

## Bacterial Metabolism of 2,6-Xylenol

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Strain DM1, a *Mycobacterium* sp. that utilizes 2,6-xylenol, 2,3,6-trimethylphenol, and *o*-cresol as sources of carbon and energy, was isolated. Intact cells of *Mycobacterium* strain DM1 grown with 2,6-xylenol cooxidized 2,4,6-trimethylphenol to 2,4,6-trimethylresorcinol. 4-Chloro-3,5-dimethylphenol prevents 2,6-xylenol from being totally degraded; it was quantitatively converted to 2,6-dimethylhydroquinone by resting cells. 2,6-Dimethylhydroquinone, citraconate, and an unidentified metabolite were detected as products of 2,6-xylenol oxidation in cells that were partially inactivated by EDTA. Under oxygen limitation, 2,6-dimethylhydroquinone, citraconate, and an unidentified metabolite were released during 2,6-xylenol turnover by resting cells. Cell extracts of 2,6-xylenol-grown cells contained a 2,6-dimethylhydroquinone-converting enzyme. When supplemented with NADH, cell extracts catalyzed the reduction of 2,6-dimethyl-3-hydroxyquinone to 2,6-dimethyl-3-hydroxyhydroquinone. Since a citraconase was also demonstrated in cell extracts, a new metabolic pathway with 2,6-dimethyl-3-hydroxyhydroquinone as the ring fission substrate is proposed.

Dimethyl-substituted phenols or xylenols are by-products of coal conversion processes, such as carbonization, direct hydrogenation, or extraction (5), and thus constitute components of waste streams of this industry. Previous studies have shown that five of the six structural isomers were metabolized by fluorescent or nonfluorescent *Pseudomonas* species (3). Their metabolic pathways have been examined in some detail. The degradation of 2,3- and 3,4-xylenol by two *Pseudomonas putida* strains is initiated by hydroxylation of the benzene nucleus, thus forming 3,4-dimethylcatechol (1,2-dihydroxy-3,4-dimethylbenzene) (3, 18). The benzene nucleus is cleaved by a *meta* fission mechanism (9) analogous to the metabolism of 3-methyl- (1,2-dihydroxy-3-methyl-) and 4-methylcatechol (1,2-dihydroxy-4-methylbenzene) (1, 7, 8). In contrast, the first step in 2,4-xylenol degradation is the oxidation of the methyl group *para* to the hydroxyl group and subsequent attack of the second methyl group (4). The ring fission substrate protocatechuic acid is subject to *ortho* cleavage (4). 2,5- and 3,5-xylenol are metabolized by *Pseudomonas alcaligenes* 25X and *P. putida* 35X by an initial oxidation of one methyl group of carboxyl. After subsequent hydroxylation of the ring, the resulting 3-methyl- or 4-methylglutaric acid serves as the substrate for 1,2-ring fission (13, 14).

Little is known about the degradation of the sixth isomer, 2,6-xylenol. To date, only the disappearance of 2,6-xylenol by phenol-adapted bacteria has been observed (24). The present paper describes the isolation of *Mycobacterium* sp. strain DM1 that grows with 2,6-xylenol as the sole carbon and energy source. A number of intermediates of 2,6-xylenol metabolism were isolated and identified. A pathway for the degradation is suggested.

### MATERIALS AND METHODS

**Organisms.** The 2,6-xylenol-degrading organism was isolated from a soil sample originating from a benzene washing plant in Dortmund-Dorstfeld, Federal Republic of Germany. It was enriched in batch and continuous culture by elective growth with 2,6-xylenol in the mineral salts medium de-

scribed below. The strain was identified by N. Weiß, German Culture Collection of Microorganisms, Braunschweig, Federal Republic of Germany, on the basis of mycolic acid composition of the cell wall and biochemical reactions as a *Mycobacterium* species (personal communication). Strain AO3 (*Moraxella osloensis*) was used for the production of methylmaleylpyruvate (2-methyl-4,6-dione-hept-2-en-diacid).

**Media and culture conditions.** For cultivation in batch and continuous culture, a mineral medium was used, containing the following (per liter): KH<sub>2</sub>PO<sub>4</sub>, 1 g; Na<sub>2</sub>HPO<sub>4</sub> · 2H<sub>2</sub>O, 3.5 g; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1 g; Fe(III)NH<sub>4</sub> citrate, 10 mg; MgSO<sub>4</sub> · 7H<sub>2</sub>O, 200 mg; Ca(NO<sub>3</sub>)<sub>2</sub> · 4H<sub>2</sub>O, 50 mg; trace element solution (described by Pfennig and Lippert [16]), 10 ml; vitamin solution (described by Genthner et al. [11]), 10 ml; 2 mM 2,6-xylenol (batch culture) or 10 mM 2,6-xylenol (continuous culture) as the carbon source. Solid media were prepared by the addition of 1.5% agar no. 1 (Oxoid Ltd., London, England) to the mineral salts medium with 2,6-xylenol or other appropriate carbon sources. Stock cultures were maintained on agar slants with 2,6-xylenol. The medium for strain AO3 was prepared as described by Crawford et al. (6).

Small quantities of cells were grown in 500-ml baffled Erlenmeyer flasks containing 50 ml of medium. The flasks were incubated at 30°C on a rotary shaker at 100 rpm. For experimental use, cells were continuously grown in a 2-liter fermentor (B. Braun, Melsungen, Federal Republic of Germany) with a working volume of 0.7 to 1.8 liters. The aeration rate was 1.2 liter/min, and the culture was agitated at 250 rpm. The dilution rate was varied between 0.018 and 0.080 h<sup>-1</sup>. Oxygen limitation experiments were also conducted in the 2-liter fermentor as batch cultures. Growth was monitored by measuring the turbidity at 546 nm.

**Analytical methods.** For oxygen limitation experiments, the oxygen saturation of the culture fluid was determined with an oxygen electrode (Ingold, Steinbach, Federal Republic of Germany). 2,6-Xylenol, 2,3,6- and 2,4,6-trimethylphenol, and the metabolites in the culture liquid were analyzed by reverse-phase high-pressure liquid chromatography (HPLC) (chromatograph with pumps 655 A-12, solvent programmer, and autosampler [E. Merck AG, Darmstadt, Federal Republic of Germany]; RP 8 Lichrospher

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TABLE 1. Solvent systems used as mobile phase for HPLC of 2,6-xyleneol, trimethylphenols, and metabolites<sup>a</sup>

Phenol or metabolite	% Vol in mobile phase in stock solution <sup>b</sup> :					Retention time <sup>c</sup> (min)
	A	B	C	D	E	
2,6-Xyleneol	80	20				1.52
	40	60				4.70
2,3,6-Trimethylphenol	80	20				1.15
2,4,6-Trimethylphenol	80	20				1.15
2,4,6-Trimethylresorcinol	80	20				0.42
	40	60				1.29
Citraconic acid	40	60				0.47
		100				7.56
Unidentified metabolite	40	60				0.70
		100				25.10
			70	30		1.46
3-Methylgentisic acid	40	60				1.87
2-Hydroxy-2-methylbenzaldehyde	40	60				6.38
3-Methylsalicylic acid	40	60				4.16
Methylmaleylpyruvic acid		100				10.90
2,6-Dimethyl-3-hydroxyquinone	50				50	6.00
2,6-Dimethyl-3-hydroxyhydroquinone	50				50	1.28

<sup>a</sup> For experimental conditions of HPLC, see text. The mobile phase was composed from stock solutions with a solvent programmer.

<sup>b</sup> Stock solution A contained 1 ml of H<sub>3</sub>PO<sub>4</sub> (85%) and 1 liter of acetonitrile; stock solution B contained 1 ml of H<sub>3</sub>PO<sub>4</sub> (85%) and 1 liter of water; stock solution C contained 9.8% (vol/vol) methanol, 88.2% (vol/vol) water, and 2% (vol/vol) PIC reagent A (Millipore Corp., Bedford, Mass.); stock solution D contained 88.2% (vol/vol) methanol, 9.8% (vol/vol) water, and 2% (vol/vol) PIC reagent A; stock solution E contained 1 ml of H<sub>3</sub>PO<sub>4</sub> (85%) and 75% (vol/vol) methanol; stock solution F contained 1 ml of H<sub>3</sub>PO<sub>4</sub> (85%) and 1 liter of water.

<sup>c</sup> Netto retention times refer to a flow rate of 1 ml/min.

column, 125 by 4.6 mm, 5- $\mu$ m-diameter particles [Bischoff, Leonberg, Federal Republic of Germany]). The mobile phases are described in footnote *b* of Table 1. Samples of the culture fluid (10  $\mu$ l) were injected after cells had been removed by centrifugation in 1.5-ml microtest tubes for 2 min at 20,000  $\times$  *g*. For quantification of substrates and metabolites, a UV detector (200 or 327 nm) and a computing integrator (Merck) were used.

UV spectra were scanned *in situ* with a Merck multichannel photo detector (200 to 520 nm) or an Uvikon 860 spectrophotometer (180 to 500 nm; Kontron, Eching, Federal Republic of Germany). Infrared spectra of metabolites were recorded in tetrachloromethane with a Perkin-Elmer 580B spectrophotometer (Perkin-Elmer, Überlingen, Federal Republic of Germany). Mass spectra of the isolated intermediates were measured with a Finnigan MAT 44 mass spectrophotometer. The nuclear magnetic resonance (NMR) spectra were recorded with a Varian 60- or 80-mHz instrument (Varian, Darmstadt, Federal Republic of Germany) with tetramethylsilane as the internal standard.

**Isolation of metabolites.** Cells of *Mycobacterium* strain DM1 were grown on 2,6-xyleneol in continuous culture. After centrifugation, they were suspended in 25 mM phosphate buffer (pH 7.0) and incubated in the presence of the appropriate substrate at 30°C. The excretion of metabolites was monitored by HPLC. For the isolation of 2,4,6-trimethylresorcinol, the acidified culture fluid was extracted with diethylether. The organic phase was dried over Na<sub>2</sub>SO<sub>4</sub>. The products were separated by thin-layer chromatography (plates, 20 by 20-cm<sup>2</sup>, 0.1-cm thickness, silica gel 60 PF<sub>254</sub> [Merck]) with toluene-chloroform-acetone, 40:25:35. 2,4,6-

Trimethylresorcinol (*R<sub>f</sub>*, 0.72) was eluted with ethylacetate. For the isolation of 2,6-dimethylhydroquinone, the culture fluid was extracted with *n*-hexane to remove excess 2,6-xyleneol. After acidification, the solution was extracted with diethylether. The extract was dried over Na<sub>2</sub>SO<sub>4</sub>, dissolved with ether, and chromatographed with cyclohexanol-ethylacetate, 70:30 (*R<sub>f</sub>*, 0.62). 2,6-Dimethylhydroquinone was eluted with ethylacetate. To isolate citraconate, the culture fluid was concentrated fourfold. The bulk of inorganic salts was precipitated by repeated additions of methanol (20 ml), and the solution was concentrated by vacuum distillation. After the precipitated salts were removed, the solution was finally concentrated to 5 ml and the product was purified by thin-layer chromatography with 96% ethanol-25% ammonia-water (78:12.5:9.5). The band (*R<sub>f</sub>*, 0.76) which contained citraconate was located by observation with UV light at 254 nm and eluted with methanol.

**Cell extracts.** Before cell disintegration, 400 ml of continuously grown cells of *Mycobacterium* sp. was shaken in the presence of 1.5 mM 2,6-xyleneol for 1 h at 30°C. After centrifugation, the cells were washed and suspended in 20 ml of phosphate buffer. Extracts were prepared by using a French pressure cell (Aminco, Silver Spring, Md.) at 7.7 mPa and 0°C. Cell debris was removed by centrifugation at 16,000  $\times$  *g* (10°C, 30 min). For preparation of cell extracts from strain AO3, centrifugation was carried out at 100,000  $\times$  *g* for 60 min at 10°C in a Sorvall ultracentrifuge (Du Pont Instruments, Newton, Conn.). The protein content of cell suspensions was assayed by the method of Schmidt (22), whereas the protein content of cell extracts was measured by the method of Schleif and Wensink (21).

**Enzyme assays.** All assays were carried out at 25°C with a spectrophotometer (Uvikon 860; Kontron, Eching, Federal Republic of Germany) in silica cuvettes with 10-mm path-length. Specific activities are expressed as micromoles of products per minute and milligram of protein.

The 2,6-dimethylhydroquinone-converting enzyme was determined by measuring the decrease of A<sub>340</sub> of NADH. The test solution contained the following in 1 ml of water: 100  $\mu$ mol of phosphate buffer (pH 7.5), 1  $\mu$ mol of 2,6-dimethylhydroquinone, 0.1  $\mu$ mol of NADH, and 5 to 20  $\mu$ l of cell extract (diluted 1:50). 2,6-Dimethyl-3-hydroxyquinone reductase was determined by measuring the decrease of substrate and increase of product by HPLC. The test solution contained the following in 1 ml of water: 10  $\mu$ mol of phosphate buffer (pH 7.0), 1  $\mu$ mol of (NH<sub>4</sub>)<sub>2</sub>Fe(SO<sub>4</sub>)<sub>2</sub>, 1  $\mu$ mol of NADH, 0.1  $\mu$ mol of 2,6-dimethyl-3-hydroxyquinone, and 10  $\mu$ l of cell extract (316  $\mu$ g of protein). Oxidation of the product by Fe<sup>3+</sup> under acidic conditions regenerated 2,6-dimethyl-3-hydroxyquinone. 3-Methylgentisate 1,2-dioxygenase and methylmaleylpyruvate hydrolase were determined by the method of Pooh and Bayly (17). Citraconate (citraconate hydrolyase) was measured by the method of Hopper and Kemp (14). Controls were run so that reactions without active protein, NADH, or substrate were excluded.

**Oxygen uptake experiments.** Continuously grown cells (200 ml) were incubated in the presence of 1.5 mM 2,6-xyleneol for 1 h at 30°C. After centrifugation, the cells were suspended in 10 ml of 25 mM phosphate buffer (pH 7.0). The oxygen uptake was determined with an oxygen electrode (YSI model 53; Yellow Springs Instrument Co. Inc., Yellow Springs, Ohio) at 25°C. The test solution contained 2.8 ml of air saturated phosphate buffer, 0.1 ml of cell suspension (4.03 mg of protein per ml), and 0.1 ml of substrate solution (5 mM).

**Chemicals.** 3-Methylgentisic acid was prepared as described by Hopper and Chapman (13). 2-Hydroxy-3-methylbenzaldehyde was produced by the method of Gillespie (12). 2,6-Dimethylhydroquinone was obtained from 3,5-dimethylphenol, potassium nitrosodisulfonate, and sodium acetate, followed by reduction with sodium boranate as described by Frank et al. (10). 2,6-Dimethyl-3-hydroxyquinone was prepared by the method of Schill et al. (20) and Wilgus and Gates (25). The  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ) data for 1,2,4-triacetoxy-3,5-dimethylbenzene were:  $\delta = 2.0$  (s, 3H,  $\text{CH}_3$ ), 2.15 (s, 3H,  $\text{CH}_3$ ), 2.27 (s, 3H,  $\text{CH}_3$ ), 2.30 (s, 3H,  $\text{CH}_3$ ), 2.35 (s, 3H,  $\text{CH}_3$ ), and 7.0 (s, 1H, CH). The data for 2,6-dimethyl-3-hydroxyquinone (red needles, melting point 91 to 93°C) were:  $\delta = 1.96$  (s, 3H,  $\text{CH}_3$ ), 2.11 (d, 3H,  $\text{CH}_3$ ) 5J 2 Hz, 6.64 (q, 1H, OH) 5J 2 Hz, and 6.95 (s, 1H, CH).

Methylmaleylpyruvate was prepared from 3-methylgentisic acid by using a partial purified cell extract of strain AO3 (6). All commercial chemicals were of analytical grade.

## RESULTS

**Isolation of strains.** A mineral salts medium containing 2,6-xyleneol was inoculated with a soil sample from a benzene washing plant. Initially, the doubling time of the culture was about 4 to 5 days. After 10 months of adaptation, the doubling time of the culture decreased to approximately 9 h. From this mixed culture, an organism (strain DM1) was isolated by plating on selective agar plates. It was identified as *Mycobacterium* sp. In batch culture, the orange cells formed large flocs. Under continuous culture conditions, however, growth was homogeneous. Strain DM1 grew with 2,6-xyleneol, 2,3,6-trimethylphenol, or *o*-cresol as carbon and energy sources. Other methyl-substituted phenols tested did not serve as growth substrates.

**Experiments with resting cells.** 2,6-Xyleneol-grown cells instantaneously oxidized 2,6-xyleneol, 2,3,6-trimethylphenol, or 2,6-dimethylhydroquinone at high rates without measurable accumulation of intermediates. In contrast 2,4,6-trimethylphenol was almost quantitatively converted to a single metabolite. This product was isolated as described in Materials and Methods and identified by  $^1\text{H}$  NMR and mass spectra as 2,4,6-trimethylresorcinol.

1,2-Dihydroxybenzene, 1,2-dihydroxy-3-methylbenzene, 1,2-dihydroxy-3,5-dimethylbenzene, 3-methylgentisic acid, 3-methylsalicylic acid, and 2,6-dimethyl-3-hydroxyhydroquinone were not oxidized by whole cells.

**Isolation of metabolites.** From the results of the resting cell experiments, it was concluded that none of the known metabolic pathways was used for 2,6-xyleneol degradation by *Mycobacterium* sp. The first step of the metabolism of 2,6-xyleneol was expected to be a hydroxylation of the ring, thus forming 2,6-dimethylhydroquinone. In fact, this intermediate was produced by resting cells during turnover of 2,6-xyleneol in the presence of 4-chloro-3,5-dimethylphenol as a structural analog of 2,6-dimethylhydroquinone. After 2,6-xyleneol concentration decreased to nearly zero, the accumulated 2,6-dimethylhydroquinone was taken up again and further metabolized (Fig. 1). Two other intermediates were formed in minor amounts. The procedure of isolation of the metabolites is described in Materials and Methods. 2,6-Dimethylhydroquinone was identified by  $^1\text{H}$  NMR and mass spectra and comparison with an authentic compound.

If 2,6-xyleneol-grown resting cells of *Mycobacterium* strain DM1 were incubated with 2,6-xyleneol in the presence of EDTA, the same metabolites as mentioned above accumulated in the culture fluid. Besides 2,6-dimethylhydroquinone,

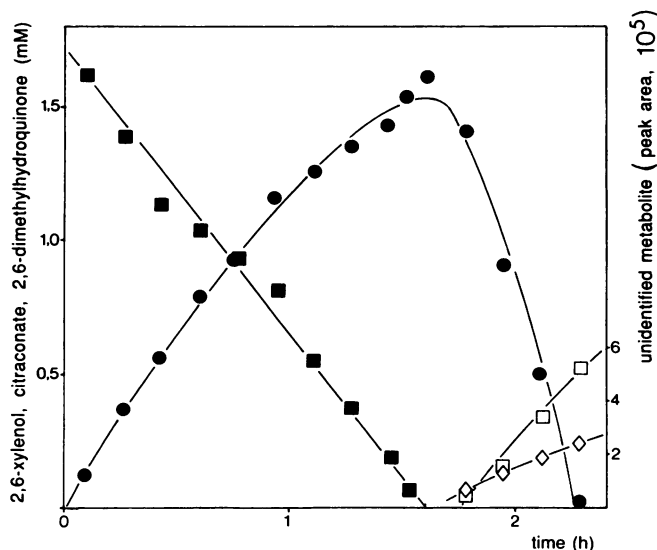


FIG. 1. 2,6-Xyleneol oxidation in the presence of 4-chloro-3,5-dimethylphenol by resting cells of *Mycobacterium* strain DM1. Experimental conditions are described in Materials and Methods. The culture fluid contained 0.48 mg of protein per ml, 1.7 mM 2,6-xyleneol, and 50 mM 4-chloro-3,5-dimethylphenol. Symbols: ■, 2,6-xyleneol; ●, 2,6-dimethylhydroquinone; □, citraconate; ◇, unidentified metabolite.

a second intermediate which exhibited a low retention time during reverse-phase HPLC was observed. This and its behavior during extraction with ethylacetate pointed to a metabolite of high polarity. For thin-layer chromatography, a mobile phase was chosen that would separate dicarboxylic acids (23). The isolated metabolite was identical with authentic citraconic acid with respect to HPLC properties and UV absorbance measured *in situ*.

The third unidentified metabolite was excreted in very small amounts and hence it was not possible to isolate the pure substance. Similar to citraconic acid, this metabolite was very polar and had a noncharacteristic adsorption maximum at approximately 200 nm in both acid and neutral conditions.

On the basis of the HPLC data, it could be concluded that the unidentified metabolite was not the ring fission product of dimethylhydroquinone, which should possess a conjugated diene chromophore and thus exhibit an absorbance maximum at a longer wave length.

During growth of *Mycobacterium* strain DM1 in batch culture, 2,6-dimethylhydroquinone, citraconic acid, and the unidentified metabolite were accumulated if the oxygen content of the medium varied between 1 and 60% saturation.

**Measurement of enzyme activities.** Neither 3-methylgentisate 1,2-dioxygenase nor methylmaleylpyruvate hydrolase activity could be measured in cell extracts of 2,6-xyleneol-grown cells of *Mycobacterium* sp. Instead of methylgentisate pathway enzymes, *Mycobacterium* strain DM1 exhibited some 2,6-dimethylhydroquinone-converting enzyme activity. The reaction was NADH- and  $\text{O}_2$ -dependent and must be correlated with a hydroxylation of the benzene nucleus. However, only part of the NADH consumption activity (16 U/mg of protein) results from the activity of the 2,6-dimethylhydroquinone-converting enzyme, a 2,6-dimethylhydroquinone hydroxylase (monooxygenase). Since 2,6-dimethyl-3-hydroxyhydroquinone is very unstable under aerobic physiological conditions and subject to rapid autox-

idation, NADH may be oxidized for the reduction of the quinone.

2,6-Dimethyl-3-hydroxyhydroquinone was synthesized as described in Materials and Methods and identified by  $^1\text{H}$  NMR spectroscopy. 2,6-Dimethyl-3-hydroxyquinone, the oxidized form of the possible ring fission substrate, was incubated with cell extract and NADH. The decrease of substrate concentration and the generation of 2,6-dimethyl-3-hydroxyhydroquinone were monitored by HPLC. The reaction product was readily oxidized with  $\text{Fe}^{3+}$ , yielding 2,6-dimethyl-3-hydroxyquinone. This was proof for the existence of a 2,6-dimethyl-3-hydroxyquinone reductase in 2,6-xyleneol-grown cells. With a 20-fold concentration of protein from cell extract, 2,6-dimethylhydroxyquinone disappeared in 1 min. Protein from cell extract converted 2,6-dimethyl-3-hydroxyhydroquinone very slowly with the generation of the unidentified metabolite.

Citraconic acid was converted by cell extracts and citraconase activity could be measured (0.047 U/mg of protein). Fructose-grown cells exhibited neither 2,6-dimethylhydroxyquinone-converting enzyme activity nor citraconase activity.

## DISCUSSION

The xyleneol-utilizing microorganisms described in the literature (3, 18) initiate degradation either by methyl group oxidation leading to protocatechuic acid or methylgentisic acid or by ring hydroxylation yielding 3,4-dimethylcatechol. The present *Mycobacterium* strain DM1 converted 2,4,6-trimethylphenol into 2,4,6-trimethylresorcinol. If this reaction were due to an unspecific hydroxylation by the initial enzyme of 2,6-xyleneol degradation, then the first metabolite of this pathway should be 2,6-dimethylhydroquinone. Actually this compound was quantitatively formed from 2,6-xyleneol by resting cells in the presence of its structural analog, 4-chloro-3,5-dimethylphenol. It was also accumulated during 2,6-xyleneol conversion under oxygen limitation. In addition, two other intermediates, citraconate and an unidentified metabolite, were produced. In the presence of EDTA, citraconate was accumulated in such high amounts that it could be isolated. Although citraconate is an intermediate of the 3-methylgentisic acid pathway (13), this route could be excluded for 2,6-dimethylphenol degradation for the following reasons: 2-hydroxy-3-methylbenzaldehyde, 3-methylsalicylic acid, and 3-methylgentisic acid, potential metabolites of such a pathway, were not oxidized or only slowly oxidized by whole cells of strain DM1. In addition, cell extracts exhibited neither 3-methylgentisic acid 1,2-dioxygenase nor methylmaleylpyruvate hydroxylase activity. Instead, 2,6-dimethylhydroquinone was readily oxidized by intact cells. A 2,6-dimethylhydroquinone-converting enzyme was present and seemed to exhibit high activity levels in cell extracts of the strain. However, NADH consumption in the presence of 2,6-dimethylhydroquinone was due to a 2,6-dimethylhydroquinone hydroxylase and to a gratuitous reduction of 2,6-dimethyl-3-hydroxyquinone. The latter is readily formed from 2,6-dimethyl-3-hydroxyhydroquinone by autoxidation and reconverted into the hydroquinone by an NADH-dependent reaction. A similar redox equilibrium was observed during thymol degradation. The oxidized state of the ring fission substrate 2-hydroxy-6-isopropyl-3-methylquinone was reduced in the presence of EDTA to the corresponding hydroquinone (2). The supposed ring fission reaction with 2,6-dimethyl-3-hydroxyhydroquinone in cell extracts of strain DM1 was very slow so that the reaction

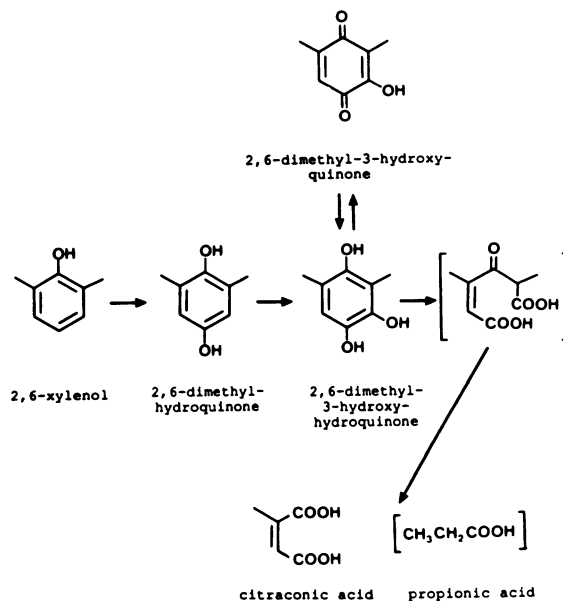


FIG. 2. Proposed pathway for 2,6-xyleneol degradation. When reactions could not be rigorously demonstrated by product analysis, tentative metabolites are shown in brackets.

product could not be isolated. An unidentified metabolite was observed during HPLC analysis. Theoretically, 2,6-dimethyl-3-hydroxyhydroquinone can be subject to ring fission by an intradiol or extradiol cleavage mechanism. *ortho* cleavage analogous to 1,2,4-trihydroxybenzene ring fission (15) would yield a methylmaleylpyruvic acid which might be cleaved by a hydrolase or thiolase to citraconic and propionic acid. Products of the alternative extradiol cleavage (19) between C-4 and C-5 or C-2 and C-3 might give rise to methylmalonic acid and  $\alpha$ -ketobutyric acid, on the one hand, and to pyruvic acid and  $\alpha$ -keto-*n*-valeric acid on the other. Since none of the latter metabolites could be detected but citraconic acid accumulated during inhibition experiments and a citraconic acid hydrolyase could be demonstrated in cell extracts, the pathway in Fig. 2 is proposed.

The 2,6-xyleneol-utilizing *Mycobacterium* strain DM1 could be established in a bacterial mixed culture which totally degraded synthetic coal conversion wastewater containing 2,6-xyleneol as a minor component (manuscript in preparation). Nevertheless, 2,6-xyleneol seems to be a critical constituent of multicomponent phenolic wastes, because it is the first compound appearing in the effluent of the continuous culture if the function of the reactor is disturbed. Therefore, 2,6-xyleneol accumulation may function as an indicator for early diagnosis of a shock load during wastewater treatment in practice.

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## LITERATURE CITED

1. Bayly, R. C., S. Dagley, and D. T. Gibson. 1966. The metabolism of cresol by species of *Pseudomonas*. *Biochem. J.* **101**:293-301.
2. Chamberlain, E. N., and S. Dagley. 1968. The metabolism of

- thymol by a *Pseudomonas*. *Biochem. J.* **110**:755–763.
3. Chapman, P. J. 1972. An outline of reaction sequences used for the bacterial degradation of phenol compounds, p. 17–55. *In* Degradation of synthetic organic molecules in the biosphere. National Academy of Sciences, Washington, D.C.
  4. Chapman, P. J., and D. J. Hopper. 1968. The bacterial metabolism of 2,4-xyleneol. *Biochem. J.* **110**:491–498.
  5. Cochran, N. P. 1976. Oil and gas from coal. *Sci. Am.* **234**(5): 24–29.
  6. Crawford, R. L., S. W. Hutton, and P. J. Chapman. 1975. Purification and properties of gentisate 1,2-dioxygenase from *Moraxella osloensis*. *J. Bacteriol.* **121**:794–799.
  7. Dagley, S., and M. P. Patel. 1957. Oxidation of p-cresol and related compounds by a *Pseudomonas*. *Biochem. J.* **66**:227–233.
  8. Evans, W. C. 1947. Oxidation of phenol and benzoic acid by some soil bacteria. *Biochem. J.* **41**:373–382.
  9. Feist, C. J., and G. D. Hegeman. 1969. Phenol and benzoate metabolism by *Pseudomonas putida*: regulation of tangential pathways. *J. Bacteriol.* **100**:869–877.
  10. Frank, B., J. Stöckigt, U. Zeidler, and G. Franckowiak. 1973. Stereospezifische Synthese der 10-Methyl-10-desmethoxycarbonyl-hemiseccalonsäure. *Chem. Ber.* **106**:1198–1220.
  11. Genthner, B. R., C. L. Davis, and M. P. Bryant. 1981. Features of rumen and sewage sludge strains of *Eubacterium limosum*, a methanol- and H<sub>2</sub>/CO<sub>2</sub>-utilizing species. *Appl. Environ. Microbiol.* **42**:12–19.
  12. Gillespie, M. B. 1973. *Biochem. Prep.* **3**:79.
  13. Hopper, D. J., and P. J. Chapman. 1970. Gentisic acid and its 3- and 4-methyl-substituted homologues as intermediates in the bacterial degradation of m-cresol, 3,5-xyleneol and 2,5-xyleneol. *Biochem. J.* **122**:19–28.
  14. Hopper, D. J., and P. D. Kemp. 1980. Regulation of enzymes of the 3,5-xyleneol-degradative pathway in *Pseudomonas putida*: evidence for a plasmid. *J. Bacteriol.* **142**:21–26.
  15. Larway, P., and W. C. Evans. 1965. Metabolism of quinol and resorcinol by soil *Pseudomonas*. *Biochem. J.* **95**: 52 p.
  16. Pfennig, N., and K. D. Lippert. 1966. Über das Vitamin B12-Bedürfnis phototropher Schwefelbakterien. *Arch. Mikrobiol.* **55**:245–256.
  17. Poh, C. L., and R. C. Bayly. 1980. Evidence for isofunctional enzymes used in m-cresol and 2,5-xyleneol degradation via the gentisate pathway in *Pseudomonas alcaligenes*. *J. Bacteriol.* **143**:59–69.
  18. Ribbons, D. W. 1970. Specificity of monohydric phenol oxidations by meta cleavage pathways in *Pseudomonas aeruginosa* T1. *Arch. Mikrobiol.* **74**:103–115.
  19. Ribbons, D. W., and P. J. Chapman. 1968. Bacterial metabolism of orcinol. *Biochem. J.* **106**:44p–45p.
  20. Schill, G., C. Zürcher, and E. Logemann. 1975. 2,6-Di-n-alkyl-1,4-benzochinon mit langen Alkylketten. *Chem. Ber.* **108**:1570–1579.
  21. Schleif, R. F., and P. C. Wensink. 1981. Practical methods in molecular biology, p. 359–360. Springer-Verlag, New York.
  22. 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.
  23. Stahl, E. 1960. Thin layer chromatography, p. 651–653. Springer-Verlag, New York.
  24. Tabak, H. H., C. W. Chambers, and P. W. Kabler. 1964. Decomposition of phenolic compounds and aromatic hydrocarbons by phenol-adapted bacteria. *J. Bacteriol.* **87**:910–919.
  25. Wilgus, H. J., III, and J. W. Gates. 1967. Thiele acetylation of substituted benzoquinones. *Can. Chem.* **45**:1975–1980.