring, Md.). Cell debris was removed by centrifugation at 100,000 × *g* for 1 h at 4°C.

Enzyme assays. Catechol 2,3-dioxygenase (EC 1.13.11.2) was assayed by the method of Nozaki (30), and catechol 1,2-dioxygenase (EC 1.13.11.1) was assayed by the procedure of Dorn and Knackmuss (14, 15). If catechol 2,3-dioxygenase and catechol 1,2-dioxygenase were simultaneously induced, the former enzyme was inactivated by treating the extract with H_2O_2 for 10 min before adding the test substrate (29). 2-Hydroxymuconic semialdehyde hydrolase was assayed as described by Williams and Murray (50). Specific activities are expressed as micromoles of substrate converted or product formed per minute per gram of protein at 25°C. Protein was determined by the Bradford procedure (4).

Activity measurements with whole cells. Chlorophenol hydroxylase activity was assayed by measuring the rate of oxygen uptake polarographically with a Clark-type electrode (37). The measured rates of oxygen uptake represent the combined activities of chlorophenol hydroxylase and catechol 1,2- or 2,3-dioxygenase. High-pressure liquid chromatography (HPLC) measurements gave no hints for accumulation of the respective catechols, indicating phenol-hydroxylating activities to be always rate limiting. Since 2 mol (±10%) of oxygen was consumed per mol of phenol by phenol-grown cells of *A. eutrophus* JMP 134, the actual phenol hydroxylase activity can be regarded to be 50% of that measured by oxygen uptake. Therefore, the determined activity was divided by 2 to obtain the actual phenol hydroxylase activity. The reliability of this method was verified by comparing values obtained by this method with those determined by quantifying substrate disappearance by HPLC as described below. Those values differed by no more than 10%. For determination of low activities, harvested cells were suspended to an A_{546} of 10 to 20 (corresponding to 1.15 to 2.3 g of protein per liter) in 50 mM phosphate buffer (pH 7.4) and the disappearance of substrates and the formation of products were quantified by HPLC. Protein concentration was measured by the method of Schmidt et al. (46).

Extraction and derivatization of metabolites. For transformation experiments with resting cells, JMP 134-1 was grown on fructose plus 2,4-D as inducer, as described above. Cells (3 liters) were harvested and suspended to A_{546} = 10 to

TABLE 1. Specific activities of catabolic enzymes from cells of *A. eutrophus* JMP 134 induced with 2,3- or 3,4-dimethylphenol or 2-methylphenol

Enzyme activity	Assay substrate	Sp act (U/g of protein) after induction with[a]:			
		2,3-Dimethylphenol	3,4-Dimethylphenol	2-Methylphenol	No inducer[b]
Phenol hydroxylase	Phenol	85	70	130	<10
	2-Methylphenol	80	60	95	<10
	2,3-Dimethylphenol	70	50	60	<10
	2,4-Dimethylphenol	15	10	20	<10
	2,5-Dimethylphenol	<10	<10	<10	<10
	3,4-Dimethylphenol	75	55	65	<10
	3,5-Dimethylphenol	<10	<10	<10	<10
Catechol 1,2-dioxygenase	Catechol	20	15	45	5
	3-Chlorocatechol	<5	<5	5	5
Catechol 2,3-dioxygenase	Catechol	80	100	630	<1
Hydroxymuconic semialdehyde hydrolase	2-Hydroxy-6-oxohepta-2,4-dienoate	70	120	220	<10

[a] Cells were harvested after 15 h of induction with the appropriate substrate at 2 mM. Phenol-hydroxylating activities were determined with whole cells. All other activities were determined with cell extracts.
[b] Growth with fructose.

15 (corresponding to 1.15 to 1.75 g of protein per liter) in phosphate buffer. 2,3-, 2,5- or 3,4-dimethylphenol (0.5 mM) was added, and transformation was monitored by HPLC. After complete conversion another 0.5 mM substrate was added. Cells were removed by centrifugation, and the supernatant was acidified (to pH 2) with phosphoric acid and extracted three times with 500 ml of ethyl acetate. H_2O was removed from the ethyl acetate fractions by $MgSO_4$ before evaporation. When necessary, products were separated by preparative HPLC and the effluent fractions were extracted with ethyl acetate as described above.

Analytical methods. Bioconversion of dimethylphenols and accumulation of metabolites were determined with the HPLC system described by Pieper et al. (37), equipped with an SC125/Lichrospher 5-μm column (Bischoff, Leonberg, Germany). The aqueous solvent system contained 1 ml of H_3PO_4 (85%) and 150 to 500 ml of methanol per liter. The column effluent (flow rate, 1 ml/min) was monitored at 210 nm. Samples of culture fluid (2 to 10 μl) were injected after cells had been removed by centrifugation. For purification of product mixtures, a Lichrosorb 5-μm column (16 by 250 mm; Knauer, Bad Homburg, Germany) was operated at a flow rate of 6 ml/min with an aqueous solvent system containing 1 ml of H_3PO_4 (85%) and 200 ml of methanol per liter.

Spectrophotometric analysis was performed with an Uvikon 810 spectrophotometer (Kontron Instruments, Eching, Germany). ^1H nuclear magnetic resonance (NMR) spectra were recorded on a CXP 300 spectrometer (Bruker, Rheinstetten, Germany) with Aspect 2000 software, using tetramethylsilane as the internal standard and D_6-acetone as the solvent. Mass spectra (electron impact at 70 eV) were recorded on a MAT711 mass spectrometer (Varian, Palo Alto, Calif.). Gas chromatography-mass spectrometry (electron impact at 70 eV) recordings were performed on a GC-17A gas chromatograph coupled to a QP-5000 mass spectrometer (Shimadzu). Samples were derivatized with trimethylchlorosilane-hexamethyldisilazane and chromatographed on an SE-54-CB column (50 m by 0.32 mm). Optical activity was measured in aqueous solution with a model 241 polarimeter (Perkin-Elmer Corp., Norwalk, Conn.). Melting points were measured with a heatable microscope (Reichert Thermovar).

Chemicals. Chemicals were purchased from Aldrich Chemie, Steinheim, Germany; Fluka AG, Buchs, Switzerland; and Merck AG, Darmstadt, Germany. 2-Methyl-, 3-methyl-, 4-methyl-, and 2,4-dimethylmuconolactones were prepared as previously described (23, 34, 36).

RESULTS

Metabolism of dimethylphenols by *A. eutrophus* JMP 134. *A. eutrophus* JMP 134 has been reported to use 2-methyl- but not 2,4-dimethylphenol as a growth substrate (35, 36). Cultures exposed to 2,3- and 3,4-dimethylphenols produced high levels of phenol-hydroxylating as well as catechol *meta*-cleavage activities (Table 1) but only low levels of *ortho*-cleavage activity. 2,5- and 3,5-dimethylphenols, like 2,4-dimethylphenol (36), did not serve as inducers. As expected from their induction properties, only 2,3- and 3,4-dimethylphenols served as growth substrates (growth rates of $\mu = 0.1$ to $0.12\ h^{-1}$). Presumably, these compounds are mineralized via *meta* cleavage of intermediate dimethylcatechols as described by Ribbons (42) and Shingler et al. (48).

To analyze substrate flux into the two types of pathway, cometabolism of dimethylphenols by JMP 134 cells of different induction status was assessed. Previous studies showed that 2,4-D-grown cells exhibited high levels of dichlorophenol hydroxylase, phenol hydroxylase, and enzymes of the modified *ortho* pathway for chlorocatechol metabolism (37). JMP 134-1 exhibited constitutive activities of aforementioned enzymes plus dichlorophenol hydroxylase (35). 2-Methylphenoxyacetate-grown cells of JMP 134 were reported to exhibit high activity against 2- and 4-methylphenols as well as catechol *meta*-cleavage activity and, to a lower extent, *ortho*-cleavage activity. The same was true for 2-methylphenol-grown cells of JMP 134, which, however, expressed even lower *ortho*-cleavage activities (37). In this study, we found activities against dimethylphenol substrates always lower than 10 U/g of protein, with the exception of 2,3- and 3,4-dimethylphenol transformation by 2-methylphenoxyacetate- and 2-methylphenol-grown cells (Table 2). HPLC analysis showed that when cells exhibited only *ortho*-cleavage activities, dead-end products were formed in large amounts in all cases. Only one product was observed from 2,3-, 2,5-, or 3,5-dimethylphenol, whereas two products in ratios of approximately 1:4 as judged by peak areas were formed from 3,4-dimethylphenol.

Purification and identification of products formed from dimethylphenols by resting cells. 2,4-D-induced JMP 134-1 resting cells were used to transform substrates to dead-end products as described in Materials and Methods. The product formed from 3,5-dimethylphenol was identified as 2,4-dimethylmuconolactone (4-carboxymethyl-2,4-dimethylbut-2-en-4-olide [17, 36]) by HPLC, UV, mass spectrometry, and ^1H-NMR comparisons with the authentic compound.

The dead-end product of oxidation of 2,3-dimethylphenol was identified as 2,3-dimethylmuconolactone (4-carboxymethyl-2,3-dimethylbut-2-en-4-olide) by comparison of UV, mass spectrometry, and ^1H-NMR data with those given for a product accumulated from 3,4-dimethylbenzoate by *Rhodococcus rhodochrous* N75 (47). 2,3-Dimethylmuconolactone was also shown to be the major metabolite formed during 3,4-dimethylphenol metabolism by JMP 134-1.

TABLE 2. Substrate disappearance and product formation from dimethylphenols by *A. eutrophus* JMP 134 cells of different induction status[a]

Parameter	Assay substrate	Value of parameter with following strain and growth substrate:			
		JMP134-1, Fr	JMP134, 2,4-D	JMP134, 2MPA	JMP134, 2MPhe
Rate of substrate conversion (U/g of protein)	2,3-Dimethylphenol	1.1	5.8	75	70
	2,5-Dimethylphenol	0.4	4.1	7.8	5.0
	3,4-Dimethylphenol	2.4	5.3	80	75
	3,5-Dimethylphenol	2.0	6.4	6.4	3.0
Rate of product formation (U/g of protein)	2,3-Dimethylphenol	1.4	5.6	NP[b]	NP
	2,5-Dimethylphenol	0.6	4.0	8.2	4.4
	3,4-Dimethylphenol I	1.9	3.9	NP	NP
	3,4-Dimethylphenol II	0.4	0.8	ND[c]	ND
	3,5-Dimethylphenol	2.2	5.8	6.1	2.9
Extent of product formation (% of theoretical value)	2,3-Dimethylphenol	>90	>90	0	0
	2,5-Dimethylphenol	>90	>90	50	30
	3,4-Dimethylphenol I	80	80	0	0
	3,4-Dimethylphenol II	20	20	5	10
	3,5-Dimethylphenol	>90	>90	>90	>90

[a] Cells were grown with the indicated carbon source (Fr, fructose; 2,4-D, 2,4-dichlorophenoxyacetate; 2MPA, 2-methylphenoxyacetate; 2MPhe, 2-methylphenol) and incubated with 0.2 mM dimethylphenol. Substrate transformation and product formation were analyzed by HPLC. Products were quantified after preparation of standards. Two products (I and II) were formed from 3,4-dimethylphenol.

[b] NP, no product.

[c] ND, not determined.

The minor metabolite of 3,4-dimethylphenol showed a mass spectrum very similar to that observed for 2,3-dimethylmuconolactone, i.e., a molecular ion with an accurate mass of 170.0580, indicative of $C_8H_{10}O_4$, *m/e* = 170.0579, and prominent fragments with *m/e* = 152 (M^+-H_2O), 125 (M^+-COOH), 124 ($M^+-HCOOH$), 111 (M^+-CH_2COOH), 110 ($M^+-CH_2COOH-H$), and 83 ($M^+-CH_2COOH-CO$). The mass spectrum of the trimethylsilyl derivative (Fig. 1) is in accordance with a dimethylmuconolactone structure. Comparison of ^{1}H-NMR data (Table 3) with those reported for various monomethyl- and dimethylmuconolactones (8, 9, 17, 23, 28, 34, 36, 47) clearly identifies this new metabolite as 3,4-dimethylmuconolactone (4-carboxymethyl-3,4-dimethylbut-2-en-4-olide). Two protons of a methylene group resonate at 2.66 and 2.88 ppm, respectively, which is in close agreement with literature data on methylmuconolactones. A vinylic proton appears at δ = 5.79 ppm, indicative of protons situated at C-2 of muconolactones. Two methyl groups were identified at C-3 and C-4.

In contrast to ^{1}H-NMR data of previously characterized muconolactones (8, 9, 17, 23, 28, 34, 36, 44, 47), the typical spectrum of two diastereotopic methylene protons is missing in the ^{1}H-NMR spectrum of the product formed from 2,5-dimethylphenol (Table 4). This indicates the presence of a substituent on the 4-carboxymethyl side chain as described for 5-chloro-3-methylmuconolactone (38). A single proton with a chemical shift of δ = 2.83 ppm was observed. Vicinal couplings occurred with a methyl substituent on C-5 (7.1 Hz) and a proton situated on C-4 (5.5 Hz). Chemical shifts of protons of a second methyl substituent (1.87 Hz) and two single protons (5.25 and 7.32 Hz), as well as coupling constants, were very similar to those observed for 2-methylmuconolactone (4-carboxymethyl-2-methylbut-2-en-4-olide) (δ = 1.83, 5.26, and 7.34 ppm, respectively [23]), indicating the identity of the product with 2,5-dimethylmuconolactone [4-(1-carboxyethyl)-2-methylbut-2-en-4-olide]. Mass-spectrometric data of the underivatized product formed from 2,5-dimethylphenol (M^+ = 170.0578 and prominent fragments with *m/e* = 124 [$M^+-HCOOH$], 97 [M^+-CHCH_3COOH], and 69 [$M^+-CHCH_3COOH-CO$]) as well as of the trimethylsilyl derivative (Fig. 1) are in accordance with the postulated structure. The prominent fragment of *m/e* = 97 in both spectra demonstrated only one methyl substituent to be present directly on the lactone ring. The product was shown to be optically active, with $[\alpha]_{D25}$ = +20.9°, *c* 0.43 in H_2O (9.4 mg/ml). The melting point was 75°C.

Quantification of metabolite production. Comparison of the amounts of products formed by differently induced cells (Table 2) showed that independent of the presence of *meta*-cleavage activity, 2,4-dimethylmuconolactone was always quantitatively produced from 3,5-dimethylphenol, indicating that *meta* cleavage is not effective on the assumed intermediate 3,5-dimethylcatechol (Fig. 2). In contrast, 2,5-dimethylmuconolactone formation from 2,5-dimethylphenol and 2,3-dimethylmuconolactone formation from 2,3-dimethylphenol was quantitative only in the absence of *meta*-cleavage activity. The amount of 2,3- plus 3,4-dimethylmuconolactone formed from 3,4-dimethylphenol made up >90% yields by cells exhibiting only *ortho*-cleavage activities. No accumulation of 2,3-dimethylmuconolactone was observed in the presence of *meta*-cleavage activities, which is in accordance with an absence of accumulation of this compound from 2,3-dimethylphenol by 2-methylphenoxyacetate- or 2-methylphenol-grown cells. Accumulation of 2,5-dimethylmuconolactone from 2,5-dimethylphenol and of 3,4-dimethylmuconolactone from 3,4-dimethylphenol was detectable in the presence of *meta*-cleavage activities but significantly lower than by cells exhibiting only *ortho*-cleavage activities.

Biological activity of dimethylmuconolactones. *A. eutrophus* can use 4-methyl- and 3-methylmuconolactone as growth substrates (34). 2-Methylmuconolactone, in contrast, was used as a growth substrate only by the spontaneous mutant *A. eutrophus* JMP 134-2 (33a). Because the 2-methyl isomer was not converted by 3-methylmuconolactone-grown cells and the 3-methyl isomer was not converted by 2-methylmuconolactone-grown cells, distinct metabolic pathways are obviously responsible for degradation of such isomers (41a). None of the isolated dimethylmuconolactones was metabolized by 4-methylmuconolactone-grown cells of JMP 134 or by 2-methylmuconolactone-grown cells of JMP 134-2, which is consistent with their nature as dead-end products.

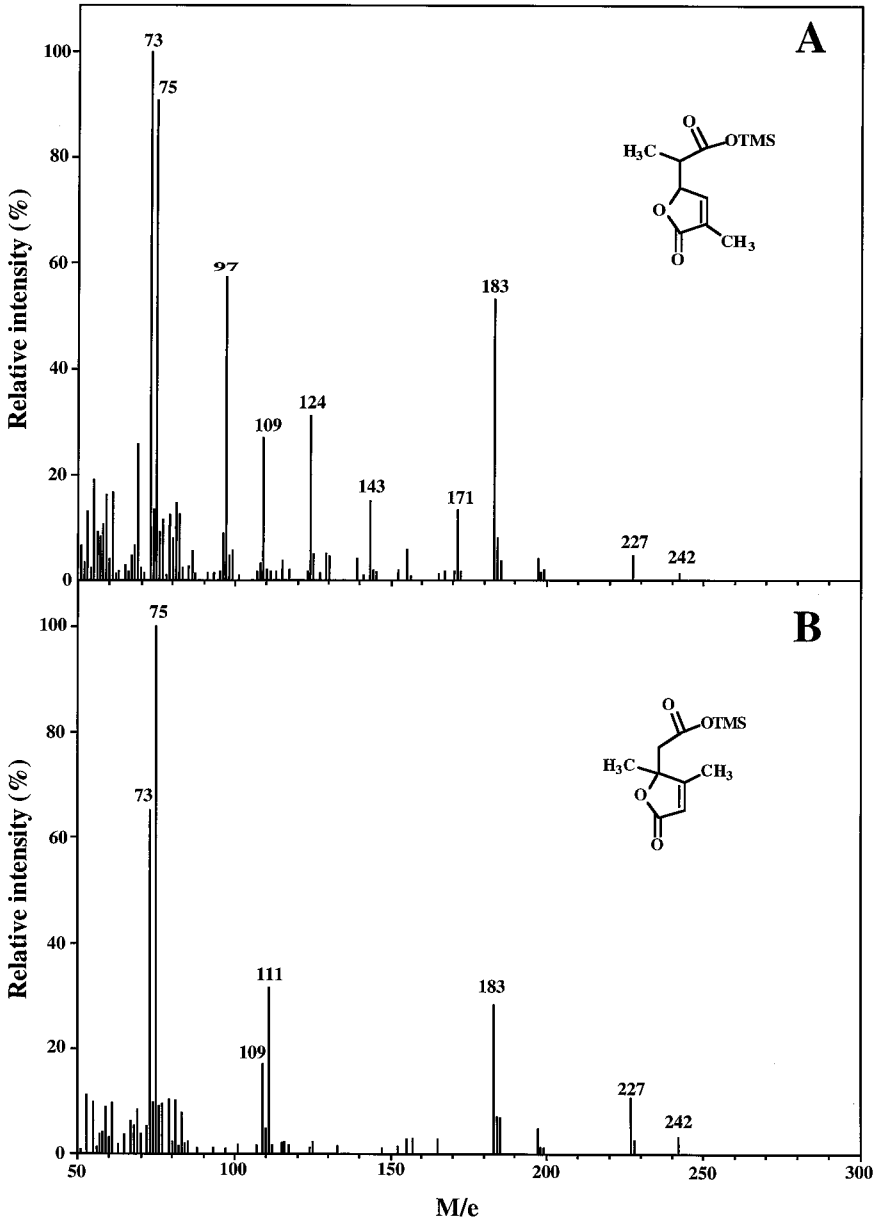

FIG. 1. Electron ionization mass spectra of the trimethylsilyl (TMS) derivatives of 2,5-dimethylmuconolactone (A) and 3,4-dimethylmuconolactone (B).

DISCUSSION

Different pathways have been described for the metabolism of methyl-substituted phenols. The action of a phenol hydroxylase will lead to formation of the corresponding catechols, which can be subject to either *ortho* or *meta* cleavage. As an alternative to ring hydroxylation, the metabolism of methylphenols can be initiated by side chain oxidation, and the corresponding hydroxybenzoates (18) will finally be formed. However, such an activity was not detected in *A. eutrophus* JMP 134. Mineralization of methylphenols via ring hydroxylation and subsequent *meta* cleavage has been reported for monomethylphenols and 2,3- and 3,4-dimethylphenol (2, 7, 42, 48). Remarkably, mineralization of polymethylaromatics via polymethylcatechols, followed by *meta* cleavage, has been reported only for compounds such as *o*-xylene (1) and 1,2,4-trimethylphenol (3, 26), which were initially transformed into 3,4-dimethylcatechol. No description of the metabolism of the isomeric dimethylcatechols via *meta* cleavage has been reported.

Phenol has been reported to be mineralized via hydroxylation and *ortho* cleavage of the intermediate catechol. In the

TABLE 3. ^{1}H-NMR data for 3,4-dimethylmuconolactone

Proton	Chemical shift (ppm)	Coupling assignment	Coupling constant (Hz)
2-H	5.79	^4J (2-H, 3-CH_3)	1.5
3-CH_3	2.08	^4J (2-H, 3-CH_3)	1.5
4-CH_3	1.54		
5-H_A	2.69	^2J (5-H_A, 5-H_B)	14.7
5-H_B	2.88	^2J (5-H_A, 5-H_B)	14.7

TABLE 4. ^{1}H-NMR data for 2,5-dimethylmuconolactone

Proton	Chemical shift (ppm)	Coupling assignment	Coupling constant (Hz)
2-CH_3	1.87	^4J (2-CH_3, 3-H)	1.8
		^5J (2-CH_3, 4-H)	1.8
3-H	7.32	^4J (2-CH_3, 3-H)	1.8
		^3J (3-H, 4-H)	1.8
4-H	5.25	^5J (2-CH_3, 4-H)	1.8
		^3J (3-H, 4-H)	1.8
		^3J (4-H, 5-H)	5.5
5-H	2.83	^3J (4-H, 5-H)	5.5
		^3J (5-H, 5-CH_3)	7.1
5-CH_3	1.12	^3J (5-H, 5-CH_3)	7.1

case of *ortho* cleavage of methylcatechols, however, methylmuconolactones are usually formed as dead-end products (9, 23). There are only three reports on mineralization of the 4-methylcatechol after *ortho* cleavage (5, 34, 41). A 4-methylmuconolactone methylisomerase, which transforms 4-methyl- into 3-methylmuconolactone, has been described as the key enzyme of this apparently rare route in bacteria (6, 39), and a metabolic route analogous but not identical to the classical 3-oxoadipate pathway has been postulated for further metabolism of 3-methylmuconolactone as a result of the identification of 4-methyl-3-oxoadipate as an intermediate (34). On the basis of this new metabolic route, an organism able to mineralize 4-methylphenol via *ortho* cleavage of intermediate 4-methylcatechol and therefore able to simultaneously degrade otherwise incompatible substrate mixtures of methylaromatics and chloroaromatics could be constructed (43). In contrast to the situation with 3- and 4-methylmuconolactone metabolism, there is little information about 2-methylmuconolactone metabolism. An organism able to degrade this compound and toluene via *ortho* cleavage of the 3-methylcatechol intermediate has been described by Pettigrew et al. (33), although its metabolic fate has not been reported. Metabolism of dimethylaromatics via dimethylcatechols, followed by *ortho* cleavage, has been described only for 2,4-dimethylphenoxyacetic acid and for 2,4-dimethylphenol (36) and 3,4- and 3,5-dimethylbenzoate (17, 47); 2,4- and 2,3-dimethylmuconolactone were reported to be dead-end metabolites of the 3,5- and 3,4-dimethylcatechol intermediates. In this report, we describe the identification of two new dimethylmuconolactones (3,4- and 2,5-dimethylmuconolactone) which result from the metabolism of 2,5- and 3,4-dimethylphenol via *ortho* cleavage.

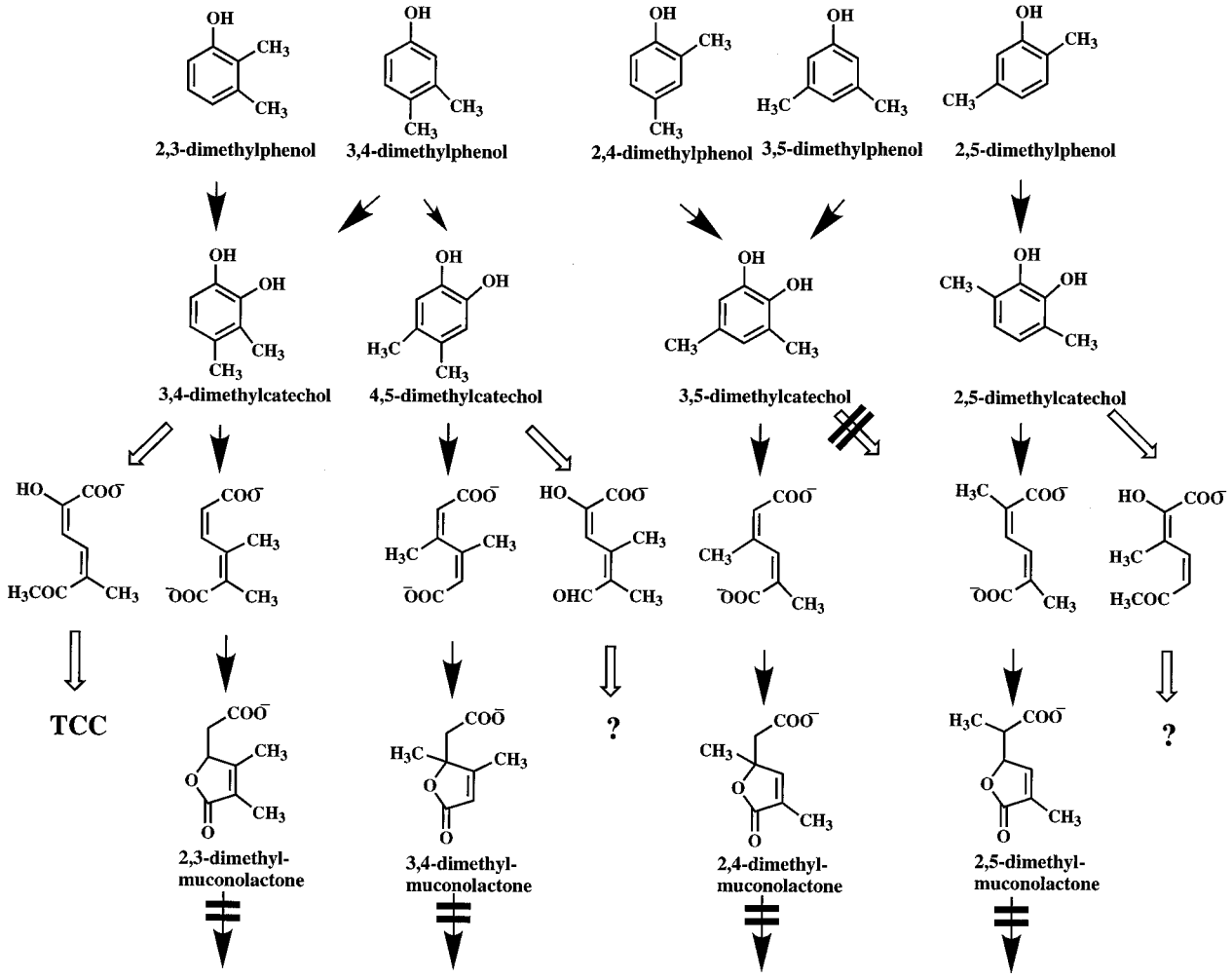

FIG. 2. Metabolism of dimethylphenols by *A. eutrophus* JMP 134. →, phenol-hydroxylating activities or activities of *ortho*-cleavage pathways. ⇨, activities of *meta*-cleavage pathways.

Two major problems in the metabolism of methylmuconolactones have been identified. 4-Methylmuconolactones cannot be degraded via a route analogous to the classical 3-oxoadipate pathway, because muconolactone isomerase-catalyzed abstraction of a proton from the C-4 position followed by isomerization to an enol-lactone (31) is not possible. Both previously described 4-methylmuconolactone methylisomerases, which circumvent this pathway bottleneck in the case of 4-methylmuconolactone, were reported to be highly specific and not to attack the 2,4-dimethyl structure (36, 47). Moreover, even if attack on the 3,4-dimethylisomer were possible, no net structural change of the molecule could be thereby achieved. Consequently, degradation of this compound would require a completely new metabolic sequence. The isomerization of 2- and 3-methylmuconolactones and/or hydrolysis of the corresponding enol-lactones is also reported to be problematic (9, 23, 34). Even when appropriately induced cells of *A. eutrophus* JMP 134 or its derivative JMP 134-2 are able to degrade these latter monomethylmuconolactones, they are not able to attack 2,3- or 2,5-dimethylmuconolactone, indicating restricted specificity of the 2- and 3-methylmuconolactone degradative pathways. It would seem worthwhile to analyze the metabolic fate of 2- and 3-methylmuconolactones and to identify enzymes involved in the metabolic route and the pathway bottlenecks.

The accumulation of dimethylmuconolactones simplifies analysis of the regioselectivity of phenol hydroxylation. Whereas in the case of *ortho*-methylsubstituted phenols, hydroxylation is directed at the 6-position, both 2- and 6-hydroxylation seems to be possible in *meta*- and *para*-substituted phenols like 3,4-dimethylphenol. As described by Peelen et al. (32) for the hydroxylation of 3-fluorophenol by phenol hydroxylase from *Trichosporon cutaneum* and by Knackmuss and Hellwig (22) for the hydroxylation of 3-methylphenol by phenol hydroxylase from *Pseudomonas* sp. strain B 13, we have shown here that C-2 hydroxylation of 3,4-dimethylphenol by *A. eutrophus* JMP 134 phenol-hydroxylating enzymes is preferred over C-6 hydroxylation.

Our experiments also provide some insights into the substrate range of *meta*-cleavage activities, since the extent of dimethylmuconolactone accumulation in the presence of both *ortho*- and *meta*-cleavage activities can be regarded as an indication of the effectiveness of catechol 2,3-dioxygenase. Thus, in the presence of both *ortho*- and *meta*-cleavage activities, 3,4-dimethylcatechol is quantitatively channeled into the *meta*-cleavage pathway, whereas 3,5-dimethylcatechol is quantitatively subjected to *ortho* cleavage even when only low levels of catechol 1,2-dioxygenase are induced. 3,6- and 4,5-dimethylcatechol are routed into both pathways; the flux of these substrates into *ortho* and *meta* pathways will be governed mainly by the affinities of the types of cleavage enzymes toward the substrate, because in no case was accumulation of catechols observed. Analysis of the kinetic properties of the purified enzymes will provide further insight into parameters controlling substrate flux.

Environmental pollutants usually exist as complex mixtures. As is the case for methylsubstituted phenols, multiple distinct pathways will often be involved in the catabolism of structural isomers. Understanding the degradation of such mixtures and exploiting this understanding to improve degradation qualitatively and quantitatively will necessitate detailed analysis of substrate flux into the different productive and unproductive pathways and identification of critical metabolic steps.

ACKNOWLEDGMENTS

We are grateful to K. H. Engesser and V. Wray for valuable and stimulating discussions and to R.-M. Wittich for critical reading of the manuscript. We thank W. Rozdzinski for mass-spectrometric analyses and J. Rebell and P. Fischer for performing the NMR measurements.

K. N. Timmis thanks the Fonds der Chemischen Industrie for generous support.

REFERENCES

1. **Baggi, G., P. Barbieri, E. Galli, and S. Tollari.** 1987. Isolation of a *Pseudomonas stutzeri* strain that degrades *o*-xylene. Appl. Environ. Microbiol. **53:** 2129–2132.
2. **Bayly, R. C., S. Dagley, and D. T. Gibson.** 1966. The metabolism of cresols by species of *Pseudomonas*. J. Bacteriol. **101:**294–301.
3. **Bestetti, G., and E. Galli.** 1987. Characterization of a novel TOL-like plasmid from *Pseudomonas putida* involved in 1,2,4-trimethylbenzene degradation. Appl. Environ. Microbiol. **169:**1780–1783.
4. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:** 248–254.
5. **Bruce, N. C., and R. B. Cain.** 1988. β-Methylmuconolactone, a key intermediate in the dissimilation of methylaromatic compounds by a modified 3-oxoadipate pathway evolved in nocardioform actinomycetes. FEMS Microbiol. Lett. **50:**233–239.
6. **Bruce, N. C., R. B. Cain, D. H. Pieper, and K.-H. Engesser.** 1990. Purification and characterization of 4-methylmuconolactone methylisomerase, a novel enzyme of the modified 3-oxoadipate pathway in nocardioform actinomycetes. Biochem. J. **262:**303–312.
7. **Buswell, J. A.** 1975. The metabolism of phenol and cresols by *Bacillus stearothermophilus*. J. Bacteriol. **124:**1077–1083.
8. **Cain, R. B., G. W. Kirby, and G. V. Rao.** 1989. Stereochemistry of enzymic cyclisation of 3-methyl-*cis*,*cis*-muconic acid to form 3- and 4-methylmuconolactone. J. Chem. Soc. Chem. Commun. **21:**1629–1631.
9. **Catelani, D., A. Fiecchi, and E. Galli.** 1971. (+)-γ-Carboxymethyl-γ-methyl Δ^{a}-butenolide, a 1,2-ring-fission product of 4-methylcatechol by *Pseudomonas desmolyticum*. Biochem. J. **121:**89–92.
10. **Cochran, N. P.** 1976. Oil and gas from coal. Sci. Am. **234:**24–29.
11. **Don, R. H., and J. M. Pemberton.** 1981. Properties of six pesticide degradation plasmids isolated from *Alcaligenes paradoxus* and *Alcaligenes eutrophus*. J. Bacteriol. **145:**681–686.
12. **Don, R. H., A. J. Weightman, H.-J. Knackmuss, and K. N. Timmis.** 1985. Transposon mutagenesis and cloning analysis of the pathways for degradation of 2,4-dichlorophenoxyacetic acid and 3-chlorobenzoate in *Alcaligenes eutrophus* JMP 134(pJP4). J. Bacteriol. **161:**85–96.
13. **Dorn, E., M. Hellwig, W. Reineke, and H.-J. Knackmuss.** 1974. Isolation and characterization of a 3-chlorobenzoate degrading pseudomonad. Arch. Microbiol. **99:**61–70.
14. **Dorn, E., and H.-J. Knackmuss.** 1978. Chemical structure and biodegradability of halogenated aromatic compounds: two catechol 1,2-dioxygenases from a 3-chlorobenzoate grown pseudomonad. Biochem. J. **174:**73–84.
15. **Dorn, E., and H.-J. Knackmuss.** 1978. Chemical structure and biodegradability of halogenated aromatic compounds: substituent effects on 1,2-dioxygenation of catechol. Biochem. J. **174:**85–94.
16. **Harker, A. R., R. H. Olsen, and R. J. Seidler.** 1989. Phenoxyacetic acid degradation by the 2,4-dichlorophenoxyacetic acid (TFD) pathway of plasmid pJP4: mapping and characterization of the TFD regulatory gene, *tfdR*. J. Bacteriol. **171:**314–320.
17. **Hartmann, J., W. Reineke, and H.-J. Knackmuss.** 1978. Metabolism of 3-chloro-, 4-chloro-, and 3,5-dichlorobenzoate by a pseudomonad. Appl. Environ. Microbiol. **37:**421–428.
18. **Hopper, D. J., and P. J. Chapman.** 1971. Gentisic acid and its 3- and 4-methyl-substituted homologues as intermediates in the bacterial degradation of *m*-cresol, 3,5-xylenol and 2,5-xylenol. Biochem. J. **122:**19–28.
19. **Hopper, D. J., P. J. Chapman, and S. Dagley.** 1971. The enzymatic degradation of alkylsubstituted gentisates, maleates and malates. Biochem. J. **122:**29–40.
20. **Kabisch, M., and P. Fortnagel.** 1990. Nucleotide sequence of the metapyrocatechase I (catechol 2,3-dioxygenase I) gene *mpcI* from *Alcaligenes eutrophus* JMP 222. Nucleic Acids Res. **18:**3405–3406.
21. **Kabisch, M., and P. Fortnagel.** 1990. Nucleotide sequence of the metapyrocatechase II (catechol 2,3-dioxygenase II) gene *mpcII* from *Alcaligenes eutrophus* JMP 222. Nucleic Acids Res. **18:**5543.
22. **Knackmuss, H.-J., and M. Hellwig.** 1978. Utilization and cooxidation of chlorinated phenols by *Pseudomonas* sp. B 13. Arch. Microbiol. **117:**1–7.
23. **Knackmuss, H.-J., M. Hellwig, H. Lackner, and W. Otting.** 1976. Cometabolism of 3-methylbenzoate and methylcatechols by a chlorobenzoate utilizing *Pseudomonas*: accumulation of (+)-2,5-dihydro-4-methyl- and (+)-2,5-dihydro-2-methyl-5-oxo-furan-2-acetic acid. Eur. J. Appl. Microbiol. **2:**267–276.
24. **Kuhm, A. E., M. Schlömann, H.-J. Knackmuss, and D. H. Pieper.** 1990. Purification and characterization of dichloromuconate cycloisomerase from *Alcaligenes eutrophus* JMP134. Biochem. J. **266:**877–883.
25. **Kukor, J. E., R. H. Olsen, and J. S. Siak.** 1989. Recruitment of a chromosomally encoded maleylacetate reductase for degradation of 2,4-dichlorophenoxyacetic acid by plasmid pJP4. J. Bacteriol. **171:**3385–3390.
26. **Kunz, D. A., and P. J. Chapman.** 1981. Catabolism of pseudocumene and

3-ethyltoluene by *Pseudomonas putida* (*arvilla*) mt2: evidence for new functions of the TOL (pWW0) plasmid. J. Bacteriol. **146:**179–191.
27. **Liu, T., and P. J. Chapman.** 1984. Purification and properties of a plasmid encoded 2,4-dichlorophenol hydroxylase. FEMS Microbiol. Lett. **173:**314–318.
28. **Miller, D. J.** 1981. Toluate metabolism in nocardioform *Actinomycetes*: utilization of the enzymes of the 3-oxoadipate pathway for the degradation of methyl-substituted analogues. Zentralbl. Bakteriol. Suppl. **11:**355–360.
29. **Nakazawa, T., and T. Yokota.** 1973. Benzoate metabolism in *Pseudomonas putida* (*arvilla*) mt-2: demonstration of two benzoate pathways. J. Bacteriol. **115:**262–267.
30. **Nozaki, M.** 1970. Metapyrocatechase (*Pseudomonas*). Methods Enzymol. **17A:**522–525.
31. **Ornston, L. N., and R. Y. Stanier.** 1966. The conversion of catechol and protocatechuate to β-ketoadipate by *Pseudomonas putida*. I. Biochemistry. J. Biol. Chem. **241:**3776–3786.
32. **Peelen, S., I. M. C. M. Rietjens, W. J. H. van Berkel, W. A. T. van Workum, and J. Verwoort.** 1993. ^{19}F-NMR study on the pH-dependent regioselectivity and rate of the *ortho*-hydroxylation of 3-fluorophenol by phenol hydroxylase from *Trichosporon cutaneum*. Implications for the reaction mechanism. Eur. J. Biochem. **218:**345–353.
33. **Pettigrew, C. A., B. E. Haigler, and J. S. Spain.** 1991. Simultaneous biodegradation of chlorobenzene and toluene by a *Pseudomonas* strain. Appl. Environ. Microbiol. **57:**157–162.
33a. **Pieper, D. H.** 1986. Ph.D. thesis. University of Wuppertal, Wuppertal, Germany.
34. **Pieper, D. H., K.-H. Engesser, R. H. Don, K. N. Timmis, and H.-J. Knackmuss.** 1985. Modified *ortho*-cleavage pathway in *Alcaligenes eutrophus* JMP 134 for the degradation of 4-methylcatechol. FEMS Microbiol. Lett. **29:**63–67.
35. **Pieper, D. H., K.-H. Engesser, and H.-J. Knackmuss.** 1989. Regulation of catabolic pathways of phenoxyacetic acids and phenols in *Alcaligenes eutrophus* JMP134. Arch. Microbiol. **151:**365–371.
36. **Pieper, D. H., K.-H. Engesser, and H.-J. Knackmuss.** 1990. (+)-4-Carboxymethyl-2,4-dimethylbut-2-en-4-olide as dead-end metabolite of 2,4-dimethylphenoxyacetic acid or 2,4-dimethylphenol by *Alcaligenes eutrophus* JMP134. Arch. Microbiol. **154:**600–604.
37. **Pieper, D. H., W. Reineke, K.-H. Engesser, and H.-J. Knackmuss.** 1988. Metabolism of 2,4-dichlorophenoxyacetic acid, 4-chloro-2-methylphenoxyacetic acid and 2-methylphenoxyacetic acid by *Alcaligenes eutrophus* JMP134. Arch. Microbiol. **150:**95–102.
38. **Pieper, D. H., K. Stadler-Fritzsche, K.-H. Engesser, and H.-J. Knackmuss.** 1993. Metabolism of 2-chloro-4-methylphenoxyacetate by *Alcaligenes eutrophus* JMP134. Arch. Microbiol. **160:**169–178.
39. **Pieper, D. H., K. Stadler-Fritzsche, H.-J. Knackmuss, K.-H. Engesser, N. C. Bruce, and R. B. Cain.** 1990. Purification and characterization of 4-methylmuconolactone methylisomerase, a novel enzyme of the modified 3-oxoadipate pathway in the Gram-negative bacterium *Alcaligenes eutrophus* JMP 134. Biochem. J. **271:**529–534.
40. **Pieper, D. H., K. Stadler-Fritzsche, M. Schlömann, and H.-J. Knackmuss.** 1992. Metabolism of 2-chloro-4-methylphenoxyacetate by *Alcaligenes eutrophus* JMP134: implications for the degradation of chloro- and methylaromatics via *ortho* cleavage, p. 277–291. *In* E. Galli, S. Silver, and B. Withold (ed.), *Pseudomonas*: molecular biology and biotechnology. American Society for Microbiology, Washington, D.C.
41. **Powlowski, J. B., and S. Dagley.** 1985. β-Ketoadipate pathway in *Trichosporon cutaneum* modified for methylsubstituted metabolites. J. Bacteriol. **163:**1126–1135.
41a. **Prucha, M., K. N. Timmis, and D. H. Pieper.** 1993. The role of muconolactone isomerases in the degradation of substituted aromatic compounds, p. 183. *In* Abstracts of the Fourth International Symposium on *Pseudomonas*. Vancouver, Canada.
42. **Ribbons, D. W.** 1970. Specificity of monohydric phenol oxidations by *meta* cleavage pathways in *Pseudomonas aeruginosa* T1. Arch. Mikrobiol. **74:**103–115.
43. **Rojo, F., D. H. Pieper, K.-H. Engesser, H.-J. Knackmuss, and K. N. Timmis.** 1987. Assemblage of *ortho* cleavage route for simultaneous degradation of chloro- and methylaromatics. Science **238:**1395–1398.
44. **Schlömann, M., P. Fischer, E. Schmidt, and H.-J. Knackmuss.** 1990. Enzymatic formation, stability, and spontaneous reactions of 4-fluoromuconolactone, a metabolite of the bacterial degradation of 4-fluorobenzoate. J. Bacteriol. **172:**5119–5129.
45. **Schlömann, M., E. Schmidt, and H.-J. Knackmuss.** 1990. Different types of dienelactone hydrolase in 4-fluorobenzoate-utilizing bacteria. J. Bacteriol. **172:**5112–5118.
46. **Schmidt, K., S. Liaaen Jensen, and H.-G. Schlegel.** 1963. Die Carotinoide der Thiorhodaceae. I. Okenon als Hauptcarotinoid von *Chromatium okenii* Perty. Arch. Mikrobiol. **46:**117–126.
47. **Schmidt, S., R. B. Cain, G. V. Rao, and G. W. Kirby.** 1994. Isolation and identification of two novel butenolides as products of dimethylbenzoate metabolism by *Rhodococcus rhodochrous* N75. FEMS Microbiol. Lett. **120:**93–98.
48. **Shingler, V., C. H. Franklin, M. Tsuda, D. Holroyd, and M. Bagdasarian.** 1989. Molecular analysis of a plasmid-encoded phenol hydroxylase from *Pseudomonas* CF600. J. Gen. Microbiol. **135:**1083–1092.
49. **Vollmer, M. D., K. Stadler-Fritzsche, and M. Schlömann.** 1993. Conversion of 2-chloromaleylacetate in *Alcaligenes eutrophus* JMP 134. Arch. Microbiol. **159:**182–188.
50. **Williams, P. A., and K. Murray.** 1974. Metabolism of benzoate and the methylbenzoates by *Pseudomonas putida* (*arvilla*) mt-2: evidence for the existence of a TOL plasmid. J. Bacteriol. **120:**416–423.